"Estructuració genètica de les baldrigues del gènere *Calonectris* i els seus ectoparàsits"

Tesi Doctoral

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Estructuració genètica de les baldrigues del gènere *Calonectris* i els seus ectoparàsits

Memòria presentada per

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La Doctoranda

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Els directors de la tesi

Dr. Jacob González-Solís

Dr. Miguel Àngel Peinado

El tutor de la tesi

Dr. Santiago Mañosa

Al papa i la mama

A la Irene

Al lleó

"Ocell: crec que és millor que obris els ulls i
fugis de la meva espatlla. Aprofita avui
per a creuar extensions marines i
encendre't d'estrelles"

"Música d'Arpa", Joan Brossa.

Agraïments

D'oportunitats, de les bones oportunitats, no se't presenten gaires. La meva bona oportunitat ha estat aquesta tesi. Representa un punt i a part, una nova etapa carregada d'amistats, de viatges impressionants i d'evolució personal i professional. És curiós, però hi ha vegades que el destí posa en el teu camí persones absolutament excepcionals i et fa pensar com has pogut sobreviure fins aleshores sense elles. Per això, el meu agraïment a la gent que hi és i ha estat al meu costat, va molt mes enllà. Però no pretenc acomiadar-me, això no és més que un punt i seguit, i només vull dir; Gràcies a tots.

Primer que tot el meu director de tesi, el Jacob González-Solís. Un anunci seu en un fòrum d'ornitologia en té la culpa. Ocells marins, viatges a illes oceàniques, i la possibilitat de treballar en un laboratori de genètica molecular....la idea era massa atractiva com per a deixar-la escapar. I no ho vaig fer, gràcies a tu, que em vas donar l'oportunitat. Gràcies per la teva generositat al ensenyar-me i pel teu entusiasme en tot moment. Ja saps que m'encanta aprendre però amb una "*llesta*" com jo m'imagino que no haurà estat fàcil. No trobaria mai la manera d'agrair-te tota la bellesa que hem compartit i que m'omple l'esperit. Totes i cadascuna de les campanyes plegats, dures i intenses però trepidants, plenes de perills i aventures però sempre en indrets incomparables. Gràcies per regirar-me la vida de cap a peus i de peus a cap..i per que no puc agrair-te res professionalment sense agrair-t'ho també a nivell personal.

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Quan un pensa en agraïments es fan dos grups: els companys de feina i els amics. No és el meu cas. A mi la feina m'ha donat també bons amics. Tot i així, com a persona dividida que soc, biòloga "*de bata*" i "*de bota*" amb un peu en l'ecologia i un altre en la genètica; els meus companys i amics també ho estan de dividits. Començaré pels "ocellaires" (uns mes que d'altres per això). El Joan el meu "germà de

tesi". Varem néixer, científicament parlant, al mateix temps. I hem compartit gairebé tot el doctorat. Durant aquest temps ens hem barallat, reconciliat però sobretot ens hem ajudat molt. Tot i ser un empipador, gràcies per la teva motivació i confiança. A Barcelona vaig arribar sola com una "*mussola*" però vaig tenir la sort de conèixer a l'Antigoni a qui li agraeixo la seva amistat. Gràcies per escoltar-me i recolzar-me en els moments difícils. Al Raül, vull agrair-li el seu ajut i bons consells orientals, esperem que no se'ns instal·li a la Xina . En l'última etapa també li dono les gràcies a la Maria i al Jose. A la Maria pel seu optimisme i simpatia. Al Jose per la seva capacitat d'adaptació, dels falcons a la contaminació passant per la baldriga petita a la Palma, tota una proesa això si sempre amb alegria. També gràcies al Fran, el Sergi, i la Rocío. I no em puc oblidar dels gloriosos veterans; al Marc, el meu instructor d'escalada favorit, i al bohemi del Víctor, per la seva grandiloqüència insuperable, tota una llegenda del "*pbk*". Gràcies a tots dos.

I continuaré pels "*moleculars*", en primer lloc a l'Eli, una font d'optimisme inesgotable, vull agrair-li el seu ajut. Encara no se com va anar, però per sort t'he tingut a prop. Per ser com ets; Independent, sincera, i extravertida...tot plegat un cocktail que et fa ser molt especial. I un gràcies immens pels moments, bons i també els dolents, a Cap Verd. A la Regina li vull agrair la seva alegria i el seu entusiasme, mai s'esgota, és com una font il·limitada de felicitat. Sempre t'enveja't per això. Per aquesta mena de dolça innocència que desprens. Pels bons moments a Madeira, que van ser molts. Gràcies xavala. A la Mar, li agraeixo tots els cops de mà al laboratori, ja n'he perdut el compte. Sempre disposada ha ajudar. La teva hiperactivitat treballant aconsegueix posar-nos les piles a tots. Gràcies per tot el que m'has ensenyat durant aquest temps, i pels partits d'esquaix que m'has deixat guanyar (dissimuladament, això si). I que puc dir del Jairo, "l'il·luminat", el "*crac catacrac"* del laboratori. Gràcies per instruir-me i acollir-me com la teva alumna. Gràcies company pel teu ajut a Almeria, per la dura lluita contra la son i la fúria de les tempestes. A la Gemma, la meva mestressa d'extraccions de ADN. Sempre t'ho he dit, que de gran volia ser com tu. A ella vull agrair-li el seu ajut i bon saber. A la Cristina i a la Mireia, gràcies també per auxiliar-me en diverses ocasions, tants favors he demanat a tothom... No em vull oblidar del Jordi, el teu kiwi d'Austràlia m'ha portat sort, ho reconec. Gràcies pels bons consells. Tenim pendent alguna campanya plegats, potser un dia d'aquets...I per últim la Maria li agraeixo el seu dinamisme i sinceritat. Tot i que amb ella he coincidit poc temps, és impossible oblidar-se de ella per que deixa empremta.

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ambdós, els hi agraeixo l'amabilitat, l'hospitalitat i l'ajut rebut durant aquest temps a l'estranger. També vull agrair a tota la gent que ens ha ajudat en les diferents campanyes o ha recollit mostres per nosaltres, la seva col·laboració i ajut. És un goig haver conegut a tanta gent apassionada i altruista. Especialment, en aquest mon de competitivitat ferotge. A la Verònica Neves, al John Scopel, al Francis Zino, a la Marta Cerdà, al Pascual Calabuig, etc... A tots ells gràcies.

A la meva família no li puc agrair res en concret; Ser qui són i estar sempre al meu costat, potser?. La meva mare que m'estima tant i tant, i que tant pateix també. Li vull agrair la seva dedicació i entrega tot i la llunyania. I es que la distància no ens ha posat les coses fàcils. Al meu pare, amb el que sempre hi he mantingut certa complicitat. Serà perquè som "pastats" (això diu la meva mare). Li agraeixo el suport incondicional. A la meva germana, la Irene "*terratrèmol*", tot un caràcter, gràcies per cuidar-me tant com em cuida, tot i ser la petita. Ha estat una sort poder conviure i compartir amb tu la meva tesi.

Això es tot, crec?!...*À bientôt*.

"En el fons, els científics som gent amb sort: podem jugar al que vulguem durant tota la vida"

Lee Smolin

índex

Introducció

Marc Conceptual

La Teoria de la Coevolució Hoste- Paràsit

Els sistemes hoste- paràsit són interessants des de un punt de vista de la Biologia Evolutiva i la Sistemàtica ja que representen una associació llarga i intima entre dos o més grups d'organismes sovint distants evolutivament i molt diferents en quant a les seves característiques biològiques. Aquesta llarga historia d'associació pot conduir a una evolució conjunta entre ambdós llinatges. Així doncs, la coevolució hoste- paràsit descriu el procés de canvi evolutiu recíproc en dos organismes, hoste i paràsit, que interactuen. En aquest context, tot i que el què esperaríem és que la filogènia del paràsit reflectís la filogènia de l'hoste (Brooks 1985), al menys fins a quatre escenaris evolutius alternatius són possibles: Canvi d'hoste (*Host-switching*), pèrdua o extinció del paràsit (*Missing the boat*), duplicació o especiació intrahoste, i fracàs del paràsit per especiar en resposta a l'hoste (*Failure to speciate*) (Page 1994, Page & Charleston 1998, Johnson *et al.* 2003) (Fig. 1). Tots ells explicarien una incongruència entre les histories evolutives d'hoste i paràsit.

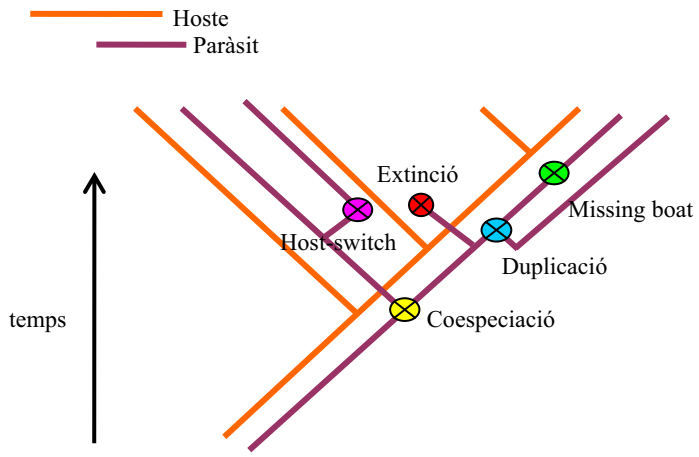

Fig 1. En els sistemes hoste- paràsit 4 escenaris evolutius alternatius a la coespeciació poden conduir a una incongruència filogenètica entre ambdós organismes.

Microcoevolució en el context de la Filogeografia i la Genètica de poblacions

La coevolució hoste- paràsit s'ha estudiat fonamentalment a nivell interespecífic (e.g. Paterson *et al.* 1993, Hafner *et al.* 1994, Page 1994, Johnson *et al.* 2003), i encara que processos similars poden donar-se també a nivell intraspecific, en l'estadi evolutiu previ a l'especiació de

l'hoste, hi han molt pocs estudis en aquesta línia (Nadler *et al.* 1990, Demastes *et al.* 1998, Hafner *et al.* 1998, Johnson *et al.* 2002).

La dinàmica i la coevolució de les interaccions hoste- paràsit pot dependre en última instancia de la variabilitat genètica i de la seva estructuració en ambdós grups. I per tant, factors que actuïn a aquest nivell poden jugar un paper important com a factors causals en el procés coevolutiu. En un context de coespeciació, qualsevol factor que provoqui l'aïllament de l'hoste resultarà en l'aïllament del paràsit i per tant afavorirà que ambdós, hoste i paràsit, codivergeixin. Si això és així, esperaríem trobar certa congruència en els patrons d'estructuració genètica d'hoste i paràsit. Al contrari, si els factors que aïllen l'hoste són diferents als que actuen en l'aïllament del paràsit, l'estructuració genètica d'hoste i paràsits serà incongruent. Finalment, en el cas en que ambdós, l'hoste i els paràsits, mostrin una estructuració genètica concordant amb algun patró espacial, com per exemple la geografia, aquesta estructura pot ser més o menys congruent (Thompson 1994).

A nivell microevolutiu, l'aïllament físic i genètic sincrònic d'hoste i paràsit pot ocórrer a tres nivells diferents: A nivell de subespècies, en fenòmens d'especiació simultanis que eliminen el flux gènic entre espècies d'hostes i paràsits incipients conduint a filogènies idèntiques. Entre poblacions, quant la creació simultània de barreres al flux gènic entre poblacions fundadores donant lloc a arbres poblacionals equivalents en hoste i paràsit. O entre individus hoste amb la transmissió per descendència de paràsits entre hostes emparentats, el què portaria a una similitud en l'arbre genealògic d'hoste i paràsit (Rannala & Michalakis 2003).

Factors i possibles mecanismes implicats

Diversos factors com l'especificitat del paràsit, la distribució geogràfica de l'hoste així com la capacitat de dispersió d'hoste i paràsit, entre d'altres, poden influenciar la congruència entre l'estructuració genètica d'hoste i paràsit (Blouin 1995, Dybdahl & Lively 1996, Johnson *et al.* 2002, McCoy *et al.* 2003, Weckstein 2005). En primer lloc, només observarem congruència a aquest nivell sempre que la dispersió del paràsit estigui lligada a la dispersió de l'hoste. És a dir, les barreres per al flux gènic de l'hoste haurien d'actuar també com a barreres per flux gènic

del paràsit (McCoy *et al.* 2003). Segon, el grau de congruència depèn en gran mesura de la especificitat d'hoste, la qual està fortament influenciada per l'ecologia i l'habilitat de dispersió del paràsit (Clayton 1990, Clayton *et al*. 1992, Hahn *et al.* 2000, Whiteman *et al.* 2004). Per tant, paràsits que difereixen en la seva habilitat de dispersió diferiran també en el seu grau d'especificitat per l'hoste, i en conseqüència en el grau de congruència observat (Johnson *et al.* 2002). Alternativament, *Ne* (mida poblacional efectiva) pot afectar a diversos procesos microevolutius en els paràsits i que poden determinar el grau de congruència entre ambdòs llinatjes (Nadler 1995, Rannala & Michalakis 2003, Criscione & Blouin 2005). Per exemple, fenòmens d'extinció i colonització freqüents poden influir en la mida efectiva de les poblacions reduint l'estructuració genètica de les poblacions d'ectoparàsits i per tant reduint també el grau de congruència observat (Nadler 1995). Finalment, l'ecologia d'hoste i paràsit també juga un paper molt important. Així, factors ecològics que afectin a la distribució i l'abundància d'hostes i paràsits poden influenciar els patrons de similitud entre ambdós organismes (Clayton *et al.* 2004, Rannala & Michalakis 2003). En aquest context, inclús seria possible predir la congruència genètica d'hoste i paràsit a partir d'informació relativa a la seva ecologia (Clayton *et al.* 2004).

El Model d'estudi

Els ocells marins (Ordre: Procelariformes)

El terme "ocell marí" inclou un ampli rang de famílies d'ocells i espècies, totes elles amb una sola cosa en comú: l'adaptació a la vida al mar. Els ocells marins s'agrupen en 4 ordres d'ocells amb aproximadament 313 espècies: Esfenisciformes (pingüins), Procel·lariformes (albatros, petrells i baldrigues), Pelecaniformes (cormorans, fragates, mascarells) i Caradriformes (gavines, xatracs, paràsits i alques) (Gaston 2004). Entre ells, les espècies de l'Ordre dels Procel·lariformes, els petrells, baldrigues, albatros i ocells de tempesta, són els ocells marins d'alta mar per excel·lència. I donat que tots ells són veritablement marins, només van a terra per criar, han estat sempre rodejats de mite. Malgrat la diversitat d'espècies i

d'adaptacions a la vida pelàgica, els petrells presenten certes característiques comuns. Per exemple, la coloració acostuma a ser poc vistosa, el dimorfisme sexual és poc acusat (en quant al plomatge tot i ser freqüent en el cas de la mida), la maduresa sexual és retardada, la fecunditat baixa, la cura de la progènie és biparental, i són espècies de vida llarga (Warham 1990, Brooke 2004). Tot i passar la major part de la seva vida a mar obert, el coneixement que en tenim d'ells és notable. Primer, per que nidifiquen en denses colònies que ofereixen una oportunitat magnífica per la recollida d'un munt de dades en poc temps. Segon, per que la tecnologia moderna ens ofereix els medis pel seguiment remot de l'activitat dels ocells marins a l'oceà fora de l'època de reproducció. Tot plegat fa que els ocells marins siguin un model d'estudi excepcional dins del camp de l'Ecologia Evolutiva.

En el context d'aquest estudi, els ocells marins, en particular els petrells i les baldrigues, representen un model d'hoste idoni per investigar els factors (com la dispersió, l'especificitat o la distribució geogràfica de l'hoste), que poden influenciar la dinàmica coevolutiva d'hoste i paràsit a diferents escales. Tot i que els ocells marins tenen una habilitat de dispersió enorme, sovint les distribucions de cria són força restringides. I donat que nidifiquen en illes oceàniques i passen part de la seva vida en mar obert, les oportunitats per la transmissió i la dispersió dels paràsits esta força limitada. A més a més, són monògams de parella i sovint també monògams sexuals (Bried & Jouventin 2002), i moltes espècies manifesten filopatria natal i una fidelitat molt forta vers els seus llocs de cria la qual cosa pot limitar la dispersió i promoure l'aïllament genètic i la diferenciació entre les poblacions (Bried & Jouventin 2002, Brooke 2004). No obstant, el grau d'estructuració genètica i filogeogràfica pot variar àmpliament entre espècies (Friesen *et al.* 2007).

Les baldrigues (Calonectris sp.)

Les baldrigues del gènere *Calonectris* són espècies d'ocells marins pelàgiques de mars i oceans subtropicals de l'hemisferi Nord que nidifiquen en illes oceàniques. Les baldrigues, al igual que molts altres procel·lariformes, són filopàtriques i fidels a la colònia de cria. Tot i que la filopatria sembla que és més forta en els mascles, mentre que les femelles tendeixen més a

dispersar (Thibault *et al.* 1997). Les dues espècies reconegudes són la baldriga canosa (*Calonectris leucomelas*), que nidifica entorn a Japó, Taiwan i l'est de la Xina i Corea, i la baldriga cendrosa (*C. diomedea*) que cria en illes del Nord Est Atlàntic i el Mediterrani (Warham 1990) (Fig 2.). Actualment la baldriga cendrosa compren dues espècies separades geogràficament: la baldriga cendrosa Mediterrània (*C. d. diomedea)*, que nidifica en illes de la costa ibèrica i nord d'Àfrica fins al mars Adriàtic i Egeu; i la baldriga cendrosa Atlàntica (*C. d. borealis*), que cria a Madeira, illes Selvagens, i als arxipèlags de les illes Canàries i de les Açors (Fig. 3).

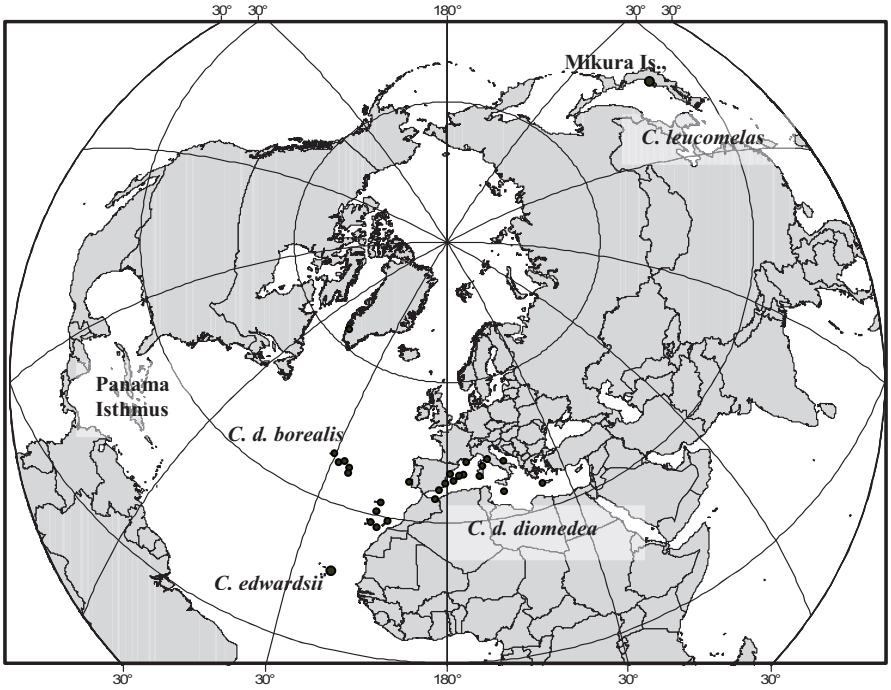

Fig. 2. Mapa de distribució de les poblacions de les espècies de baldriga del gènere *Calonectris.*

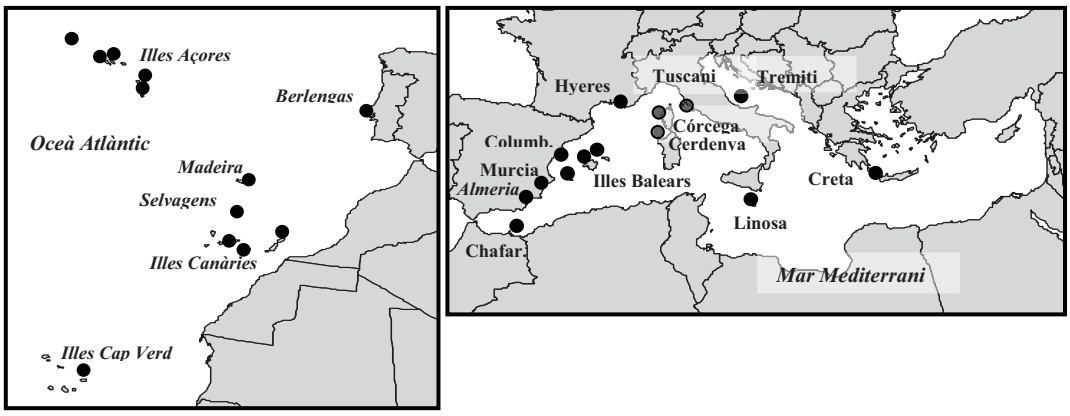

Fig. 3. Poblacions de baldriga cendrosa Mediterrània i Atlàntica i de la baldriga de Cap Verd incloses en aquest estudi.

Fins fa poc, la baldriga endèmica de Cap Verd (*C. edwardsii)* que cria exclusivament a l'arxipèlag de Cap Verd, havia estat considerada com una subespècie de la baldriga cendrosa però ara es considera com una espècie separada (Hazevoet 1995), tot i que fins al moment no existia cap estudi genètic que ho corroborés. De fet, diferències evidents en la coloració, la mida i les vocalitzacions entre la baldriga de Cap Verd i ambdues subespècies de baldriga cendrosa, han portat a diversos autors a considerar-les espècies diferents (Bourne 1955, Cramps & Simmons 1977, Porter *et al.* 1997). No obstant, les similituds en la biologia reproductora, l'ecologia i sobretot la manca d'anàlisis genètics fan que el seu status taxonòmic es mantingui encara controvertit.

Quan a l'espècie de baldriga cendrosa, s'ha provat que existeixen diferències genètiques entre les dues subespècies que coincideixen amb les diferencies morfològiques i de coloració (Randi *et al.* 1989, Wink *et al.* 1993, Heidrich *et al.* 1998, Gómez-Díaz *et al.* 2006). Dins de cada subespècie s'ha detectat també certa variació geogràfica en la morfometria (Granadeiro 1993), però estudis genètics basats en al·lozims, DNA mitocondrial i microsatèl·lits no han trobat cap estructuració espacial de les poblacions (Randi *et al.* 1989, Wink *et al.* 1993, Heidrich *et al.* 1996, Carneiro da Silva and Granadeiro 1999, Rabouam *et al.* 2000). En aquest context, és necessària una re-avaluació de l'estructuració genètica de les poblacions de baldriga cendrosa al llarg de tot el seu rang de distribució per tal d'assolir un coneixement més acurat dels patrons de variabilitat genètica i per entendre els mecanismes implicats en la diferenciació genètica de l'espècie.

Fig. 4. Per ordre de fotografia: la baldriga canosa (*Calonectris leucomelas*), la baldriga cendrosa Mediterrània (*C. d. diomedea*), la baldriga cendrosa Atlàntica (*C. d. borealis*) i la baldriga de Cap Verd (*C. edwardsii*).

Els ectoparàsits: Els polls de la ploma i la puça

Les plomes dels ocells marins proporcionen un ambient heterogeni que acull una àmplia fauna d'ectoparàsits (Janovy Jr 1997), i que inclou tant espècies fortament hoste- específiques com els polls de la ploma (Phthiraptera) i els àcars (Acari), fins a espècies més o menys generalistes com la puça (Siphonaptera), o hoste- inespecífiques com la paparra (Acari) (Janovy Jr 1997). A nivell macroevolutiu nombrosos estudis suggereixen una llarga història de coevolució entre els polls de la ploma dels ocells marins i els seus hostes (Paterson *et al.* 2000, Page *et al.* 2004), i per tant ens ofereixen un context de coespeciació idoni per testar les nostres hipòtesis.

La baldriga cendrosa Atlàntica i Mediterrània i la baldriga de Cap Verd comparteixen tres espècies de polls pels quals són l'hoste principal (Price *et al.* 2003): *Halipeurus abnormis* (Piaget 1885) (Ischnocera: Philopteridae), *Saedmunssonia peusi* (Eichler 1949b) (Ischnocera: Philopteridae), i *Austromenopon echinatum* (Edwards 1960) (Amblycera: Menoponidae).

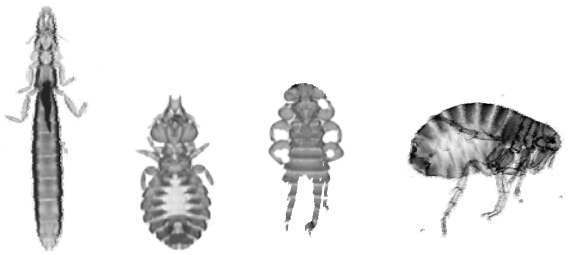

Fig. 5. Les tres espècies de polls de la ploma i la puça que parasiten la baldriga cendrosa i la baldriga de cap verd: *Halipeurus abnormis*, *Saedmunssonia peusi*, *Austromenopon echinatum* i *Xenopsilla gratiosa*, respectivament.

Per la seva elevada especificitat, els polls de la ploma (Insecta: Phthiraptera) s'han considerat sempre com un model d'estudi ideal per entendre les relacions coevolutives d'hostes i paràsits (Hafner & Nadler 1988, Paterson *et al.* 1993, Page 1994, Hafner *et al.* 1994, Page 1996, Paterson et al. 1995, Paterson *et al.* 2000). Les espècies de polls estan sovint restringides a una única espècie o genera d'hoste en el que transcorre tot el seu cicle vital i on s'alimenten principalment de plomes, pell morta, sang o secrecions. En alguns casos, inclús poden ser específics d'un lloc concret dintre de l'hoste. Tant és així que moltes espècies mostren adaptacions morfològiques i comportamentals a un microhabitat particular de l'hoste, fins al

grau que alguns polls de la ploma presenten cicles vitals que estan sincronitzats amb el del seu hoste (Marshall 1981). Els polls de la ploma fugen de la llum mentre que són atrets per l'escalfor i l'olor de l'hoste, i a més es veuen profundament afectats per variacions de temperatura i humitat prop del cos de l'hoste. Tenen un cicle hemimetàbol que inclou un ou, tres estadis nimfals, i l'adult. Cada estadi nimfal requereix de tres a dotze dies per ser completat mentre que l'adult pot viure fins a un mes. De fet, poques espècies poden sobreviure més d'uns pocs dies fora de l'hoste (Marshall 1981). La manca de mobilitat independent fa que la transmissió es produeixi per dispersió passiva durant els períodes de contacte directe entre hostes. Així doncs, per espècies com els ocells marins, la transmissió vertical al llarg d'un llinatge d'hoste és més freqüent que la transmissió horitzontal entre diferents llinatges d'hoste (Page 1996). Tot i així, existeixen diferències en el grau d'especificitat entre els diferents gèneres de polls de la ploma (Johnson *et al.* 2002). Així per exemple, els polls de la ploma que pertanyen a la família Amblycera o "*body lice*" , polls corporals o del cos, mostren una especificitat d'hoste més elevada en comparació als polls de la família Ischnocera o "*wing lice*", polls alars o de l'ala. No obstant, existeixen pocs estudis comparatius al respecte (Johnson *et al.* 2002, Clayton *et al.* 2004).

La baldriga cendrosa i la de Cap Verd també comparteixen una espècie de puça (Siphonaptera: Pulicidae) - *Xenopsylla gratiosa* (Jordan et Rothschild 1923), que es considera especifica de tres gèneres diferents d'ocells marins: *Calonectris*, *Puffinus,* i *Hydrobates*, que compren un total de 8 espècies distribuïdes al llarg de l'Atlàntic i el Mediterrani (Beaucournu *et al.* 2005). Les puces són paràsits hematòfags obligats estretament associats a l'ambient de l'hoste, en aquest cas el niu (Marshall 1981). De fet, sovint totes les etapes del cicle vital transcorren fora de l'hoste, excepte en el cas dels adults que s'alimenten intermitentment a l'hoste. Tot i que presenten un cert grau d'especificitat d'hoste, no existeixen evidències de coespeciació entre ocells i puces. En comparació amb els polls de la ploma, l'especificitat és menor en la puça ja que només passa un estadi del seu cicle vital a l'hoste, el qual coincideix amb l'època de reproducció. Al contrari que en el cas anterior, les puces amb una habilitat de dispersió major, poden transmetre's tant verticalment, de pares a fills, com horitzontalment

entre hostes veïns de la mateixa espècie així com entre hostes d'espècies relacionades criant en simpatría. En efecte, nombrosos estudis suggereixen que l'hàbitat de l'hoste influeix fortament en els patrons de distribució i abundància de les espècies de puces (Krasnov et al. 1997, Krasnov *et al.* 2004, Krasnov *et al.* 2005b, Krasnov *et al.* 2005a).

Tot i que molts estudis han estudiat la congruència entre els polls de la ploma i els seus hostes vertebrats, pocs estudis han investigat les diferències en el grau de congruència entre espècies de polls diferents dins una mateixa espècie d'hoste (Johnson *et al.* 2002), i cap estudi fins ara ha explorat aquests patrons de codivergència incloent també altres tipus d'ectoparàsits simultàniament. En aquest context, les puces ofereixen una oportunitat única per investigar la influència de l'habilitat de dispersió del paràsit en l'especificitat d'hoste el que explicaria les diferències en el grau de congruència entre diferents espècies d'ectoparàsits.

Hipòtesis i prediccions

El nostre model d'estudi: les baldrigues (*Calonectris sp*.) i els seus ectoparàsits, tres espècies de polls de la ploma i una espècie de puça, representen un sistema hoste- paràsit ideal per investigar la influencia de l'ecologia i l'evolució de l'hoste en l'estructuració genètica de les poblacions d'ectoparàsits. Dues hipòtesis principals es deriven. En primer lloc, la filopatria i la fidelitat de la baldriga vers els seus llocs de cria pot afavorir l'aïllament genètic i la diferenciació entre poblacions, la qual cosa limitaria el flux gènic. Tanmateix, l'aïllament genètic de l'hoste hauria de limitar el flux gènic dels seus ectoparàsits. Per tant, esperaríem trobar una estructuració genètica congruent així com distàncies genètiques i taxes de canvi evolutiu equivalents en hoste i ectoparàsits. En segon lloc, les diferents espècies d'ectoparàsits mostren una especificitat diferencial que es pot reflectir en diferències en el grau de congruència amb l'hoste. És a dir, mentre que en espècies d'ectoparàsits hoste- específics com els polls de la ploma, ectoparàsits obligats i amb transmissió vertical, esperarem una estructuració genètica idèntica a la de l'hoste; la similitud hauria de ser menor en la puça, una espècie més generalista, amb transmissió tant vertical com horitzontal i que exhibeix una gran capacitat de dispersió.

Implicacions per a la conservació

Resoldre les incerteses taxonòmiques i les relacions evolutives entre poblacions dins d'una mateixa espècie, es crític per la seva conservació. Tradicionalment, les unitats de la biologia de la conservació han estat les espècies. Però, les espècies sovint són difícils de delimitar. A més a més, moltes d'elles exhibeixen una gran diversitat genètica entre les seves poblacions. En aquest cas, si les poblacions mostren una diferenciació genètica suficient, estaria justificat gestionar-les com llinatjes evolutius separats per pròposits de conservació. Així doncs, l'estudi de la filogeografia ens pot ajudar a delinear correctament les espècies, a identificar les entitats amb significat evolutiu dins de cada espècie, i a definir-ne les unitats de conservació apropiades (Frankham *et al.* 2002).

El manteniment de la diversitat genètica també es un objectiu primordial per la gestió de les poblacions d'espècies amenaçades. La pèrdua de diversitat genètica implica una reducció del potencial evolutiu i s'associa amb una disminució de l'eficàcia biològica de l'espècie. Mentre que poblacions grans connectades per flux gènic, la diversitat genètica és gran i es manté estable al llarg del temps; la pèrdua de diversitat genètica en poblacions petites i aïllades genèticament, és ràpida i molt severa (Frankham *et al.* 2002). En aquest sentit, l'estudi de l'estructuració genètica de les poblacions, ens pot ajudar a avaluar el grau de diversitat genètica així com a entendre la connectivitat i flux gènic entre les poblacions.

En el cas dels ocells marins, diverses característiques biològiques i una vida pelàgica fan que la seva conservació sigui particularment problemàtica, al mateix temps però, que s'ha convertit en prioritaria. Les interaccions entre els ocells marins i les activitats humanes, com la pesca de palangre, els parcs eòlics i els vessaments de petroli, sovint resulten en la mort de molts ocells. Les amenaces associades a l'activitat humana, com la pesca de palangre, els parcs eòlics i els vessaments de petroli, s'han incrementat dràsticament les darreres dècades. Globalment, la pesca de palangre és una de les amenaces més importants pels ocells marins pelàgics (Lewison *et al.* 2005). Les interaccions dels ocells marins amb les pesqueries del palangre causen la mort accidental de molts individus que es queden enganxats al intentar

prendre l'esquer (Brothers *et al.* 1999). Tanmateix, els vessaments de petroli recents, com la del Prestige o l'Erika, han estat directament responsables de la mort de centenars de milers d'ocells (Cadiou *et al.* 2003, García *et al.* 2003, Carter 2003). Finalment, la construcció de parcs eòlics marins arreu d'Europa esdevindrà una de les infraestructures amb major desenvolupament tècnic al medi marí. A Espanya per exemple estan previstes tres plantes eòliques algunes d'elles amb un impacte potencial molt fort com la prevista al Delta de l'Ebre). Però tot i així no existeixen encara dades fiables del impacte que aquesta activitat pot tenir en els ocells marins (Garthe & Hüppop 2004, Hüppop *et al.* 2006). En aquest context, valorar i entendre el impacte de la mortalitat d'ocells marins al mar, requereix no només quantificar el número d'ocells morts, sinó també determinar el seu origen.

Avui en dia, la mortalitat d'ocells marins al mar es pot analitzar mitjançant l'aplicació de diversos mètodes d'assignació basats en marcadors intrínsecs, ja sigui de tipus biològic, com els marcadors genètics i els biomètrics, o de tipus biogeoquímic, com els isòtops estables i els elements traça. A més a més, i en relació amb aquest estudi, els paràsits també es poden utilitzar com a etiquetes o traces biològiques per determinar l'origen dels seus hostes (Criscione *et al.* 2005b, Criscione *et al.* 2006). No obstant, la utilitat de cadascun d'ells dependrà de l'estructuració geogràfica de les poblacions així com de l'existència de gradients i de patrons de variabilitat espacial suficientment precisos. Sovint, la combinació de diversos marcadors aporta beneficis i n'incrementa la utilitat (Royle & Rubenstein 2004, Kelly *et al.* 2005).

Mentre que la situació de conservació de la baldriga cendrosa Atlàntica està definit com vulnerable, la baldriga cendrosa Mediterrània es considera amenaçada. En el cas de la baldriga de Cap Verd, aquesta espècie endèmica està considerada en perill. En el context d'aquesta tesi, la baldriga cendrosa és una de les espècies més afectades pel palangre en el Mediterrani occidental (Gonzalez-Solís, J., *unpublished*). Mentre que el problema del palangre està ben documentat al Hemisferi Sud (Furness & Tasker 2000); hi ha força desconeixement del impacte d'aquesta pesqueria en els ocells marins del Mediterrani i Atlàntic nord (Belda & Sanchez 2001, Cooper *et al.* 2003). La pesca de palangre també opera al voltant de les illes de Cap Verd però encara no hi han dades disponibles (Cooper *et al*. 2003). Algunes dades preliminars suggereixen

que la mortalitat de baldrigues cendroses als palangres pot estar afectant del 4 al 6% de la població total (Belda & Sanchez 2001). Tot i així, no en sabem res respecte a l'origen d'aquets individus així com del impacte de la mortalitat d'ocells marins al palangre en les poblacions d'origen. A més a més, i com hem mencionat avans, resoldre'n la taxonomia i avaluar el grau de diversitat genètica i la connectivitat entre les poblacions és essencial per qualsevol esforç de conservació futur per l'espècie.

objectíus

En el present projecte de tesi, hem escollit les baldrigues cendrosa Atlàntica i Mediterrània (*Calonectris diomedea*) i la baldriga de Cap Verd (*C. edwardsii*), dues espècies d'ocells marins properes que comparteixen tres especies de polls de la ploma (*Halipeurus abnormis*, *Austromenopon echinatum* and *Saemundssonia peusi*) i una puça (*Xenopsylla gratiosa*); com a model d'estudi.

L'objectiu principal d'aquesta tesi és investigar l'estructuració genètica de les baldrigues del gènere *Calonectris* i els seus ectoparàsits. Com a objectius específics ens proposem:

1. Estudiar l'estructura filogeogràfica de les espècies del complex *Calonectris* combinant la filogènia, la morfologia i la biogeografia (Capítol 1).
2. Estudiar l'estructuració genètica de les poblacions de l'espècie de baldriga cendrosa al llarg de tot el seu rang de distribució i identificar-ne els mecanismes de diferenciació implicats (Capítol 2).
3. Estudiar l'estructuració ecològica de la comunitat d'ectoparàsits de la baldriga cendrosa a tres nivells diferents d'organització (comunitat regional, comunitat component i infracomunitats) (Capítol 3).
4. Investigar la congruència en l'estructuració filogeogràfica i l'estructuració genètica poblacional d'hoste i ectoparàsits. Tanmateix, examinar si una especificitat diferencial per l'hoste es reflecteix en diferències en el grau de similitud de les relacions genètiques d'hoste i ectoparàsits (Capítol 4).
5. Avaluar la utilitat dels marcadors intrínsecs com a eines per assignar les baldrigues a les seves poblacions d'origen. Valorar l'aplicabilitat d'aquest tipus d'aproximacions per l'anàlisi de la mortalitat d'ocells al mar (Capítol 5).

Resultats

Phylogeography of the *Calonectris* shearwaters using molecular and morphometric data

Elena Gómez-Díaz, Jacob González-Solís, Miguel Àngel Peinado, Roderic D. M. Page

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Capítol 1

En qualsevol estudi coevolutiu una de les premises necessaries és comptar amb una filogenia acurada d'hoste i paràsits. Per tant, a l'hora d'assolir el principal objectiu d'aquesta tesi hi havia un pas previ evident, i era la necessitat d'avaluar les relacions filogenètiques del complex d'espècies d'hoste, les baldrigues, en relació a la seva distribució geogràfica i resoldre'n la taxonomia. Per això varem escollir el genoma mitocondrial com a marcador molecular. A més a més, varem combinar la informació molecular amb les dades biomètriques. El resultat és un anàlisi comparat dels mecanismes d'especiació i de divergència que ens va permetre establir les bases filogenètiques i taxonòmiques sobre les quals desenvolupar les nostres hipòtesis coevolutives.

Filogeografia de les baldrigues del complex *Calonectris* mitjançant dades moleculars i biomètriques

Varem investigar les relacions filogenètiques i l'historia biogeogràfica del complex d'espècies *Calonectris*, mitjançant dades moleculars i biomètriques d'una població de la baldriga de Cap Verd *C. edwardsii* (Illes de Cap Verd), una de la baldriga canosa *C. leucomelas* (Oceà Pacífic occidental) i 26 poblacions de baldriga cendrosa distribuïdes al llarg del Atlàntic (*C. d. borealis*) i del Mediterrani (*C. d. diomedea*). La baldriga canosa apareixia com el clade més basal i distant mentre que les divergències genètiques entre els tres clades principals dins del Paleàrtic varen ser similars. El calibratge a partir del rellotge molecular situava el primer fenomen d'especiació dins de *Calonectris* coincidint amb la formació de l'Istme de Panamà, i per tant suggeria que la divergència entre els clades Pacific i Paleartic s'hauria produït per vicariancia. La separació entre els clades Mediterrani i Atlàntic s'hauria produït en al·lopatria per una contracció de rang seguit per una adaptació local durant els fenòmens biogeogràfics del Pleistocè. La forma endèmica de Cap Verd probablement va evolucionar per divergència ecològica de la subespècie Mediterrània. Finalment, una població Mediterrània s'agrupava en el clade de la subespècie Atlàntica, tant amb els anàlisis genètics com els morfomètrics, assenyalant el front oceanogràfic Almeria-Orà (AOOF) com el límit real entre les dues subespècies de baldriga cendrosa. Els nostres resultats posen de relleu d'importància de les fronteres oceanogràfiques com a barreres efectives influenciant l'estructura poblacional i filogeogràfica d'ocells marins pelàgics.

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Phylogeography of the *Calonectris* shearwaters using molecular and morphometric data

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Abstract

We investigated phylogenetic relationships and the biogeographic history of the *Calonectris* species complex, using both molecular and biometric data from one population of the Cape Verde shearwater *Calonectris edwardsii* (Cape Verde Islands), one from the streaked shearwater *C. leucomelas* (western Pacific Ocean) and 26 from Cory's shearwater populations distributed across the Atlantic (*C. d. borealis*) and the Mediterranean (*C. d. diomedea*). The streaked shearwater appeared as the most basal and distant clades, whereas the genetic divergences among the three main clades within the Palearctic were similar. Clock calibrations match the first speciation event within *Calonectris* to the Panama Isthmus formation, suggesting a vicariant scenario for the divergence of the Pacific and the Palearctic clades. The separation between the Atlantic and Mediterranean clades would have occurred in allopatry by range contraction followed by local adaptation during the major biogeographic events of the Pleistocene. The endemic form from Cape Verde probably evolved as a result of ecological divergence from the Mediterranean subspecies. Finally, one Mediterranean population (Almeria) was unexpectedly grouped into the Atlantic subspecies clade, both by genetic and by morphometric analyses, pointing out the Almeria-Oran oceanographic front (AOOF) as the actual divide between the two Cory's shearwater subspecies. Our results highlight the importance of oceanographic boundaries as potentially effective barriers shaping population and species phylogeographical structure in pelagic seabirds.

Keywords: Phylogeography; Seabirds; Biogeography; Mitochondrial DNA; Morphology; Speciation

1. Introduction

Recent phylogenetic studies highlight the role of Pliocene and Pleistocene geological and climate events in shaping most of the present seabird species distributions, as glacial cycles promoted geographic splitting and bottlenecking of populations (Ball and Avise, 1992; Klicka and Zink, 1997; Avise et al., 1998; Moum and Árnason, 2001; Steeves et al., 2003, 2005b). Although major paleogeographic events explain most genus or species level speciation processes, phylogeographic patterns among closely related species would also reflect more complex spatial and temporal ecological interactions. Indeed, both physical and non-physical boundaries have been suggested to have an important role as speciation barriers. Thus, not only would landmasses promote divergence (Avise, 2000; Steeves et al., 2003, 2005b), but strong breeding fidelity and natal philopatry among seabirds would have favoured allopatric distributions (Dearborn et al., 2003; Burg and Croxall, 2004; Steeves et al., 2005a). However, little is known about the nature of those non-physical barriers and only recently, local adaptation hypothesis have been suggested to explain divergence among some low-latitude seabird populations (Dearborn et al., 2003). In this context, the relative roles of oceanographic features and local selection pressures in delineating the phylogeographic patterns of seabird species remain poorly understood.

Among seabirds, many closely related species and conspecific populations show obvious geographic differences in

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vocalizations, body size and plumage (Newton, 2003). Those diversification patterns can be further examined from an ecological perspective. Habitat heterogeneity may promote diversity depending on how closely seabird species would be tied to their particular marine environment. And climate and oceanography may act as effective barriers promoting ecological speciation by habitat specialization.

In addition, phylogeographic hypothesis can be tested by investigating not only the patterns, but also the times of divergence. Depending on the ages of species divergences and timing of major geological and climate events, assessments can be made as regards which speciation scenario better explains the observed distributions. Given that the distribution of the taxa, considered from either an ecological or historical perspective, is linked to their evolutionary history, phylogeographic analyses based on mitochondrial DNA can help to test biogeographic hypotheses. Indeed, mtDNA has been proved to be a useful tool for testing biogeographic hypotheses and estimating divergence times in a wide range of animal species (Avise et al., 1987; Avise, 2000) including birds (Ball and Avise, 1992; Klicka and Zink, 1997).

Since pelagic seabirds are usually associated with specific marine habitat features (Zotier et al., 1999; Weimerskirch, 2002), they represent a suitable model for assessing the importance of the oceanographic environment from both historical and ecological perspectives. The ecological and historical interplay between the Atlantic and the Mediterranean is particularly interesting for investigating phylogeographic patterns among marine species. Even though the Mediterranean communicates with the Atlantic Ocean through a 14-km wide channel, their physical and biological oceanography is clearly distinct (Longhurst, 1998) and species diversity greatly differs between them (Fredj et al., 1992; Naranjo et al., 1998; Almada et al., 2001), including the diversity of seabird communities (Zotier et al., 1999). In the past, the Mediterranean has been completely separated from the Atlantic Ocean on a number of occasions and in the Late Miocene the Mediterranean was a desiccated deep. When the Messinian salinity crisis ended (5.3 My ago), the Atlantic waters poured into the Mediterranean after the tectonic collapse of the Gibraltar Strait (Krijgsman et al., 1999). According to the fossil record, shearwater and storm petrel species colonized the Mediterranean by that time in the Pliocene (Alcover et al., 1992; Tyrberg, 1999). However, recent studies suggest that it would not be until the Pleistocene, during the biogeographic events of the last glaciations, when closely related species and subspecies began to diverge (Avise and Walker, 1998).

In the present study, we investigate the phylogeographic relationships of the *Calonectris* species complex comparing phylogenetic, morphologic and biogeographic patterns of differentiation. We aim (i) to investigate the relative importance of historical processes, i.e. geological and climate events, driving the speciation processes of seabirds and (ii) to evaluate the role of oceanographic features as effective barriers shaping the phylogeographic structure of pelagic seabirds.

2. Materials and methodology

2.1. The study species

Calonectris shearwaters are pelagic species of northern subtropical seas nesting on isolated islands. The two well-recognized species in this genus are the streaked shearwater (*Calonectris leucomelas*), which breeds around Japan, Taiwan and the east of China and Korea, and Cory's shearwater (*C. diomedea*) breeding on North Atlantic and Mediterranean islands (Warham, 1990). Currently, Cory's shearwater comprises two geographically separated subspecies: the Mediterranean Cory's shearwater (*C. d. diomedea*), nesting from the Iberian coast islands to the Adriatic and Aegean; and the Atlantic Cory's shearwater (*C. d. borealis*), breeding on the Madeira, Salvagens, Canary and Azores islands. Formerly, the endemic Cape Verde shearwater (*C. edwardsii*), breeding exclusively in the Cape Verde archipelago, was also considered a subspecies of Cory's shearwater but it is now regarded as a full species (Hazevoet, 1995), although genetic analyses have not been performed so far.

2.2. Sampling

From 2001 to 2005, blood samples as well as morphometric measures were collected from adult birds on 26 breeding colonies of Cory's shearwater across the Mediterranean and Atlantic region, one breeding colony of the Cape Verde shearwater and one of the streaked shearwater (Fig. 1). Genetic analyses were performed on 57 Cory's shearwaters, 10 Cape Verde shearwaters and 3 streaked shearwaters. In addition, an individual sample from the Manx shearwater *Puffinus puffinus* was included for outgroup comparisons as a sister taxon of the *Calonectris* species (Nunn and Stanley, 1998).

2.3. DNA isolation, amplification and sequencing

DNA was isolated from ethanol-preserved whole blood using the salting-out extraction protocol from Bruford et al. (1998). We checked DNA quality and concentration by 0.7% agarose gel electrophoresis, and only templates without degradation signals were included in the analyses. For the mitochondrial Cytochrome *b* gene, we designed four specific primers (L14987/H15685 and L15562/H16025) using previously published sequences of various shearwater species. The Cytochrome *b* gene was amplified in two fragments of approximate lengths of 420 and 680 bp using each of the two following pairs of primers: L14987 (5′CATCTC CGCCTGATGAAACT3′) and H15685 (5′TGCTGGAG TGAAGTTTTCTGG3′); L15562 (5′CCCATTTCACCC CTATTTCA3′) and H16025 (5′CTAGAGCTCCGATAA TGGGGA3′), respectively. We amplified Domain I of the mitochondrial control region of all three *Calonectris* species using the three specific primers that we designed using a few published sequences of various seabird species; either CAL2H (5′CATCCCATCCAACTTAAG3′) or CAL4H

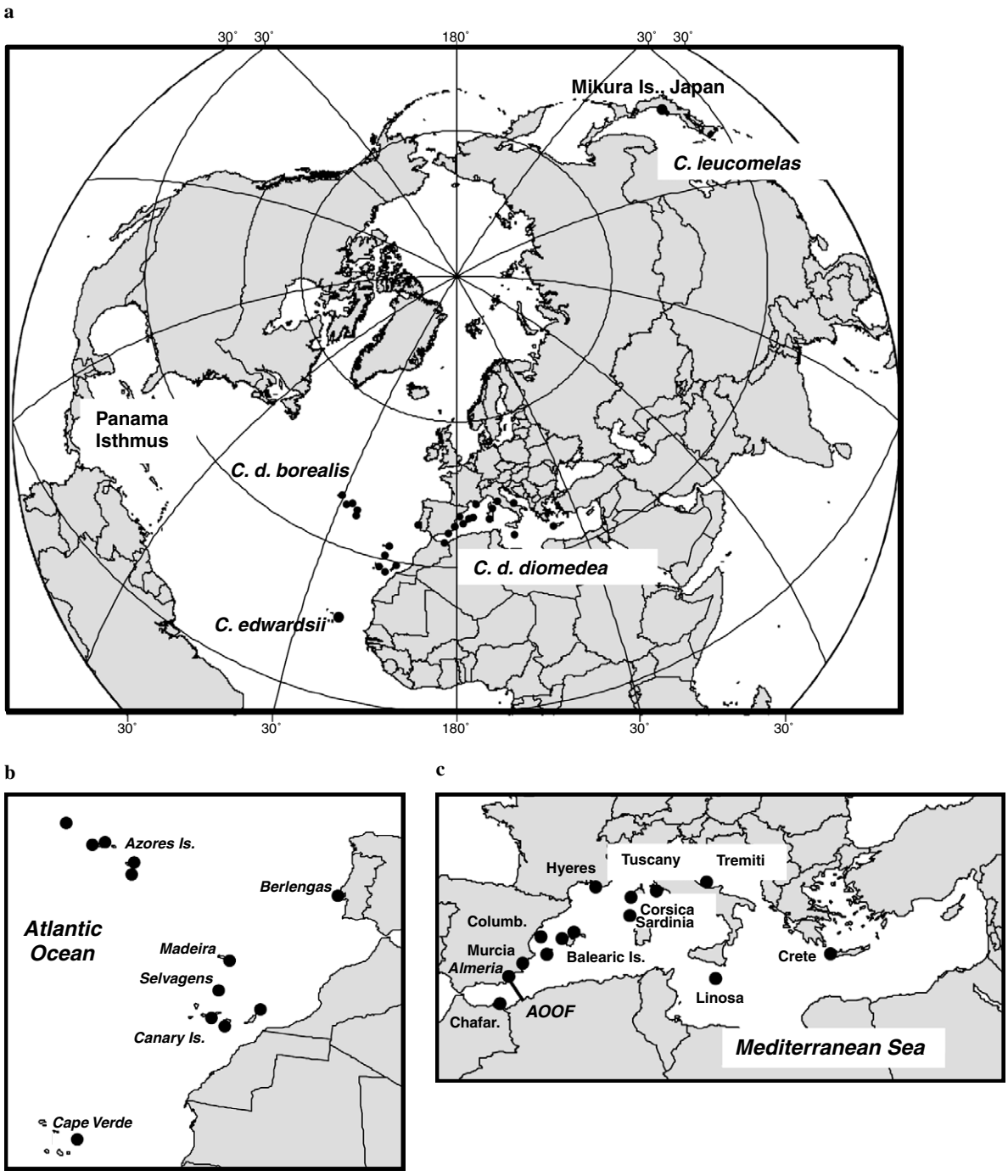

Fig. 1. (a) Geographic distributions of all three *Calonectris* species across the Mediterranean, the Atlantic and the Pacific Oceans. (b) Island populations of the Cory's shearwater and the Cape Verde shearwater sampled across the Atlantic. (c) Island populations of Cory's shearwater sampled in the Mediterranean; the AOOF (Almeria Oran Oceanographic Front) is showed as a more realistic divide between the Cory's shearwater subspecies. Almeria is also highlighted in italics. Balearic Islands include Mallorca, Cabrera, Menorca and Ibiza Islands; Azores Islands include Flores, Sao Miguel, Faial, Graciosa, Corvo and Sta.Maria; Canary Islands include Gran Canaria, Tenerife and Lanzarote Islands.

(5′AGCCTATGTATGGATGTGCAT3′) was used in conjunction with CAL1L (5′GGTCCTGAAGCTAGTAA TAC3′). The PCR was carried out in a total volume of 25 μL containing 40 mM Tris (pH 8.0), 200 mM KCl, 6 mM $MgCl_2$, 0.01% gelatin, 0.4 mM of each primer, 0.15 mM of each dNTP, 2 mM of $MgCl_2$, 0.5 U Bio*Taq* DNA polymerase (Bio-Rad Laboratories) and 40–60 ng of DNA template. Each reaction started with 4 min at 94 °C, then the amplification was carried out for 40 cycles of denaturation at 94 °C for 45 s, annealing at 60 or 56 °C (for L14987/H15685 or L15562/H16025 Cytochrome *b* primer pairs) or 58 or 60 °C (for CAL1L/CAL2R or CAL1L/CAL4R control region primer pairs) for 45 s, and extension at 72 °C for 1 min 30 s. A final extension step at 72 °C for 5 min was performed. Amplification products were separated by electrophoresis in 30% acrylamide gels, stained using ethidium

bromide and visualized under UV. PCR products were purified using the JETquick PCR Product Purification Spin Kit (Genomed, Inc., St. Louis, USA). PCR products were sequenced with the same amplification primers on an automated ABI-301 DNA Sequencher (Applied Biosystems, Foster city, USA) using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems). We used Bioedit version 5.0.1 (Hall, 1999) to assemble, edit and align sequences. To assess the reliability of the data, we compared the sequences with previously published data on the studied species. For the Cytochrome *b* gene, two sequences from Cory's shearwater (AY139626 and CDU74356) and one from the streaked shearwater (AF076045) were obtained from GenBank and included in the alignment as a reference sequence for each species, and all variable sites were confirmed by visual inspections of the chromatograms. No genetic data for the *Calonectris* species were available for the control region, and no genetic data were available for the Cape Verde shearwater for either of the two mitochondrial genes. All sequences have been submitted to GenBank (GenBank Accession Nos. DQ372022–DQ372050 and DQ371968–DQ372021).

2.4. Sequence data analyses

For the population level analyses, we defined three groups based on the clustered haplotypes in the phylogenetic analysis corresponding to the Mediterranean, the Atlantic and the Cape Verde populations, respectively (see Results). We calculated genetic statistics at the intraspecific level as the gene diversity index, the number of haplotypes and the number of polymorphic sites using DNAsp 4.0 package (Rozas and Rozas, 1999). Genetic distances for both intraspecific and interspecific levels were calculated using MEGA version 3.0 (Kumar et al., 2005).

2.5. Phylogenetic analyses

We used the partition homogeneity test (Farris et al., 1994; Swofford, 2002) to examine whether there was evidence for different phylogenetic signals between Cytochrome *b* and the control region. No significant differences were found between mitochondrial markers ($P = 0.123$). Thus, we performed all the phylogenetic analysis for a composite sequence of 1250 bp (Appendix 1). However, to test reliability of the phylogenetic signal we also performed independent phylogenetic analysis in each partition set (Page, 1996; Cunningham, 1997).

First, we tested for neutrality for the entire data set and for each gene partition separately using the Tajima's test included in the DNAsp package (Rozas and Rozas, 1999). For both genes and for the combined data set, we used MODELTEST 3.6 (Posada and Crandall, 1998) to search for the best-fit model of nucleotide substitution for our sequence data. Given the likelihood scores of the hierarchical likelihood ratio test, for both genes we selected the model TrN + G using the empirically determined base frequencies, the discrete gamma distribution and the substitution model for among site variation. In the case of the combined data set, the selected model was TrN + I + G. Such models were applied in a maximum likelihood phylogenetic analysis using PAUP*4.0b10 (Swofford, 2002). We also performed a Bayesian analysis using MrBayes v3.0B4 (Huelsenbeck and Ronquist, 2001) for Markov-chain Monte–Carlo Bayesian posterior probabilities. The maximum likelihood model employed six substitution types (Nst = 6). Rate variation across sites was modelled using a gamma distribution, with a proportion of sites being invariant (rates invgamma). The Markov-chain Monte–Carlo search was run with four chains for 2,000,000 generations. Finally, we carried out a parsimony analysis using a heuristic search, TBR branch swapping and random addition of taxa for 100 replicates. For both gene partitions, we differentially weighted substitution types based on inverse of their observed frequency in the original data set. Reliability of the MP trees was assessed by bootstrap analysis (Felsenstein, 1985), involving 1000 replications.

In the case of the Cytochrome *b* gene, to estimate approximate times of cladogenic events in the phylogeny, we first performed a maximum likelihood ratio test to determine whether the sequences were evolving according to a molecular clock. We compared the log likelihood of the tree without enforcing the molecular clock with the log likelihood of the tree constructed under molecular clock assumptions. The likelihood ratio test ($-2(\log \mathrm{Lik1} - \log \mathrm{Lik0})$) was used to test for significance of the difference between the likelihoods of the two trees. We then calculated 'net' sequence divergence (Kimura two parameters model; Kimura, 1980) between main clades using MEGA 3.0 (Kumar et al., 2005). Divergence rates were converted to absolute times by using a rate of 0.90% sequence divergence per My estimated for the Cytochrome *b* gene for the intermediate-sized *Procellaridae* (Nunn and Stanley, 1998). This rate was estimated from first-appearance fossils and the maximum divergence within the group which would make their rate estimates biased in the direction of slower rates. Nevertheless, dates based on this calibration should be the most useful approximations to estimate the time between branching points on the phylogeny (García-Moreno, 2004).

2.6. Morphometric analysis

For the morphometric analysis, we performed a clustering analysis on the biometric data to assess the population structure and the degree of morphologic differentiation among Cory's shearwater populations as well as with the Cape Verde and the streaked shearwaters. The average value of four biometric measures (bill depth at nostril and tarsus, bill and wing length) from 27 island populations was included in the analysis, combining our data with those compiled from literature (Appendix 2). Male and female means were corrected by subtracting the species mean for each sex from the corresponding mean for each population.

Then, to allow for the comparison of different biometric measures, we standardized each variable in the whole data set by subtracting the overall mean for each variable and dividing by the standard deviation. Using NTSYSpc version 2.1 (Rohlf, 1997), we calculated the average taxonomic distance for all pairwise combinations of populations and constructed a cladogram from the dissimilarity matrix using a neighbour-joining clustering analysis. To investigate the existence of latitudinal and longitudinal patterns in morphology, we tested the correlation among the population mean values for each biometric measure with the corresponding latitude and longitude coordinates of each population. We performed the correlation analyses by sex and considering each Cory's subspecies separately using the SPSS 12.0 package.

3. Results

3.1. Sequence data

For the control region data set, among the 69 sequences of 28 populations, 59 out of 79 (74.6%) variable sites were parsimony informative (Appendix 3b). We found 52 different haplotypes for the *Calonectris* species analysed: 25 for the Mediterranean Cory's shearwater; 19 for the Atlantic Cory's shearwater; 6 for the Cape Verde shearwater and 2 for the streaked shearwater. For the Cytochrome *b* gene sequences, 52 out of 114 (45.6%) variable sites were parsimony informative (Appendix 3a), resulting in 27 different haplotypes: 11 from the Mediterranean and 11 from the Atlantic Cory's shearwaters; 5 from the Cape Verde and 2 from the streaked shearwaters. In both the genes, no haplotype was shared among the two Cory's shearwater subspecies, the Cape Verde and the streaked shearwater. However, remarkable differences were found in the degree of variability obtained for each gene partition. In the control region, the average pairwise sequence divergence of all *Calonectris* populations (Kimura two parameters model; Kimura 1980) was 4.8% (S.E. 0.007; Bootstrap 1000 replicates). The streaked shearwater appeared as the most distant clade showing 12% (S.E. 1.1%) sequence divergence from the Cape Verde shearwater and 11% sequence divergence (S.E. 1.8–1.9%) from the Atlantic and the Mediterranean Cory's shearwater. The sequence divergence estimated between the Atlantic and Mediterranean shearwaters was the lowest, 5.4% (S.E. 1%); but similar to the divergence from the Mediterranean subspecies to the Cape Verde shearwater (5.7%; S.E. 1.2%), whereas the divergence between the Atlantic subspecies and the Cape Verde shearwater was slightly greater (6.6%; S.E. 1.4%). In the Cytochrome *b* gene, the average pairwise sequence divergence of all *Calonectris* populations (Kimura two parameters model; Kimura 1980) was 1.1% (S.E. 0.002; Bootstrap 1000 replicates). According to the Cytochrome *b* estimates, the streaked shearwater appeared as the most divergent clade showing 3.2% (S.E. 0.6%) sequence divergence from the Cape Verde shearwater and the Mediterranean Cory's shearwater, and a slightly greater sequence divergence (3.4%, S.E. 0.06%) from the Atlantic Cory's shearwater. The sequence divergence estimated between the Atlantic and Mediterranean shearwaters was 1.1% (S.E. 0.1%) similar to the divergence from the two Cory's shearwater subspecies to the Cape Verde shearwater (1.1%; S.E. 0.3%).

3.2. Phylogeography

Bayesian, parsimony and maximum likelihood analyses on the combined data set grouped haplotypes into four main clades corresponding to the four major taxa conventionally accepted (Fig. 2). Besides the maximum likelihood analyses for either, the Cytochrome *b* gene (Appendix 4b) and the mitochondrial control region (Appendix 4a), supported a similar tree topology. Moreover, all the analyses consistently grouped populations into four clades that were statistically well supported. The main division of the trees separated a Pacific clade (the streaked shearwater) (BS 100%; MC Bayesian posterior probabilities) from a Palearctic clade (BS 100%), which included the two Cory's shearwater subspecies together with the Cape Verde species. Within the Palearctic clade, the Mediterranean Cory's shearwater grouped together with the Cape Verde shearwater in a single clade (BS 100%), separated from the Atlantic Cory's shearwater populations in a second clade (BS 100%). Although independent phylogenetic analyses on the control region agreed with those on the combined data set, phylogenies based on Cytochrome *b* grouped the Cape Verde species, the Mediterranean and the Atlantic Cory's shearwater subspecies in three distinct poorly supported clades (BS 84%, BS 62% and BS 55%, respectively) (Appendix 4b). As regards population structuring, within the Palearctic clades a few other internal nodes were well supported (Fig. 2). For the Mediterranean clade (BS 100%), two subclades were differentiated and four groups of haplotypes (BS 100%, BS 100%, BS 99% and BS 96%) grouped apart from the main clade. The Atlantic clade (BS 100%) was slightly less structured and some Atlantic haplotypes grouped into a well-supported subclade (BS 100%). Finally, haplotypes within the Cape Verde clade (BS 100%) also appeared slightly substructured and split into two distinct subgroups (BS 100 and 98%) (Fig. 2).

Overall, the phylogeographic structure obtained for the two gene partitions and the combined data set agree with the spatially segregated distributions of the *Calonectris* clades. However, the haplotypes corresponding to one Mediterranean population (Terreros Is., Almeria, Spain) were placed within the Atlantic cluster.

The results of the Tajima's test were not significant considering both Cytochrome *b* and the control region (Tajima's $D=-1.02$; $P>0.10$ and $D=-0.89$; $P>0.10$, respectively) providing evidence that the DNA sequences of the three *Calonectris* species agree with neutrality expectations. As regards the mode of evolution, the results of the maximum likelihood ratio test for the Cytochrome *b* gene confirmed that sequences were evolving according to a

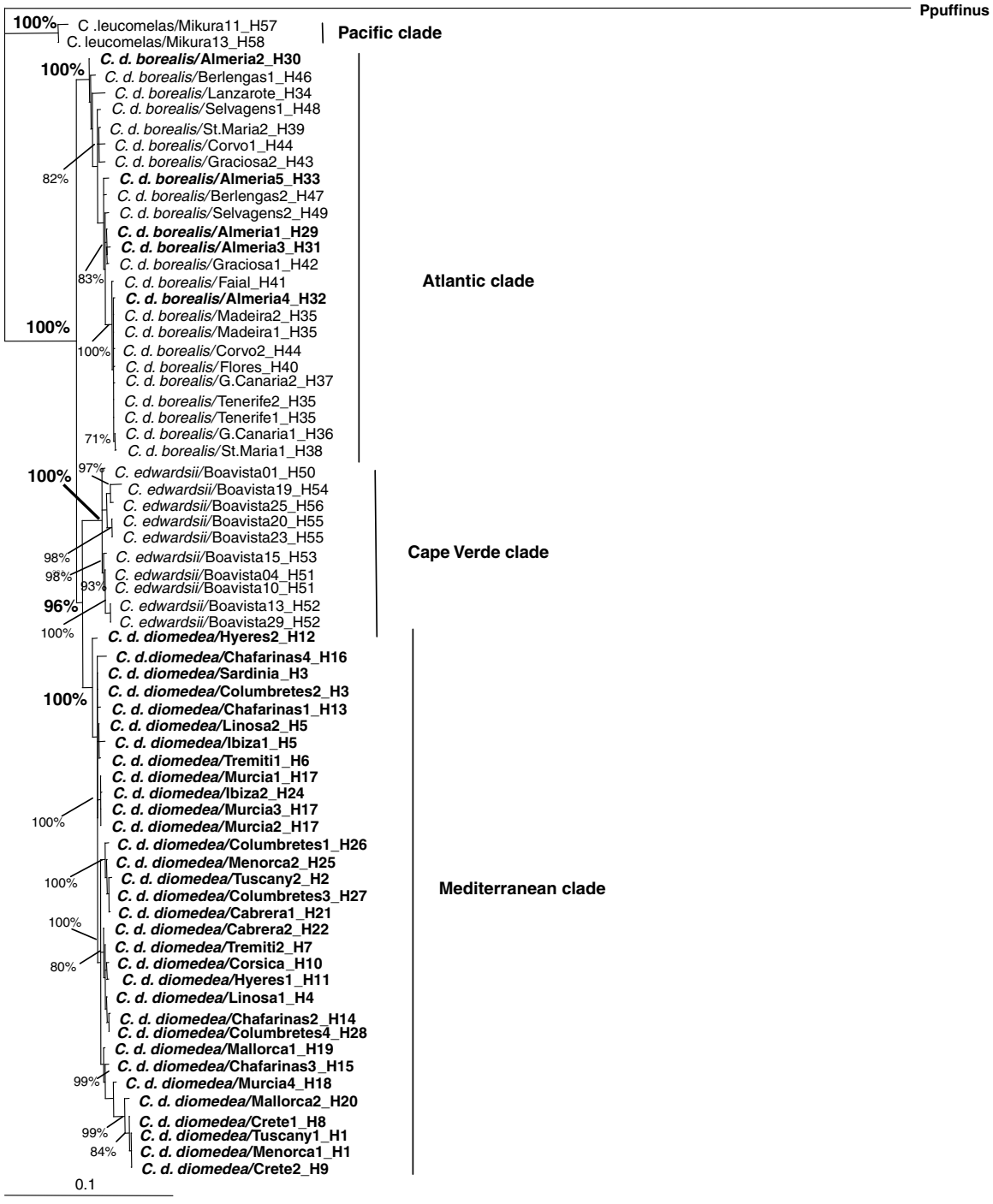

Fig. 2. Maximum likelihood phylogenetic tree for *Calonectris* shearwaters based on 1250 combined bp of the mtDNA Cytochrome *b* gene and the control region. Numbers adjacent to branches are Monte–Carlo posterior probabilities greater than 70%, and main clade support is indicated in bold. Each sequence is labelled with the species and the geographic location. Mediterranean, Atlantic and Pacific populations are indicated in bold, italics and normal font, respectively. Note that individuals from Almeria (Mediterranean) were classified within the Atlantic clade.

molecular clock. The comparison between the log likelihood value (−2154.22) of the tree without enforcing the molecular clock and the log value of the same tree constructed under molecular clock assumptions (−2170.51) did not differ significantly (likelihood ratio test statistic = 32.56; DF = 70; $P = 0.95$). For each subspecies that was phylogeographically subdivided, we estimated net sequence divergence between major phylogroups thus attempting to correct for within-group diversity (Avise et al., 1998). Then, we converted Cytochrome *b* net sequence divergence to an estimate of population separation time using the calibration rate for the *Procellariidae* family. The separation between the *Calonectris* species and its *Puffinus* sister clade would have occurred 9 My ago. The first speciation event within the *Calonectris* species complex which separated the Pacific and the Palearctic clades took place approximately

3.44 My ago. Within the Palearctic clade, the Atlantic, the Mediterranean Cory's shearwater subspecies and the Cape Verde shearwater coalesced into a common ancestor much more recently, between 900,000 and 700,000 years ago. Slight differences in divergence times among the three Palearctic subclades together with the outgroup comparisons suggest that the Atlantic Cory's shearwater was the ancient clade, whereas the Cape Verde shearwater and the Mediterranean Cory's subspecies seemed to be more recently derived clades. Nevertheless, the estimated dates based on the Nunn and Stanley (1998) fossil-calibration rate should be considered as approximate taking into account the bias associated with the method for calibrating the evolutionary rates (as is described in Methods) as well as the errors associated with the fossil record, both phylogenetic uncertainties and dating geologic errors (García-Moreno, 2004).

3.3. Biometric structure of the Calonectris species

We examined differences in body measurements among Cory's shearwater populations, the Cape Verde shearwater and the streaked shearwater as a separate species. The cladogram represents the similarity pattern of the 27 island populations included in the analysis, considering four biometric measures (Fig. 3). Agreeing with the phylogenetic analysis, four groups were clearly defined corresponding to each of the two subspecies of Cory's shearwater, the Cape Verde shearwater and the streaked shearwater. The first cluster grouped populations corresponding to the clade with the largest body size, the Atlantic Cory's shearwater. In line with the genetic analyses, one Mediterranean population, Almeria, appeared as morphologically similar to the Atlantic populations being set within the Atlantic cluster. Apart from Almeria, all Mediterranean populations grouped together in a single cluster corresponding to the intermediate in body size, the Mediterranean Cory's shearwater. Finally, the medium-sized streaked shearwater and the smallest in size Cape Verde shearwater were grouped separately. The Cape Verde and the streaked shearwaters appeared as a different morphospecies, whereas the Atlantic and the Mediterranean subspecies turned out as morphologically more similar. Moreover, the neighbour-joining

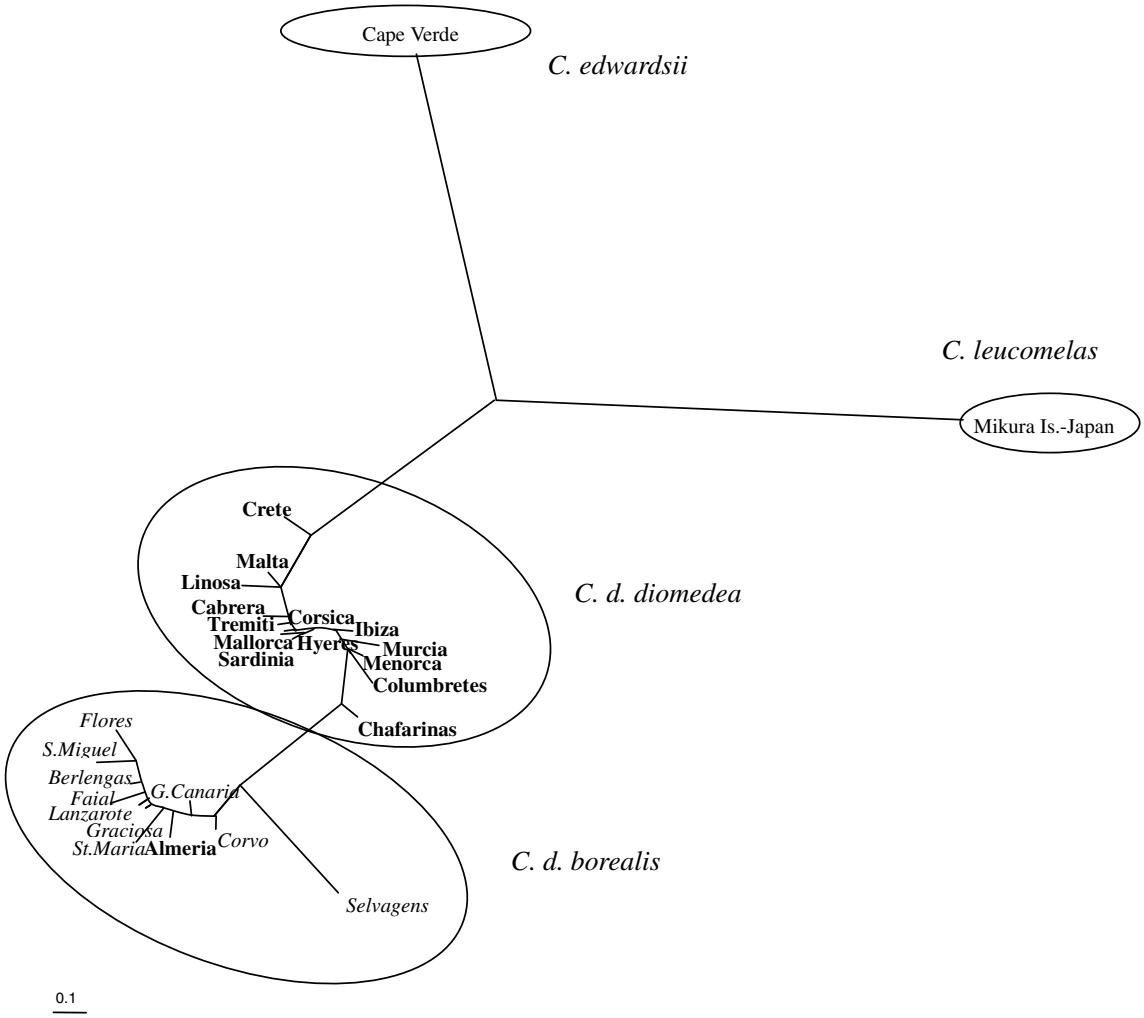

Fig. 3. Neighbour-joining tree showing the similarity pattern among the 27 populations included in the biometric analysis. Grouped populations representing different morphospecies are shown. Mediterranean populations are indicated in bold and Atlantic populations in italics.

clustering analysis suggested the existence of a biometric structure within Cory's shearwater across its geographical distribution (Fig. 3), not manifested in the phylogenetic analyses (Fig. 2). That is, we could detect a geographic gradient in morphometrics within the Mediterranean region as a slight increase in body size from east to west, that is, from the most eastern population, Crete, to the most western population, Chafarinas.

The biometric structure within the Mediterranean was also pointed out in the correlation analysis. Indeed, we found a significant positive longitudinal pattern within the Mediterranean subspecies in two of the four biometric measures, for both males and females (tarsus: $R^2 = 0.29$, $n = 14$, $P = 0.047$ and $R^2 = 0.74$, $n = 14$, $P < 0.001$; wing: $R^2 = 0.27$, $n = 14$, $P = 0.055$ and $R^2 = 0.70$, $n = 14$, $P < 0.001$, for males and females, respectively). For the Atlantic subspecies, only one significant correlation was found between the four measures and longitude. However, we found a clear trend to increase the size with latitude in the Atlantic (tarsus: $R^2 = 0.35$, $n = 11$, $P = 0.056$ and $R^2 = 0.31$, $n = 11$, $P = 0.073$; wing: $R^2 = 0.40$, $n = 11$, $P = 0.038$ and $R^2 = 0.36$, $n = 11$, $P = 0.053$, for males and females, respectively).

4. Discussion

4.1. Phylogenetic structure

Not only morphologic but also all the phylogenetic analyses performed grouped populations into four main clusters. Furthermore, general agreement among trees obtained from Cytochrome *b* and the control region separately and from combining both data partitions would give confidence to the phylogenetic accuracy of the tree topology obtained (Cunningham, 1997). Although both independent data sets supported all four *Calonectris* clades, there were some inconsistencies resolving evolutionary relationships within the Palearctic clade. Although for the Cytochrome *b* gene, phylogenetic relationships among the three main subclades within the Palearctic appeared unresolved, the control region provided a better estimate for the *Calonectris* shearwater phylogeny, elucidating inner relationships. Conflicting phylogenetic signals between the gene partitions would result from the lack of resolution in the Cytochrome *b* gene. It could be explained by a faster divergence rate of the control region compared to the Cytochrome *b* gene. Nevertheless, combining both sequence sets into a single analysis provided the greatest number of relationships receiving strong support. Moreover, the phylogenetic relationships among the three main subclades within the Palearctic agree with those suggested from morphology (Bourne, 1955; Cramp and Simmons, 1977; Porter et al., 1997; this study). Thus, congruence between gene trees together with concurrence in morphology should provide evidences that the phylogeny suggested corresponds to the species tree for the *Calonectris* species complex (Slowinksi and Page, 1999). However, as both sequence sets belong to the mitochondrial genome and could be considered as linked genes, further investigations based on nuclear markers are needed to confirm our results (Ballard and Whitlock, 2004).

The phylogenetic structure obtained agreed with the spatially segregated distributions of all four *Calonectris* clades and corresponded to the four major taxa conventionally accepted. Within the Palearctic clade, Atlantic and Mediterranean haplotypes differed from each other as much as from the Cape Verde haplotypes, suggesting long-term geographic isolation and gene flow barriers among all three clades. Besides, biometric patterns of the Mediterranean subspecies suggested an additional morphologic structure across its distribution, previously recognized by other authors (Massa and Lo Valvo, 1986; Granadeiro, 1993). That is, there is a significant increase in body size from the east to the west within the Mediterranean subspecies and a less marked pattern from south to north within the Atlantic subspecies. This pattern is probably related to the complex longitudinal and latitudinal oceanographic subzonation of the Mediterranean and the Atlantic, respectively (Longhurst, 1998). Alternatively, the morphometric gradient within the Mediterranean could be interpreted as a transition cline between the smaller Mediterranean and the larger Atlantic Cory's shearwater forms, but this hypothesis is not supported by the lack of subspecies substructure in the genetic analyses. Nevertheless, further research on population genetic structure is needed to determine the degree of isolation among geographically closer Mediterranean and Atlantic populations of Cory's shearwaters.

Although there was a major correspondence between genetic clades and geographic distributions, interestingly all sequences from five individuals analysed from Almeria (Mediterranean) corresponded to the Atlantic clade (Fig. 2). This assignment is well supported by the multiple fixed differences in the Cytochrome *b* gene differentiating the Atlantic and the Mediterranean subspecies. The Atlantic identity of the shearwaters from Almeria is further supported by the morphological analyses based on 31 individuals from this locality (Fig. 3). There are some previous reports of Atlantic pairs breeding in the Mediterranean (Lo Valvo and Massa, 1988; Sánchez, 1997; Thibault and Bretagnolle, 1998; Martínez-Abraín et al., 2002), and at least one case of hybridization between the two subspecies (Martínez-Abraín et al., 2002). Nevertheless, we found no evidence of hybridization in Almeria, and given that genetic and morphologic analyses are based on a substantial number of individuals from this colony, we can unequivocally diagnose this breeding population as the first Atlantic population found to breed in the Mediterranean. This result challenges the current view assuming the Gibraltar Strait as the distribution barrier between the two Cory's shearwater subspecies and suggests that the divide between the subspecies better corresponds to the Almeria-Oran Oceanographic Front (AOOF). In fact, the AOOF represents a major oceanographic discontinuity in the Mediterranean, being the real boundary between Atlantic and Mediterranean surface waters (Beckers et al., 1997). The biogeographic importance of this boundary is well reflected in

several genetic studies on marine species with Atlantic–Mediterranean distributions, which exhibit a phylogenetic break in open waters at the AOOF (Pannacciulli et al., 1997; Quesada et al., 1995). Although the Atlantic closest breeding colony is Chafarinas Is., which most breeders actually belong to the Mediterranean subspecies (Molina et al., 2005), Almeria would have a stronger Atlantic influence by the AOOF. Furthermore, Almeria would probably be the first breeding locality reached if we take into account the dominant winds at the Mediterranean entrance. Since Procellariforms, including *Calonectris* shearwaters, largely use winds for their oceanic movements (Schreiber and Burger, 2002), this feature is relevant because prevailing winds probably reflect connectivity among geographic locations better than absolute distances. Thus, our results underline the importance of oceanographic features and prevailing winds on the marine surface as important factors explaining the phylogeographic patterns in seabirds.

4.2. Historical biogeography and speciation mode

According to previous phylogenies (Nunn and Stanley, 1998; Kennedy and Page, 2002), the Pacific clade appeared in the base of the *Calonectris* phylogeny, closer to its *Puffinus* sister clade. Since ancient members of a taxon are expected to be found closer to its geographic origin (Crisci et al., 2003), the obtained phylogeny supports a Pacific origin for *Calonectris*. However, from a biogeographic perspective, the area of highest species diversity has been traditionally considered the centre of origin of the group (Blackburn and Gaston, 1996; Jetz et al., 2004). Thus, the greater diversity in the Atlantic compared to the Pacific Ocean, that is, the presence of two clades in geographically disjunct areas (the Atlantic Cory's and the Cape Verde shearwaters) as well as the presence of a peripheral clade in the Mediterranean, supports an Atlantic origin for the *Calonectris* group. In addition, fossil records and estimates of divergence dates can help to disentangle the biogeographic origin of the *Calonectris* group. We estimated the first speciation event between the Pacific and the Palearctic clade to about 3 My ago. However, *Calonectris* fossils have been described from South Africa (Olson, 1983) and from South Carolina (Olson and Rasmussen, 2001) dating back to the late Miocene and early Pliocene, about 5 My ago, that is, prior to the molecular clock estimate of the Pacific and the Palearctic species separation. Since fossil remains have been identified as belonging to Cory's shearwater, the *Calonectris* ancestor would have been more similar to the Palearctic form and would have been present in the Atlantic since 5 My ago, supporting an Atlantic origin for this group. Indeed, fossil records suggest an Atlantic origin for most procellariform species and subspecies with allopatric forms in the Pacific and the Atlantic oceans (Austin, 2004). However, a Pacific origin cannot be completely ruled out since few data are available as regards for fossil procellariforms in the Pacific.

The estimated separation between the Pacific and the Palearctic *Calonectris* clades dated back to 3 My ago, which corresponds to a major geological event: the separation of the Atlantic and Pacific Oceans by the Panama Land Bridge (Coates et al., 1992). These matching events suggest that vicariance played a major role explaining the speciation between the Palearctic and Pacific *Calonectris* clades. Indeed, a number of genetic studies indicate that the emergence of the Panama Isthmus led to the vicariant speciation of many tropical and south-temperate marine taxa (Avise, 2000). Furthermore, a similar study on the related *Puffinus* shearwaters also suggested the separation between Pacific and North Atlantic species took place when the Panama Land Bridge formed (Austin, 2004).

Regarding the separation between the Atlantic and the Mediterranean clades, we estimated the separation between the two Cory's shearwater subspecies at 1 Mya. Clock calibrations in the present study do not support the two former hypotheses suggesting either the colonization of the Mediterranean by an Atlantic ancestor just 10,000 years ago (Voous, 1976), or an older separation up to 5 My ago (Olson and Rasmussen, 2001). Indeed, congruent patterns and divergence times dating back to the Pleistocene ages have been found among seabirds related to the *Calonectris* clade (*Hydrobates pelagicus*, *Puffinus* spp.), also showing a similar phylogeographic boundary between Atlantic and Mediterranean regions (Austin, 2004; Cagnon et al., 2004). Within the Palearctic clade, the separation among the three main subclades since the late Pleistocene matches major paleogeographic events during the last ice ages. Although Cory's shearwater would have colonized the Mediterranean in the early Pliocene, range contractions into Pleistocene refuges (Azores, Madeira and Canary Islands, as well as various Mediterranean Islands and coastlines) may have limited gene flow among populations. In such a scenario, allopatry during the Pleistocene ages together with the highly philopatric behaviour of the species (Rabouam et al., 1998) could have favoured isolation by distance. Furthermore, it is known that Pleistocene glacial cycles and associated ecological changes probably promoted changes in climate and oceanography, those that characterise the Mediterranean today (Longhurst, 1998). In this context, the geographic pattern of genetic differentiation among populations would have been reinforced by local adaptation to each of the two basins.

According to this study, the separation of the Cape Verde lineage occurred about 700,000 years ago, providing evidence that the clade has been in the archipelago at least this long. However, the apparently recent divergence between Cory's and the Cape Verde shearwater species contrasts with the noticeable morphological differentiation. It follows that once the islands were colonized, the species may have been differentiated through local adaptation to the new oceanographic environment. In general, body size among the procellariform species decreases towards the tropical areas, probably due to the lower productivity and the lower wind speed (Newton, 2003). Indeed, the Cape

Verde waters belongs to the Afro-tropical biogeographical region and exhibits subtropical oceanographic features clearly distinct from other Macaronesic Islands where the Atlantic Cory's shearwaters breed (Longhurst, 1998). The distinct oceanographic environment may explain incongruence between genetic and morphological patterns of differentiation as regards the two Cory's shearwater subspecies.

To summarize, the phylogeographic history of the *Calonectris* species complex suggests a strong geographic component to the cladogenesis among the main species and subspecies clades. Phylogeny indicates that *Calonectris* species evolutionary history involves a vicariant speciation resulting in the Pacific and Palearctic clades after the Panama Land Bridge formed. Within the Palearctic clade, the Cape Verde shearwater probably evolved as an endemic form by ecological divergence. Similarly, the Atlantic and Mediterranean clades would have evolved in allopatry by range contraction followed by a local adaptation to specific oceanographic conditions, during the Pleistocene ice ages. Indeed, the Almeria Oran Oceanographic Front apparently represents a more significant barrier between the two Cory's subspecies than the Gibraltar strait. Overall, these results suggest that marine environment and oceanographic fronts play a major role in the phylogeography of pelagic seabirds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.05.006.

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Appendix 1. The site matrix shows variable positions on a composite sequence of 1250 bp of the cytochrome b gene (1-956 bp) and the control region (957-1250 bp) in the 54 haplotypes found in all four *Calonectris* clades.

Haplotype	Sample Sequence	Nucleotide positions 1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 0000000001 1111111112 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
C. d. diomedea		
Hap_1	Tuscany1/Menorca1	ATATGACCGG GGATAGACAA GGCGAAAGGA GGTAGGGAGT ATATAGGGGT AGAAGGGAAA AAGGGGAGGT TTTATGAAGA GATGACGGTG AGATTATAGA GAGGGATAAT CAATATCAGA
Hap_2	Tuscany2	....T.. A......... ....A...G ...G.T.... ......G... ....G....... ......C... ...C......
Hap_3	Sardinia/Columbretes2	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_4	Linosa1	.........A .... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_5	Linosa2	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_6	Tremiti1	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_7	Tremiti2	.........A .... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_8	Crete1	....
Hap_9	Crete2	....
Hap_10	Corsica	.........A .... A......... ....A.... .....T.... ......G... ....A.. ..G....... ......C... ...C......
Hap_11	Hyeres1	.........A .... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_12	Hyeres2	.... A......... ....A.... G....T.... ......G... ....G....... ......C... ...C......
Hap_13	Chafarinas1	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_14	Chafarinas2	.........A .... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_15	Chafarinas3	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_16	Chafarinas4	.C........ .... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_17	Murcia1-3	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C....A.
Hap_18	Murcia4	....G..... A......... ....A.... ....
Hap_19	Mallorca1	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_20	Mallorca2	....
Hap_21	Cabrera1	.... A......... ....A.... ...G.T.... ......G... ....G....... ......C... ...C......
Hap_22	Cabrera2	.........A .... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_23	Ibiza1	.... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_24	Ibiza2	....A.... .....T.... ......G... ....G....... ......C... ...C....A.
Hap_25	Menorca2	.... A......... ....A.... ...G.T.... ......G... ....G....... ......C... ...C......
Hap_26	Columbretes1	.... A......... ....A.... ...G.T.... ......G... ....G...C... ......C... ...C......
Hap_27	Columbretes3	.... A......... ....A.... ...G.T.... ......G... ....G....... ......C... ...C......
Hap_28	Columbretes4	.........A .... A......... ....A.... .....T.... ......G... ....G....... ......C... ...C......
Hap_29	Almeria1	....G. ....A..... .....A.... ....G. ......G... ..CG...... ......A... ..G....... ......C... ..GC......
Hap_30	Almeria2	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... ......C... ..GC......
Hap_31	Almeria3	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... ......C... ..GC......
Hap_32	Almeria4	....A..... ....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....G.. ......C... ..GC......
Hap_33	Almeria5	....G. ....A..... .....A...G ....G. ......G... ..CG...... ......A... ..G....... ......C... ..GC......
C. d. borealis		
Hap_34	Lanzarote	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... ......C... ..GC....A.
Hap_35	Tenerife1-2/Madeira1-2	....A..... ....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....G.. ......C... ..GC......
Hap_36	G.Canaria1	....A..... ....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....G.. ......C... ..GC.....G
Hap_37	G.Canaria2	....A..... ....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....G.. ......C... ..GC......
Hap_38	St.Maria1	....A..... ....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....G.. ......C... ..GC.....G
Hap_39	St.Maria2	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....C.. ......C... ..GC......
Hap_40	Flores	....A..... ....G. ....A..... .....A.... ....G. ......G.A. ..CG...... ....G....G.. ......C... ..GC......
Hap_41	Faial	....A..... ....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....G.. ......C... ..GC....A.
Hap_42	Graciosa1	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... ......C... ..GC......
Hap_43	Graciosa2	....G. ....A..... .....A.... ....G. ......G... ..CG...... ..C....... ....C... ...G......
Hap_44	Corvo1	....A..... .....A.... ....G. ......G... ..CGC..... .... .TG....... ......C... ..GC......
Hap_45	Corvo2	....A..... ....G. ....A..... .....A.... ....G. ......G..C ..CG...... ....G....G.. ......C... ..GC......
Hap_46	Berlengas1	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... ......C... ..GC......
Hap_47	Berlengas2	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... A.....C... .GGC......
Hap_48	Selvagens1	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... ......C... ..GC......
Hap_49	Selvagens2	....G. ....A..... .....A.... ....G. ......G... ..CG...... ....G....... ......C... ..GC......
C. edwardsii		
Hap_50	Boavista01	.....G.... ....G. ....A..... ...C.A..A. ..G....... ......G... ....G....... .....GC... ...C......
Hap_51	Boavista04-10	.....G.... ....G. ....A..... .....A.... ..G....... ......G... ....G.G..... .....GC... ...C......
Hap_52	Boavista13-29	.....G.... ....G. ....A..... .....A.... ..G....... ......G... ....G.G..... .....GC.G. ...C......
Hap_53	Boavista15	.....G.... ....G. ....A..... .....A.... ..G....... ......G... ....G.G..... .....GC... ...C......
Hap_54	Boavista19	.....G.... ..GC....G. ....A..... .....A.... ..G....... ......G... ....G..G.... .....G.... ...C......
Hap_55	Boavista20-23	.....G.... ..GC....G. ....A..... .....A.... ..G....... ......G... ....G....... .....GC... ...C......
Hap_56	Boavista25	.....G.... ..GC....G. ....A..... .....A.... ..G....... ......G... ....G..G.... .....G.... ...C......
C .leucomelas		
Hap_57	Mikura11	.... A.....G.G. .AAA.....G ....AC ..G..A.A.. ...G..T... G..AA.GA.C C....A..A. A..A..A... ..G.....AG ......CG.. ...CT.....
Hap_58	Mikura13	.... A.....G.G. .AA......G ....A...AC ..G..A.A.. ...G..T... G..AA.GA.C .....A..A. A..A..A... ..G.....AG ......CG.. ...CT.....
P. puffinus		
Hap_59		G.GC..TTA. .A..GA..GG .AG.TGGAAG AACGAAAG.. GGG.G.AA.. GAG.A..G.G GGA.TAG..C .G.G.TGG.G .G..GT..CA GC.A.G.... .GAAAG..GG TG.GTATT..

Appendix 1 (Continued).

Haplotype	Sample Sequence	Nucleotide positions 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 111 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 999 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 123
C. d. diomedea		
Hap_1	Tuscany1/Menorca1	GAGATGGTGG ATACAGGAGG AGCGAGAGTG TAGGTCGAGG TGTCGGTGTT AAGTGAGAGA TAATTGGTTG AAG
Hap_2	Tuscany2	AG..... ......A... .A..... ..A..... .....C. ....A.A... ......A... .G.
Hap_3	Sardinia/Columbretes2	.G..... ......G.. .A..... ..A..... ..... G...A.... ......A... .G.
Hap_4	Linosa1	.G..... ..... .A..... ..A.C.... ....A.... ......A... .G.
Hap_5	Linosa2	.G...A.... ......G.. .A..... ..A..... ..... G...A.... ......A... .G.
Hap_6	Tremiti1	.G...A.... ......G.. .A..... ..A..... ..... G...A.... ......A... ...
Hap_7	Tremiti2	.G..... ..... .A..... ..A.C.... ....A.... ......A... ...
Hap_8	Crete1	.... .G.
Hap_9	Crete2	....G.. ....A.... ....
Hap_10	Corsica	.G..... ..... .A..... ..A.C.... ....A.... ....
Hap_11	Hyeres1	.G..... ..... .A..... ..A.C....A ....A.... ....
Hap_12	Hyeres2	....G.A ....A..... ..... G...A.... ......A.C. .G.
Hap_13	Chafarinas1	AG..... ......G.. .A..... ..A..... .A..... G...A.... ......A... .G.
Hap_14	Chafarinas2	.G..... ..... .A..... ..A..... ....A.... ......A... .G.
Hap_15	Chafarinas3	.G.....A .....A...A GA..... ..A..... ....A.... ......A.C. .G.
Hap_16	Chafarinas4	.G.....A ....G..G.A .A..... ..A.....A. .....C. G...A.... ..... .G.
Hap_17	Murcia1-3	.G..... ......G.. .A..... ..A..... ..... G...A..G.. ......A... .G.
Hap_18	Murcia4	.G.....A .....A...A .A..... ..A..... .....C ....A.... ......A... .G.
Hap_19	Mallorca1	.G...A...A ..... .A..... ..A..... ....A.... ......A... .G.
Hap_20	Mallorca2	.G.....A. ....GA.... ....A..... ..... G..... ..... .G.
Hap_21	Cabrera1	AG..... .....A... .A..... ..A..... ....A.A... ......A... .G.
Hap_22	Cabrera2	.G..... ..... .A..... ..A..... ....A.... ......A... ...
Hap_23	Ibiza1	.....A...A G.....G.. .A..G..... ..A..... ..... G...A.... ......A... .G.
Hap_24	Ibiza2	.G..... ......G.. .A..... ..A..... ..... G...A..G.. ......A... .G.
Hap_25	Menorca2	AG..... ..... .A..... ..A..... .....C. ....A.A... ......A... .G.
Hap_26	Columbretes1	.G.....A ..... .A..... ..A..... .....C. ....A.A... ......A... .G.
Hap_27	Columbretes3	AG..... .....A... .A..... ..A..... .....C. ....A.A... ......A... .G.
Hap_28	Columbretes4	.G..... ..... .A..... ..A.C.... ....A... .G.
Hap_29	Almeria1	.G.....T. .....A G..... ..CA.....A .A..... G..... .....T..C. .GA
Hap_30	Almeria2	.G.....T. .....A.G.. ....CA..... ..... G..... .....T..C. .G.
Hap_31	Almeria3	.G.....T. .....A G..... ..CA.....A .A....C... G.....A. ..G..T..C. .GA
Hap_32	Almeria4	AG.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..CA .G.
Hap_33	Almeria5	.....T. G.....A G..... ..CA.....A .A.....C. G..... .....T..C. .G.
C. d. borealis		
Hap_34	Lanzarote	AG.....T. .....A.G.. .A..G..... ..CA.....A .A.T..... G...A..... .....C..C. GG.
Hap_35	Tenerife1-2/Madeira1-2	.G.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..CA .G.
Hap_36	G.Canaria1	.G.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..CA .G.
Hap_37	G.Canaria2	.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..CA .G.
Hap_38	St.Maria1	.G.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..C. .G.
Hap_39	St.Maria2	.....T. .....A.GA. GA..... ..CA.....A .A..... G..... .....T..C. .G.
Hap_40	Flores	.G.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..CA .G.
Hap_41	Faial	.G.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..C. .G.
Hap_42	Graciosa1	.G.....T. .....A G..... ..CA.....A .A....C... G.....G .....T..C. .GA
Hap_43	Graciosa2	.....T. .....A.GA. G..... ..CA.....A .A..... G..... .....T..C. .G.
Hap_44	Corvo1	.G.....T. .....A.GA. GA..... ..CA.....A .A..... G..... .....T..C. .G.
Hap_45	Corvo2	.G.....T. .....A G..... ..CA.....A ..... G...AG.... .....T..CA .G.
Hap_46	Berlengas1	.G.....T. .....A.G.. ....CA.....A ..... G...AG.... .....T..CA .G.
Hap_47	Berlengas2	.....T. .....A G..... ..CA.....A ..... G..... .....T..C. .G.
Hap_48	Selvagens1	.....A..T. .....A.G.. GA..... ..CA.....A .A..... G..... ..G..T..C. .G.
Hap_49	Selvagens2	AG.....T. .....A G....A.... ..CA.....A CA..... G..... .....T..C. .G.
C. edwardsii		
Hap_50	Boavista01	.G.G....T. .....G.A .....AC. .....AGA. ..... G...A..... .....A.... .G.
Hap_51	Boavista04-10	.G.G....T. .....G.A .....AC. .....TAGA. ..... G...A..... .....A.... .G.
Hap_52	Boavista13-29	.G.G....T. .....G.. .A.....AC. .....TAGA. ..... G...A..... .....A.... .G.
Hap_53	Boavista15	.G.G..A.T. .....A .....AC. .....AGA. ..... G...A..... .....A.... .G.
Hap_54	Boavista19	AG.....T. G.....AG.A .....AC. .....GA. ...T..C... G...A..... .....A.... .G.
Hap_55	Boavista20-23	.G.G....TA G....A.G.. .....AC. .....AGA. .....C. G...A..... .....A.... .G.
Hap_56	Boavista25	.G.G....T. .....G.. .A.....AC. .....TAGA. ..... G...A..... .....A.... .G.
C. leucomelas		
Hap_57	Mikura11	.G..CAACT. .....A.G.. .A.....AC. C.CA.....A ..GTAA.A.. ....A.A..G CTGCG...C. .G.
Hap_58	Mikura13	.G..CAA.T. .....A.G.. .A..G..AC. C.CA.....A ..GTAA.A.. ....A....G CCGCG...C. .G.
P. puffinus		
Hap_59		.GC.CACCT. .CGT..T..A .ATAGTGT.A .GTA....AA .A.T.A.AC. GTTA..A.AT GT..GA.AA. .G.

Appendix 2. Morphological measurements for all *Calonectris* populations included in the biometric analysis. Values are means ± standard deviations. Sample size for each sex is shown in brackets (males; females).

Island population	Bill length		Culmen		Bill depth		Wing	
	Males	*Females*	*Males*	*Females*	*Males*	*Females*	*Males*	*Females*
St. Maria- Azores Is. (47; 47)	59.13 ± 1.60	56.67 ± 1.30	56.57 ± 1.79	53.06 ± 1.41	16.27 ± 0.89	14.58 ± 0.61	370.74 ± 6.46	362.77 ± 8.78
S. Miguel- Azores Is. (10; 4)	59.70 ± 0.97	57.72 ± 1.75	56.39 ± 1.37	53.50 ± 1.21	16.40 ± 0.50	14.82 ± 0.62	373.50 ± 10.27	366.50 ± 3.87
Graciosa- Azores Is. (13; 13)	59.66 ± 1.42	56.69 ± 1.23	55.59 ± 1.17	51.94 ± 1.77	15.56 ± 0.45	14.14 ± 0.49	375.85 ± 6.56	361.15 ± 5.81
Flores- Azores Is. (5; 3)	60.42 ± 1.40	57.88 ± 2.24	57.50 ± 1.80	54.02 ± 0.73	15.52 ± 0.87	14.53 ± 0.70	376.80 ± 7.19	365.67 ± 4.04
Corvo- Azores Is. (12; 10)	58.75 ± 1.28	56.64 ± 2.02	55.77 ± 2.09	53.34 ± 1.59	15.43 ± 0.84	13.98 ± 0.95	373.08 ± 6.58	364.30 ± 8.42
Faial- Azores Is. (8; 5)	59.59 ± 1.24	56.65 ± 0.95	55.57 ± 2.47	52.98 ± 1.86	15.71 ± 0.49	14.33 ± 1.09	373.38 ± 6.70	361.60 ± 6.50
Selvagens- Portugal[1] (52; 60)	57.00 ± 1.20	54.60 ± 1.30	55.50 ± 1.70	52.80 ± 1.80	17.31 ± 0.59	15.52 ± 0.50	363.00	358.00 ± 4.50
Madeira- Portugal (11; 17)	59.22 ± 1.60	56.92 ± 2.34	57.17 ± 1.54	52.86 ± 2.26	15.58 ± 0.84	14.08 ± 0.88	374.82 ± 7.59	362.71 ± 7.06
Berlengas- Portugal (10; 9)	59.40 ± 1.47	57.75 ± 1.97	57.06 ± 1.51	52.98 ± 1.85	16.01 ± 0.55	14.43 ± 0.71	374.20 ± 6.78	364.11 ± 6.17
G.Canaria- Canary Is. (81; 87)	57.82 ± 1.54	56.07 ± 1.20	55.43 ± 2.08	52.38 ± 1.69	15.56 ± 0.73	14.17 ± 0.74	367.78 ± 7.66	360.98 ± 6.86
Lanzarote- Canary Is. (15; 11)	59.28 ± 1.35	56.77 ± 1.42	55.00 ± 1.92	53.35 ± 2.42	15.56 ± 1.01	14.23 ± 0.70	374.53 ± 9.47	366.91 ± 0.62
Tenerife- Canary Is. (4; 5)	57.84 ± 1.21	55.97 ± 0.97	54.78 ± 0.63	51.65 ± 0.72	15.11 ± 0.81	13.50 ± 0.47	368.25 ± 12.76	360.40 ± 2.88
Almeria- Spain (13; 17)	58.63 ± 1.77	56.31 ± 1.54	53.32 ± 2.40	51.41 ± 2.08	15.41 ± 1.15	14.41 ± 1.58	372.83 ± 6.51	366.18 ± 5.14
Mean for *C .d. borealis* (13)	58.9 ± 6.94	56.67 ± 0.87	55.82 ± 1.13	52.79 ± 0.76	15.80 ± 0.58	14.36 ± 0.47	372.21 ± 3.84	363.17 ± 2.72
Chafarinas Is.- Spain (29; 30)	56.52 ± 2.02	54.67 ± 2.43	52.93 ± 2.04	50.76 ± 3.37	15.09 ± 0.87	13.53 ± 0.99	353.86 ± 8.08	347.52 ± 12.57
Murcia- Spain (9; 4)	57.18 ± 1.04	53.89 ± 1.03	52.75 ± 0.73	47.79 ± 1.13	14.13 ± 0.92	12.14 ± 0.23	353.67 ± 7.53	346.25 ± 4.19
Columbrets Is.- Spain[1] (40; 44)	56.40 ± 0.30	53.60 ± 0.20	52.40 ± 0.30	48.10 ± 0.20	13.50 ± 0.30	11.90 ± 0.10	360.00 ± 1.60	347.20 ± 1.10
Mallorca- Balearic Is. (15; 11)	55.22 ± 1.46	53.38 ± 2.08	51.01 ± 1.85	48.45 ± 1.52	14.13 ± 0.79	13.04 ± 0.93	352.53 ± 8.29	347.73 ± 5.53
Ibiza- Balearic Is. (23; 22)	55.61 ± 1.26	53.46 ± 1.11	51.22 ± 1.70	48.40 ± 1.12	13.93 ± 0.71	12.80 ± 0.52	360.04 ± 5.70	347.55 ± 5.84
Menorca- Balearic Is. (19; 23)	55.96 ± 1.43	53.29 ± 1.21	51.58 ± 2.57	48.13 ± 1.77	13.91 ± 0.88	12.42 ± 0.56	359.47 ± 7.22	350.26 ± 5.68
Cabrera- Balearic Is. (9; 10)	53.59 ± 1.61	53.96 ± 2.18	48.96 ± 2.05	48.61 ± 1.45	13.81 ± 0.64	12.67 ± 1.05	353.22 ± 7.34	350.70 ± 6.50
Linosa- Italy[1] (59; 55)	53.60 ± 1.80	52.80 ± 1.60	53.60 ± 3.20	50.60 ± 2.10	13.70 ± 0.70	12.00 ± 0.50	356.00 ± 9.30	345.00 ± 9.60
Sardinia- Italy[1] (63; 28)	55.63 ± 1.32	53.21 ± 1.19	52.01 ± 1.32	48.47 ± 1.55	13.98 ± 0.67	12.71 ± 0.44	355.17 ± 7.93	343.30 ± 6.83
Tremiti Is.- Italy[1] (24; 19)	55.57 ± 1.49	52.54 ± 7.33	52.12 ± 0.96	48.47 ± 1.66	13.93 ± 0.41	12.65 ± 0.48	356.04 ± 6.94	341.84 ± 7.33
Malta- Greece[1] (18; 17)	55.29 ± 2.60	51.97 ± 3.95	52.81 ± 1.49	48.57 ± 1.34	13.63 ± 0.61	12.30 ± 0.42	354.11 ± 5.10	340.41 ± 7.06
Crete- Greece (13; 4)	54.14 ± 1.35	52.03 ± 1.64	51.63 ± 1.07	47.79 ± 1.08	13.18 ± 0.34	12.14 ± 0.48	352.69 ± 6.03	336.25 ± 9.98
Corsica- France[1] (246; 183)	55.17 ± 1.49	52.98 ± 1.66	52.13 ± 2.11	48.70 ± 1.2	14.55 ± 1.51	12.85 ± 0.51	354.06 ± 6.95	342.80 ± 7.31
Hyeres- France[1] (10; 10)	54.76 ± 1.58	53.88 ± 1.36	51.61 ± 2.46	48.99 ± 1.15	14.40 ± 0.46	12.82 ± 0.41	355.00 ± 8.64	348.50 ± 9.34
Mean for *C .d. diomedea* (14)	55.33 ± 1.05	53.26 ± 0.76	51.91 ± 1.11	48.70 ± 0.90	13.99 ± 0.47	12.57 ± 0.45	355.42 ± 2.62	345.38 ± 4.04
C.edwardsii								
Boavista- Cape Verde (15; 11)	48.93 ± 1.36	46.68 ± 1.35	45.20 ± 0.78	43.12 ± 1.02	11.81 ± 0.88	10.35 ± 0.31	317.60 ± 5.60	307.64 ± 4.52
C. leucomelas[2,3] Kanmuri Is.- Japan (41; 41)	53.20 ± 1.10	50.90 ± 1.50	51.50 ± 1.70	48.10 ± 1.60	13.20 ± 0.70	11.60 ± 0.60	339.00	305.00

1 Thibault, J. C., Bretagnolle, V., and Rabouam, C., 1997. Cory's shearwater. In: BWP Update. Oxford University Press, Oxford, pp. 75-98.

2 Arima, H. and Sugawa, H., 2004. Correlation between the pitch of calls and external measurements of streaked shearwaters *Calonectris leucomelas* breeding on Kanmuri Island. Japan Journal of Ornithology. 53, 40-44.

3 Cramp, S. and Simmons, K. E. L., 1977. The birds of the Western Paleartic. Oxford University Press, London, UK.

Appendix 3a. Variable sites found in a fragment of 976bp of the mitochondrial cyt b gene in 69 *Calonectris* shearwaters and related taxa corresponding to 27 different island populations along the Mediterranean and the Atlantic region and one from the western Pacific. Populations sampled and geographic locations are shown on the left. Nucleotide positions relative to the beginning of the sequence are indicated by digits on the top. These sequences have been deposited in GenBank under Accession numbers (**DQ372022-DQ372050**).

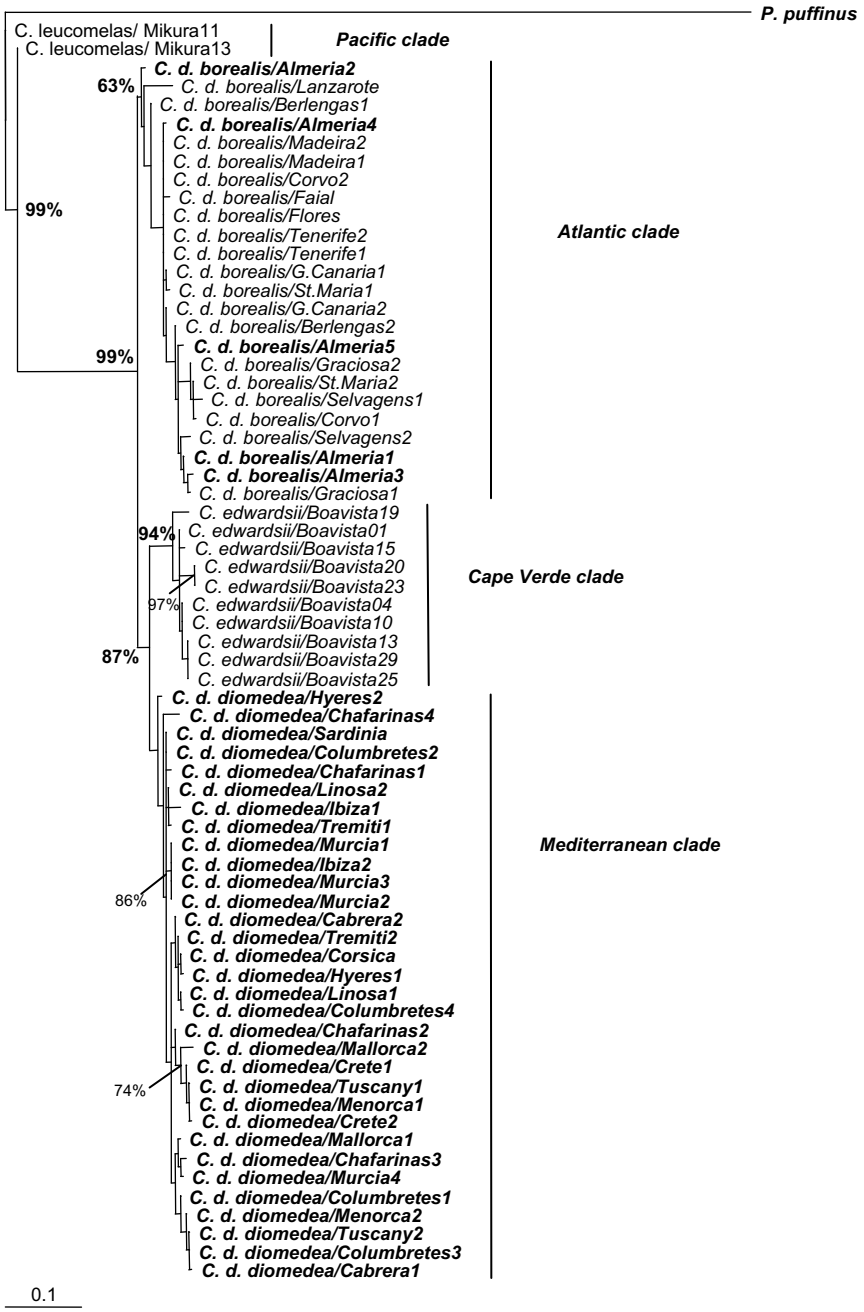
Nucleotide positions

Taxa	Samples	Geographic location	Haplotype	Accession Nos	1111111112222222222222223333333333333333344444444444444555555555555555566666667777777777888888888888888999999911223445567889902455568900011233567788900223555566778990123566677899011333445666899901245890012234679122245557888999034445542625703247578173770162302581694592346921978114581903257924043692810921624814925801581289524395140598910697359736925817023251
C. d. diomedea					
Tuscany1	Italy	1	DQ372022	ATGATGACCGGGGATAGACAGAGGGCGAAAGGAAGGTAGGGGAGTATATAGGGGTAGAAAGGGAAAAAGAAGGGAGGTTTTATGAAGAGATGACGGTGAGATTATAGAGAGGGGAGTAATCAAT
Tuscany2	"	2	DQ372023	.....T....A.....A...G....G.T.....G.....C.....
Sardinia	"	3	DQ372024	.....A.....T.....G.....C.....
Linosa1	"	4	DQ372025	.....A.....A.....T.....G.....C.....
Linosa2	"	3	DQ372024	.....A.....A.....A..T.....G.....C.....
Tremiti1	"	3	DQ372024	.....A.....A.....T.....G.....C.....
Tremitit2	"	4	DQ372025	.....A.....A.....A.....T.....G.....C.....
Crete1	Greece	1	DQ372022	.....
Crete2	"	1	DQ372022	.....
Corsica	France	5	DQ372026	.....A.....A.....A.....T.....G.....A....G.....C.....
Hyeres1	"	4	DQ372025	.....A.....A.....A.....T.....G.....C.....
Hyeres2	"	6	DQ372027	.....A.....A.....A...GGAA..T.....G.....C.....
Chafarinas1	Morocco coast	3	DQ372024	.....G.....A.A.....A.....T.....G.....C.....
Chafarinas2	"	4	DQ372025	.....A.....A.....A.....T.....G.....C.....
Chafarinas3	"	3	DQ372024	.....C.....A.....A.....T.....G.....C.....
Chafarinas4	"	7	DQ372028	.C.....C.....A.....A.....T.....G.....C.....
Murcia1	Spain	3	DQ372024	.....A.....A.....A.....T.....G.....C.....
Murcia2	"	3	DQ372024	.....C.....A.....A.....T.....G.....C.....
Murcia3	"	3	DQ372024	.....C.....A.....A.....T.....G.....C.....
Murcia4	"	8	DQ372029	.....C.....G.....A.....A.....
Mallorca1	Balearic Is	3	DQ372024	.....A.....A.....A.....T.....G.....C.....
Mallorca2	"	1	DQ372022	.....G.....
Cabrera1	"	9	DQ372030	.....A.....A.....AG.T.....G.....C.....
Cabrera2	"	4	DQ372025	.....A.....A.....A.....T.....G.....C.....
Ibiza1	"	3	DQ372024	.....A.....A.....A.....T.....G.....C.....
Ibiza2	"	10	DQ372031	.....A...TAGAAA.T.....G.....C.....
Menorca1	"	1	DQ372022	.....
Menorca2	"	9	DQ372030	.....A.....A.....G.T.....G.....C.....
Columbretes1	Spain	11	DQ372032	.....A.....A.....G.T.....G...C.....C.....
Columbretes2	"	3	DQ372024	.....A.....A.....A..T.....G.....C.....
Columbretes3	"	9	DQ372030	.....A.....A.....G.T.....G.....C.....
Columbretes4	"	4	DQ372025	.....A.....A.....A.....T.....G.....C.....
Almeria1	"	12	DQ372033	.....G.....A.....A.....G.....G....CG.....A....G.....C.....GC
Almeria2	"	13	DQ372034	.....AC.....G.....A.....A.....G.....G....CG.....G.....C.....GC
Almeria3	"	12	DQ372033	.....AC.....G.....A.....A.....G.....G....CG.....A....G.....C.....GC
Almeria4	"	14	DQ372035	.....A.C.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
Almeria5	"	15	DQ372036	.....C.....G.....A.....A...G.....G.....G....CG.....A....G.....C.....GC
C. d. borealis				
Lanzarote	Canary Is	13	DQ372034	.....G.....A.....A.....G.....G....CG.....G.....C.....GC
Tenerife1	"	14	DQ372035	.....A.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
Tenerife2	"	14	DQ372035	.....A.....G.....A.G.....A.....G.....G....CG.....G....G.....C.....GC
G.Canaria1	"	14	DQ372035	.....A.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
G.Canaria2	"	14	DQ372035	.....A.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
St.Maria1	Azores Is	14	DQ372035	.....A.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
St.Maria2	"	16	DQ372037	.....G.....A.....A.....G.....G....CG.....G....C.....C.....GC
Flores	"	17	DQ372038	.....A.....G.....A.....A.....G.....G.A...CG.....G....G.....C.....GC
Faial	"	14	DQ372035	.....A.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
Graciosa1	"	13	DQ372034	.....G.....A.....A.....G.....G....CG.....G.....C.....GC
Graciosa2	"	18	DQ372039	.....G.....A.....A.....G.....G....CG.....C.....C.....G.
Corvo1	"	19	DQ372040	.....A.....A.....A.....G.....G....CGC.....TG.....C.....GC
Corvo2	"	20	DQ372041	.....AAC.....G.....A.....A.....G.....G..C..CG.....G....G.....C.....GC
Berlengas1	Portugal	13	DQ372034	.....G.....A.....A.....G.....G....CG.....G.....C.....GC
Berlengas2	"	21	DQ372042	.....G.....A.....A.....G.....G.....G....CG.....G.....A.....C....GGC
Selvagens1	"	13	DQ372034	.....G.....A.....A.....G.....G....CG.....G.....C.....GC
Selvagens2	"	13	DQ372034	.....G.....A.....A...TA.....G.....G....CG.....G.....C.....GC
Madeira1	"	14	DQ372035	.....A.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
Madeira2	"	14	DQ372035	.....A.....G.....A.....A.....G.....G....CG.....G....G.....C.....GC
C. edwardsii				
Boavista29	Cape Verde	22	DQ372043	.....G.....G.....A.....A.....G.....G.....G.G.....G.C.G....C
Boavista15	"	23	DQ372044	.....G.....G.....A.....A.....G.....G.....G.G.....G.C.....C
Boavista19	"	24	DQ372045	.....G.....GC....G.....A.....A.....G.....G..G.....G.....C
Boavista20	"	25	DQ372046	.....G.....GC....G.....A.....A.....G.....G.....G.....G.C.....C
Boavista23	"	25	DQ372046	.....G.....GC....G.....A.....A.....G.....G.....G.....G.C.....C
Boavista13	"	22	DQ372043	.....G.....G.....A.....A.....G.....G.....G.G.....G.C.G....C
Boavista25	"	24	DQ372045	.....G.....GC....G.....A.....A.....G.....G..G.....G.....C
Boavista04	"	23	DQ372044	.....G.....G.....A.....A.....G.....G.....G.G.....G.C.....C
Boavista10	"	23	DQ372044	.....G.....G.....A.....A.....G.....G.....G.G.....G.C.....C
Boavista01	"	26	DQ372047	.....G.....G.....A.....C.A..A....G.....G.....G.C.....C
C. leucomelas				
Mikura11	Japan	27	DQ372048	.....A.....G.G....AAA.....G.....AC..G..A.A.....G..T...G....AA.GA.CC....A..A.A..A..A.....G.....AG.....CG.....C
Mikura13	"	28	DQ372049	.....A.....G.G....AA.....G.....A....AC..G..A.A.....G..T...G....AA.GA.C....A..A.A..A..A.....G.....AG.....CG.....C
P. puffinus		29	DQ372050	G..GC..TTA..A..GA..G.G..AG.TGGAAG.AACGAAA.G..GGG.G.AA..GA.G.A..G.GGA...TAG..C.G.G.TGG.G.G..GT..CAGC.A.G.....GA.AAG...GGTG.G

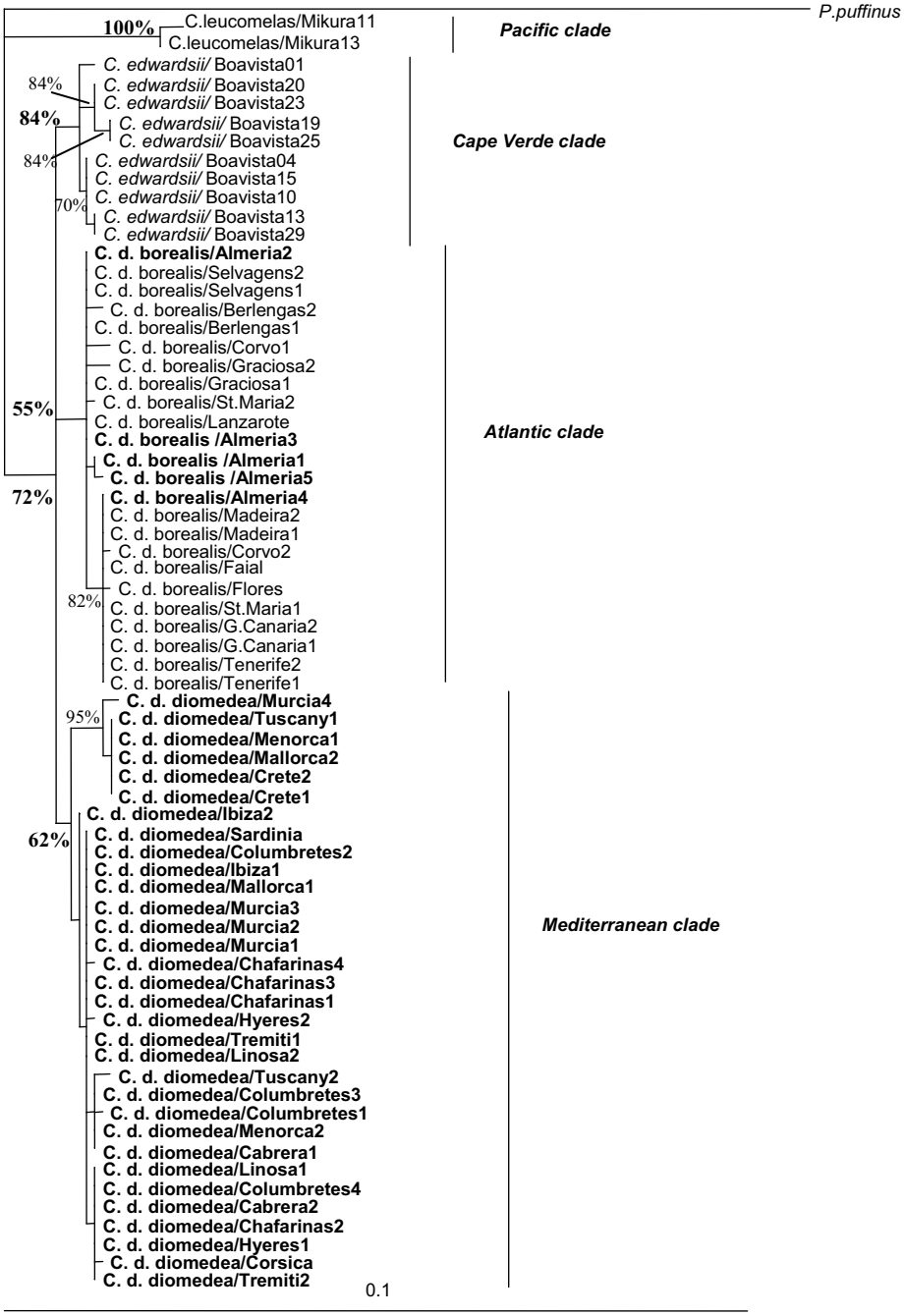
Appendix 3b. Variable sites found in a fragment of 293 bp of the domain I of the mitochondrial control region. These sequences have been deposited in GenBank under Accession numbers (**DQ371968-DQ372021**).

Taxa	Samples	Geographic location	Haplotype	Accession Nos	Nucleotide positions
					1111111111111111111111111111111111111111112222222222222222222222222222
					4566778999000001112222333444444555556666777778888111122333345555666667777888
					3262624905905678015678934501345812578457901349035734570745673678923789123434 93
C. d. diomedea					
	Tuscany1	Italy	1	DQ371968	ATCAGAGAGATGGTGGATACAGGAGGAGCGAGAGTGTAGGTCGAGGTGTCGGTGTTAAGTGAGAGATAATTGGTTGAAG
	Tuscany2	"	2	DQ371969	......AG.............A....A..........A.............C.....A.A.........A....G.
	Sardinia	"	3	DQ371970	.......G.............G...A..........A.................G...A..........A....G.
	Linosa1		4	DQ371971	.......G.................A..........A.C.................A..........A....G.
	Linosa2	"	5	DQ371972	.......G...A..........G...A..........A.................G...A..........A....G.
	Tremiti1	"	6	DQ371973	.......G...A..........G...A..........A.................G...A..........A.....
	Tremiti2	"	7	DQ371974	.......G.................A..........A.C.................A..........A.....
	Crete1	Greece	8	DQ371975	....G.
	Crete2	"	9	DQ371976	....................G...
	Corsica	France	10	DQ371977	.......G.................A..........A.C.................A.....
	Hyeres1	"	11	DQ371978	.......G.................A..........A.C....A.............A.....
	Hyeres2	"	12	DQ371979	....................G.A..........A.....................G...A..........A.C..G.
	Chafarinas1	Morocco coast	13	DQ371980	......AG.............G...A..........A.........A.......G...A..........A....G.
	Chafarinas2	"	14	DQ371981	.......G.....................A..........A.....................A..........A....G.
	Chafarinas3	"	15	DQ371982	.......G......A.....A...AGA..........A.....................A..........A.C..G.
	Chafarinas4	"	16	DQ371983	.......G......A....G..G.A.A..........A.....A.........C.G...A.....
	Murcia1	Spain	17	DQ371984	....A..G.............G...A..........A.....................G...A..G.....A....G.
	Murcia2	"	17	DQ371984	....A..G.............G...A..........A.....................G...A..G.....A....G.
	Murcia3	"	17	DQ371984	....A..G.............G...A..........A.....................G...A..G.....A....G.
	Murcia4	"	18	DQ371985	.......G.....A.....A...A.A..........A.....................C....A.....A....G.
	Mallorca1	Balearic Is	19	DQ371986	.......G...A...A.........A.A..........A.....................A..........A....G.
	Mallorca2	"	20	DQ371987	.......G.....A.....GA.....................A.....................G.....
	Cabrera1	"	21	DQ371988	......AG.............A....A..........A.....................A.A.....A....G.
	Cabrera2	"	22	DQ371989	.......G.....................A..........A.....................A.....
	Ibiza1	"	23	DQ371990	..........A...AG......G...A..G.......A.....................G...A..........A....G.
	Ibiza2	"	17	DQ371984	....A..G.............G...A..........A.....................G...A..G.....A....G.
	Menorca1	"	1	DQ371968	....
	Menorca2	"	24	DQ371991	......AG.....................A..........A.....................C.....A.A.....A....G.
	Columbretes1	Spain	25	DQ371992	.......G......A.....................A..........A.....................C.....A.A.....A....G.
	Columbretes2	"	3	DQ371970	......G.....................G...A..........A.....................G...A..........A....G.
	Columbretes3	"	2	DQ371969	......AG.....................A....A..........A.....................C.....A.A.....A....G.
	Columbretes4	"	26	DQ371993	.......G.....................A..........A.C.....................A.....
	Almeria1	"	27	DQ371994	.......G.....T..........AG.....CA.....A.A.....G.....T..C..GA
	Almeria2	"	28	DQ371995	.......G.....T......A.G.....CA.....G.....T..C..G.
	Almeria3	"	29	DQ371996	.......G.....T..........AG.....CA.....A.A....C...G.....A...G..T..C..GA
	Almeria4	"	30	DQ371997	......AG.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
	Almeria5	"	31	DQ371998	.............T.G.....AG.....CA.....A.A.....C.G.....T..C..G.
C. d. borealis					
	Lanzarote	Canary Is	32	DQ371999	....A.AG.....T.....A.G...A..G.....CA.....A.A.T.....G...A.....C..C.GG.
	Tenerife1	"	33	DQ372000	.......G.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
	Tenerife2	"	33	DQ372000	.......G.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
	G.Canaria1	"	34	DQ372001	.....G.G.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
	G.Canaria2	"	35	DQ372002	.............T..........AG.....CA.....A.....G...AG.....T..CA.G.
	St.Maria1	Azores Is	36	DQ372003	.....G.G.....T..........AG.....CA.....A.....G...AG.....T..C..G.
	St.Maria2	"	37	DQ372004	.............T......A.GA.GA.....CA.....A.A.....G.....T..C..G.
	Flores	"	33	DQ372000	......G.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
	Faial	"	38	DQ372005	....A..G.....T..........AG.....CA.....A.....G...AG.....T..C..G.
	Graciosa1	"	39	DQ372006	.......G.....T..........AG.....CA.....A.A....C...G.....G.....T..C..GA
	Graciosa2	"	40	DQ372007	.............T......A.GA.G.....CA.....A.A.....G.....T..C..G.
	Corvo1	"	41	DQ372008	.......G.....T......A.GA.GA.....CA.....A.A.....G.....T..C..G.
	Corvo2	"	33	DQ372000	A.....G.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
	Berlengas1	Portugal	42	DQ372009	.......G.....T......A.G.....CA.....A.....G...AG.....T..CA.G.
	Berlengas2	"	43	DQ372010	.............T..........AG.....CA.....A.....G.....T..C..G.
	Selvagens1	"	44	DQ372011	..........A..T......A.G..GA.....CA.....A.A.....G.....G..T..C..G.
	Selvagens2	"	45	DQ372012	......AG.....T..........AG....A.....CA.....ACA.....G.....T..C..G.
	Madeira1	"	33	DQ372000	.......G.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
	Madeira2	"	33	DQ372000	.......G.....T..........AG.....CA.....A.....G...AG.....T..CA.G.
C. edwardsii					
	Boavista01	CapeVerde	46	DQ372013	A......G.G....T.....G.A.....AC.....AGA.....G...A.....A.....G.
	Boavista04	"	47	DQ372014	.......G.G....T.....G.A.....AC.....TAGA.....G...A.....A.....G.
	Boavista10	"	47	DQ372014	.......G.G....T.....G.A.....AC.....TAGA.....G...A.....A.....G.
	Boavista13	"	48	DQ372015	.......G.G....T.....G...A.....AC.....TAGA.....G...A.....A.....G.
	Boavista15	"	49	DQ372016	.......G.G..A.T.....A.....AC.....AGA.....G...A.....A.....G.
	Boavista19	"	50	DQ372017	......AG.....T.G....AG.A.....AC.....GA....T..C...G...A.....A.....G.
	Boavista20	"	51	DQ372018	.......G.G....TAG....A.G.....AC.....AGA.....C.G...A.....A.....G.
	Boavista23	"	51	DQ372018	.......G.G....TAG....A.G.....AC.....AGA.....C.G...A.....A.....G.
	Boavista25	"	48	DQ372015	...A...G.G....T.....G...A.....AC.....TAGA.....G...A.....A.....G.
	Boavista29	"	48	DQ372015	.......G.G....T.....G...A.....AC.....TAGA.....G...A.....A.....G.
C. leucomelas					
	Mikura11	Japan	52	DQ372019	T..A...G..CAACT......A.G...A.....AC.C.CA.....A..GTAA.A......A.A..GCTGCG...C..G.
	Mikura13	"	53	DQ372020	T..A...G..CAA.T......A.G...A..G..AC.C.CA.....A..GTAA.A......A....GCCGCG...C..G.
P. puffinus			54	DQ372021	TATT...GC.CACCT..CGT..T..A.ATAGTGT.A.GTA....AA.A.T.A.AC.GTTA..A.ATGT..GA.AA..G.

Appendix 4a. Maximum Likehood phylogenetic tree for *Calonectris* shearwaters based on 293 bp of the mtDNA domain I of the control region. Mediterranean, Atlantic and Pacific populations are indicated in bold, italics and normal font, respectively. Numbers adjacent to branches are maximum parsimony bootstrap support values greater than 70%. Species and geographic location for each sequence is indicated in the phylogenetic tree.



Appendix 4b. Maximum Likehood phylogenetic tree for *Calonectris* shearwaters based on 976 bp of the mtDNA cytochrome b gene. Mediterranean, Atlantic and Pacific populations are indicated in bold, italics and normal font, respectively. Numbers adjacent to branches are maximum parsimony bootstrap support values greater than 70%. Species and geographic location for each sequence is indicated in the phylogenetic tree.



Population genetic structure in Cory's shearwaters *Calonectris diomedea*: Isolation by distance vs. habitat type

Elena Gómez-Díaz, Jacob González-Solís, Miguel Ángel Peinado

(Submitted)

Capítol 2

A nivell microevolutiu, la dinàmica de les interaccions hoste- paràsit pot dependre en ultima instancia de la variabilitat genètica i de la seva estructuració en ambdós grups. Per tant, una vegada coneixíem la filogènia de l'hoste, el següent pas era un estudi detallat de l'estructuració genètica de les poblacions de baldriga cendrosa al llarg de tot el seu rang de distribució, així com idenficar-ne els factors causals. Per això en aquest article, en primer lloc investiguem com la variabilitat genètica està distribuïda entre i dintre de cadascuna de les dues subespècies i avaluem els nivells de diferenciació genètica i flux gènic entre les poblacions. En segon lloc, examinem si diversos factors com la geografía, les característiques oceanogràfiques i la dispersió entre colònies expliquen els patrons de variabilitat genètica en la baldriga cendrosa.

Estructuració genètica de poblacions en la baldriga cendrosa *Calonectris diomedea*: Aïllament per distancia vers el tipus d'hàbitat

No només la distribució geogràfica de les poblacions sinó també les característiques oceanogràfiques del habitat marí poden jugar un paper molt important influenciant l'estructuració genètica de les poblacions en els ocells marins. En primer lloc, varem avaluar l'estructuració genètica de la baldriga cendrosa combinant dades moleculars i ecològiques de 27 colònies distribuïdes al llarg del Mediterrani (*Calonectris d. diomedea*) i l'Atlàntic (*C. d. borealis*). Segon, varem testar si la diferenciació genètica coincidia amb els patrons geogràfics i oceanogràfics de variabilitat al llarg de tot el rang de distribució de l'espècie. Els anàlisis AMOVA i Network suggerien una forta estructuració genètica entre les poblacions atlàntiques i mediterrànies de baldriga cendrosa. No obstant, varem identificar dos possibles immigrants de l'Atlantic criant al Mediterrani així com dos casos d'introgressió. Dins de cada regió, tot i ser feble, existia certa estructuració genètica en arxipèlags dins de l'Atlàntic però no en el Mediterrani. Tot i que les dades d'anellament i recuperacions assenyalaven un comportament molt fidel a la colònia de cria, les estimes de flux gènic suggerien unes taxes de dispersió importants entre colònies dins de cada regió. Dintre de l'Atlàntic, els resultats recolzaven un model d'aïllament per distància, mentre que en el Mediterrani la distància per si mateixa no explicava els patrons de variabilitat genètica observats. Contrariament, l'oceanografia si semblava jugar un paper destacat.

Population genetic structure in Cory's shearwaters *Calonectris diomedea*: Isolation by distance vs. habitat type

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Running title

Population genetic structure in Cory's shearwaters

Abstract

Not only geographic distributions of populations but also oceanographic habitat can play an important role in shaping genetic population structure in seabirds. First, we evaluated the genetic structure of Cory's shearwater combining molecular and ecological data from 27 breeding colonies distributed across the Mediterranean (*Calonectris d. diomedea*) and the Atlantic (*C. d. borealis*). Second, we assessed whether genetic differentiation agreed with geographic and oceanographic patterns of variation across the whole range. AMOVA and Network analyses suggested a strong genetic differentiation between Atlantic and Mediterranean Cory's shearwater populations. However, we identified two possible immigrants from the Atlantic breeding in the Mediterranean and two other cases of introgression were apparent. Within each region we detected a weak genetic substructure into archipelagos within the Atlantic but not within the Mediterranean subspecies. Despite ringing-recovery data indicated high site fidelity behaviour of the species, gene flow estimates suggested substantial dispersal events among colonies within regions. Within the Atlantic, results supported isolation by distance model, while within the Mediterranean distance alone did not explain observed patterns of genetic variation. Instead, some influence of oceanography was apparent.

Introduction

In principle the open ocean offers a vast landscape with few physical barriers to dispersal compared to terrestrial environments. This is particularly true for seabirds, which can easily travel vast transoceanic distances (González-Solís et al., 2007). Yet, conspecific seabird populations show obvious geographic differences in vocalizations, body size and plumage in most species (Del Hoyo et al., 1992; Del Hoyo et al., 1996). These differences should also be reflected at genetic level, since widespread behaviours among seabirds such as monogamy, strong philopatry and great fidelity to their breeding colony and nest sites, should promote genetic differentiation (Austin et al., 1994, Bried et al., 2002). However, evidences from genetic data are controversial, showing a substantial variation in the extent of population structure among different species (see review in Friesen et al., 2007).

Several factors have been suggested to play an important role in shaping genetic population structure in seabirds (Friesen et al., 2007). Two major not mutually exclusive factors include the geographic distance among colonies and the oceanographic features. Despite the potential mobility of pelagic seabirds, breeding on remote oceanic islands may ultimately lead to genetic differentiation. Under the isolation by distance model, if gene flow is related to the distance among the populations, we would expect a geographic cline in the genetic variation among populations (Rousset, 1997). Alternatively, recent evidences suggest oceanographic divides may also act as barriers to gene flow in a wide array of marine organisms (Natoli et al., 2005; Fullard et al., 2000; Jorgersen et al., 2005; Schmelzer, 2000; Zardus et al., 2006), including seabirds. Indeed, breeding distributions in some seabirds appear directly related to marine environmental traits (Zotier et al., 1999), suggesting that different habitat preferences may act as an isolating mechanism. Colony size and connectivity among populations may be somehow determined by local (colony size), and regional (colony distribution) oceanographic features of the marine habitat. Ultimately, if selection to local oceanographic characteristics occurs, we would expect some similarity between the spatial patterns of the genetic and the environmental variation.

In this study we tested these hypotheses on Cory's shearwater, a pelagic seabird species breeding on islands across the NE Atlantic and the Mediterranean. Currently, the Cory's shearwater *Calonectris diomedea* comprises the Atlantic (*C. d. borealis*) and Mediterranean (*C. d. diomedea*) subspecies. Even though the Mediterranean and the Atlantic Ocean are contiguous basins, their physical and oceanographic characteristics are clearly distinct (Longhurst, 1998). The Mediterranean where the Mediterranean (Cory's shearwater) subspecies breeds, is small and the oceanography is complex with several oceanographic discontinuities throughout its oceanic area (Zotier et al., 1999; Bricaud et al., 2002). Appropriate islands for breeding shearwaters are relatively numerous and distances among them are small. Within the North-East Atlantic, where the Atlantic subspecies breeds, habitat diversity is lower with only a few obvious oceanographic breaks (Longhurst, 1998). However, distances among populations are large and thus the effect of geographic isolation may be stronger. In view of these distinct features, we would expect some differential effects of geography and oceanography in shaping genetic structure of the species at each basin.

Both ecological and genetic data suggest population structure in Cory's shearwater may be strong. Mark-recapture data indicate a high philopatric and site fidelity behaviour, although some birds have been recovered far away from their natal colonies (Thibault et al., 1997). Nevertheless, the pattern and the extent of those movements still remain unknown. So far, in addition to marked differences in colour, size and vocalizations, the genetic differentiation between the two Cory's shearwater subspecies has been proved (Randi et al., 1989, Wink et al., 1993, Heidrich et al., 1998, Gómez-Díaz et al., 2006). Within each subspecies a geographic variation in morphology has been found (Granadeiro, 1993, Gómez-Díaz et al., 2007), but genetic studies based on allozymes, mtDNA and minisatellite DNA fingerprint failed to detect any spatial structuring of populations (Randi et al., 1989, Wink et al., 1993, Heidrich et al., 1996, Carneiro da Silva et al., 1999). In contrast, recent molecular work on microsatellites revealed some genetic structuring within the Mediterranean subspecies (Rabouam et al., 2000). However, all previous studies are restricted to a single region (i.e. Mediterranean or Atlantic) and cover a small portion of the distribution range of the species. In this context, a re-evaluation

of the genetic structure of Cory's shearwater populations across its whole distribution would provide a more reliable assessment of the patterns of differentiation and also help understanding mechanism of genetic differentiation in the species.

Here we present a comprehensive approach combining genetic and ecological data to assess the existence of spatial patterns of genetic variation across the whole breeding range of Cory's shearwater. In particular we aim, i) to investigate how genetic variation is distributed between and within subspecies, ii) to evaluate levels of genetic differentiation and gene flow among colonies, and finally iii) to examine whether geographic distances, oceanographic characteristics or dispersal patterns among colonies correlate with patterns of genetic structure in Cory's shearwater.

Materials and Methodology

Study species and sampling

Cory's shearwaters breed on islands across the Mediterranean from the Iberian coast to the Adriatic and Aegean (mainly *C. d. diomedea*), and in the northeast Atlantic, from Canary to Azores archipelagos (mainly *C. d. borealis*) (Gómez-Díaz et al., 2006, Thibault et al., 1998). From 2001 to 2006 we collected blood samples from adult birds from 27 breeding colonies of Cory's shearwater across the Mediterranean and the Atlantic region (Fig. 1).

To evaluate patterns of genetic differentiation in Cory's shearwater, we designed a hierarchical analysis of the population structure. We considered three spatial scales: first at global scale we analysed the genetic relationships between the two main taxa (the Atlantic and Mediterranean Cory's shearwaters). At a regional scale, we grouped geographically neighbour island colonies into Archipelagos for each taxa (i.e. Balearic, Azores and Canary archipelagos). Finally, at a local scale, we considered single breeding colonies.

Genetic analyses

DNA isolation, amplification and sequencing

DNA was isolated from ethanol-preserved whole blood using the salting-out extraction protocol from Bruford et al., 1998). We amplified a 293 bp fragment of Domain I of the mitochondrial control region of *Calonectris* using three specific primers that had been designed previously for the species (Gómez-Díaz et al., 2006): either CAL2H (5'CATCCCATCCAACTTAAG3') or CAL4H (5'AGCCTATGTATGGATGTGCAT3') was used in conjunction with CAL1L (5'GGTCCTGAAGCTAGTAATAC3'). Reaction conditions and automated sequencing were those described by Gómez-Díaz et al. (2006). Representative sequences for each breeding colony are available in GenBank (accession nos. *DQ371968-DQ372018*). A total of 241 samples were included (Fig. 1). Out of these, X samples were analysed in this study for the first time and they were compared with previously analysed samples (Gómez-Díaz et al., 2006) for the same loci.

Population genetics analyses

To test whether genetic variation deviated from neutral expectations, we performed Ewens-Wattersons and Chakrabortys tests (Chakraborty, 1990; Ewens, 1972; Watterson, 1978) using ARLEQUIN 3.0 (Excoffier, 2005). We calculated haplotypic diversity (H_s; Nei 1987) and nucleotide diversity (π; Nei 1987) to assess the level of genetic variation within each taxa, archipelagos and breeding colonies. We also calculated Φ_{ST} statistics (Kimura-2-parameters mutation model) among all pairwise of breeding colonies.

We evaluated population genetic structure based on the F_{ST} estimates or Wright's fixation index of population differentiation using the nested analysis of molecular variance (AMOVA) (Excoffier et al., 1992) included in ARLEQUIN 3.0 (Excoffier, 2005). We conducted two AMOVA analyses. First, by taxa region, we defined two groups of breeding colonies corresponding to each Cory's shearwater subspecies. Secondly, AMOVA analysis was conducted separately for the Mediterranean and the Atlantic subspecies, grouping breeding

colonies into archipelagos. F_{ST} estimates were tested for significance with 10,000 randomizations of the data.

To visualize the genetic relationships among colonies, we generated a population tree on Φ_{ST} pairwise distances (Kimura's 2 parameters mutation model) using SplitsTree v4.6 (Huson et al., 2006). In addition, haplotype trees were also constructed using Neighbour-Net algorithm implemented in SplitsTree.

Dispersal pattern and gene flow

We estimated gene flow between the Atlantic and the Mediterranean subspecies (M, in females per generation) using a non-equilibrium method based on coalescent theory and Bayesian statistics implemented in MDIV (Nielsen et al., 2001). We ran MDIV under the finite sites mutation model (Hasegawa et al., 1985) with three chains (length of Markov chain = 5,000,000 cycles; burn-in time = 500,000 cycles), and M_{max} and T_{max} set at 30 and 10 respectively. To test whether M or T were significantly different from zero, we used a maximum likelihood test as described in Nielsen and Wakeley (2001). Then, within each subspecies, we nested population trees based on Φ_{ST} pairwise estimates (Excoffier et al., 1992) following the rules of Templeton, 1998. That is, we pooled colonies into groups that appeared to exchange genes freely with each other but not with other such groups, and ran MDIV on the resulting nested clades. Parameters set were the same described above.

Spatial patterns of genetic variation

To investigate the existence of spatial patterns in the genetic structure of the species, we first examined isolation by distance model by measuring the correlation of genetic distances, either as pairwise Φ_{ST} or Slatkin's linearized estimates of Φ_{ST} (Φ_{ST} / (1- Φ_{ST})), with by-sea geographic distances of colony pairs (Rousset, 1997). Secondly, we examined habitat mediated differentiation by correlating genetic distances with distances in oceanographic features as indicated by differences in three oceanographic variables: chlorophyll, salinity and sea surface temperature (calculated as Euclidean distances of colony pairs). In each case, either considering

geographic and oceanographic distances across the breeding range of the study species, we first applied Multidimensional Scaling of dissimilarity data by PROXCAL analysis using SPSS 12.0. The method tries to find the underlying structure in a set of proximity measures among objects that are arranged in a two-dimensional space. Then we applied Procrustes analysis (least-squares orthogonal mapping) a non parametric approach for the comparison of the two kinds of non-linearized data sets (Jackson, 1995; Peres-Neto et al., 2001). The method is based on matching corresponding points (landmarks) from each of the two data sets, and provides a measure of fit (m^{12}), which decreases with an increasing correlation. The two sets of landmarks correspond to the two-dimensional genetic, geographic or oceanographic coordinates of each colony calculated using PROXCAL. The significance test of the m^{12} statistic was determined by employing a randomization approach to one of the data sets (Protest). Procrustes and Protest analyses were first carried out including all breeding colonies. In a second stage, we analyzed Atlantic and Mediterranean Cory's shearwater breeding colonies separately. Finally, we tested a combined effect of the oceanographic characteristics and the geographic distributions of breeding colonies influencing the genetic structure of the species. We combined the two matrices, oceanographic and geographic distances, using PROXCAL to compute the two-dimensional coordinates, and performed Procrustes analysis measuring association between the two ecological proxies together with Φ_{ST} genetic distances of colony pairs.

Biometric data

We employed principal component analysis (SPSS 12.0) to assess differences in morphometrics over the species range. We included four biometrical measures; tarsus, wing and bill length and bill depth at nostril, of 158 sexed individuals out of those included in the genetic analyses. To avoid the effect of sexual size dimorphism, morphometric variables between males and females were standardized by subtracting the mean value of each sex.

Mark- recovery data

To assess movements of birds and dispersal rates, we compiled ringing and recovery data of the Cory's shearwater from the Balearic and the Canary Archipelagos as well as from different Mediterranean Island populations across the coast of Spain. We analyzed the ringing and the recovery data of 1050 birds ringed in Spain from 1972 to 2002. We used the SPSS 12.0 package to calculate the distance between the ringing and the corresponding recovery latitude and longitude geographic positions. Only those birds re-sighted thorough the breeding period (from April to October) were included in the analysis. The distance and the direction of the movements were considered to measure the interconnectivity among Cory's shearwater populations. We represented ringing and re-sighting records as well as the corresponding distances of dispersing birds using Arc view.

Oceanographic data

Data on sea surface salinity was obtained from the National Oceanographic Data Centre (NODC; http://www.nodc.noaa.gov/). Data on chlorophyll production and sea surface temperature were obtained from the NASA Sea-viewing Wide Field-of-view Sensor database (SeaWiFS;http://seawifs.gsfc.nasa.gov/) and the NASA Aqua- MODIS satellite database (MODIS;http://modis.gsfc.nasa.gov/). Satellite tracking data on Cory's shearwaters suggested they feed within a foraging range up to 500km from the breeding colony (Navarro and González-Solís, unpublished). Thus, to define the oceanographic habitat of each breeding colony we estimated mean values of the three oceanographic variables during the breeding season of the species from April to September, from 2003 to 2006 within a radius of 100, 300 and 500 km of the colony.

Results

Sequence data

The results of Ewens-Watterson's and Chakraborty's tests were not significant (All P > 0.10) showing that the DNA sequences do no depart from neutrality expectations. Mitochondrial sequences were highly variable: among the 241 sequences of 27 colonies, there were 55 polymorphic out of 293 sites, 53 transitions and 4 transversions (Appendix 1). We found 134 different haplotypes (Appendix 1). There was no fixed mutation between the two Cory's subspecies, while the number of shared mutations was 31. Haplotypic and nucleotide diversities were high, ranging from 0.84 to 1.00 and from 1.8 to 3.4% for individual colonies, respectively. Nucleotide diversities were slightly higher in the Mediterranean than in the Atlantic (Mean ± SD = 0.1744 ± 0.0841 and 0.1593 ± 0.0769, respectively). Regarding archipelagos, diversity in Balearic Is., and Canary Is. were similar (0.0266 ± 0.0141 and 0.0256 ± 0.0136, respectively), whereas Azores Is. showed slightly smaller values (0.0233 ± 0.0124).

Population genetic structure and gene flow

AMOVA analysis was employed to describe genetic differentiation at different geographic scales (See Appendix 2 showing F_{ST} estimates among all pairwise of breeding colonies). F_{ST} estimates of breeding colony pairs ranged from 0 to 0.70 and were significantly greater than zero in most pairwise comparisons ($P<0.05$) (between some Mediterranean and Atlantic breeding colonies). In the hierarchical AMOVA, results suggest strong genetic structuring among Atlantic and Mediterranean breeding colonies ($F_{CT} = 0.56$, $P < 0.001$). Indeed, the largest variance component was due to differences among groups (56.0%). The global estimate of F_{ST} was high (0.58), and significantly greater than zero ($P<0.001$), revealing signals of population sub-structuring among colonies within groups. However, in a second AMOVA analysis, at a local scale, estimates of population differentiation were very low but still revealed some genetic structuring among breeding colonies within the Atlantic ($F_{ST} = 0.049$, $P < 0.05$). That is, there was a significant genetic differentiation between Canary and Azores archipelagos, but not among island populations within each archipelago. We did not find genetic structuring within the Mediterranean grouping populations into archipelagos nor among populations within ($F_{ST} = 0$, $P = 0.52$). The population network showed two groups of colonies

corresponding to the mostly geographically disjunct areas of the two Cory's shearwater taxa (Fig 2A). Within each taxa population trees (Fig 2B, C), breeding colonies were separated by shorter distances, showing only a weak spatial structure. Similarly, the haplotype tree also grouped most haplotypes in two groups according to each taxa (Fig 3). However, haplotypes corresponding to three Mediterranean breeders were placed within the Atlantic cluster: H3 from Chafarinas, H13 from Mallorca, and H61 from Hyeres. Conversely, haplotype H63 corresponding to one Mediterranean (Crete) and one Atlantic (Tenerife) breeder, was placed within the Mediterranean group. Private haplotypes were found in all breeding colonies. Haplotype frequencies were low and ranged between 0.67 and 12.16%. Most haplotypes appeared in only one or two populations each with few haplotypes occurring at high frequencies. Indeed, of 134 haplotypes only 32 were shared between two or more colonies, while 102 where unique to single colonies. The most representative and geographically widespread haplotypes were 70, 75, 12, 16 and 21 which occurred in ten (H70), eight (H75) and six colonies (H12, 16 and 21) (Appendix 1).

We estimated gene flow among resulting groups from the NCA analyses (Fig 2A-B-C). Gene flow between the Mediterranean and the Atlantic Cory's shearwater subspecies was estimated as less than 1 female per generation (0.48). But gene flow appeared to be asymmetrical. That is, three haplotypes typical of the Atlantic subspecies were found within the Mediterranean, while only one Mediterranean haplotype was found within the Atlantic (Fig. 3). Gene flow estimates among colonies within the Atlantic and the Mediterranean were high, ranging from 1.88 to 22.40. We found no significant divergence for most colony clade comparisons, and thus migration rates were not calculated (Table 2).

Biometrics

Principal component analysis separated two morphologically distinct groups; the larger in size *C. d. borealis* and the smaller *C. d. diomedea* subspecies, showing a clear segregation among individuals belonging to each taxa (Fig. 4). However, slight overlaps exist and a few

individuals corresponding to Mediterranean colonies were placed within the Atlantic group, while some Atlantic individuals were placed within the Mediterranean group.

Ringing- recovery data

Overall, we found a low degree of dispersal despite the high vagility of Cory's shearwaters. Off 1050 individuals re-sighted, 98.7% returned to the colony where they were ringed. Most of the individuals that were recovered far away, 5 out 14, dispersed into neighbouring breeding sites less than 300 km apart, while all the others, 9 individuals, moved distances greater than 1000 km. Ringing and recovery localities, dispersal routes and distances covered by dispersing birds are shown in Fig. 5.

Isolation by distance vs. habitat type

Procrustes analyses revealed a significant correlation between genetic and geographic distances among populations at a regional scale, considering both, the Atlantic and the Mediterranean regions ($m^{12} = 0.27$, $P < 0.001$). However, within each region, whereas spatial correlation was still significant within the Atlantic ($m^{12} = 0.71$, $P = 0.05$), significance did not hold within the Mediterranean ($m^{12} = 0.98$, $P = 0.97$). Regarding oceanographic differences across the breeding range, there was a significant association between all oceanographic variables and genetic distances among colonies when considering colonies from both taxa (chlorophyll: $m^{12} = 0.80$, $P < 0.05$, sea surface temperature: $m^{12} = 0.72$, $P < 0.01$, salinity: $m^{12} = 0.90$, $P < 0.001$). However, oceanographic characteristics did not correlate with genetic distances among breeding colonies within the Mediterranean (all $P > 0.05$) neither within the Atlantic subspecies region (all $P > 0.05$). Nevertheless, when geographic and oceanographic distances among breeding colonies were combined, we found a significant association with genetic distances within the Mediterranean (chlo: $m^{12} = 0.89$, $P < 0.05$; sst: $m^{12} = 0.62$, $P < 0.01$, sal: $m^{12} = 0.70$, $P < 0.05$), but not within the Atlantic (all $P > 0.05$).

Discussion

Population genetic structure

We found breeding colonies over the geographical range of the species were structured in two groups corresponding to each of the two Cory's shearwater subspecies. The two taxa are geographically segregated in Atlantic and Mediterranean areas, except for two small breeding sites: (1) the Almeria colony in the Mediterranean, which was genetically and phenotypically identified as belonging to the Atlantic subspecies (Gómez-Díaz et al., 2006, present study) and, (2) a few phenotypically Mediterranean Cory's shearwaters reported to breed on the Atlantic French coast (Mays et al., 2006). Genetic differentiation between the Atlantic and the Mediterranean groups was well supported by the network as well as by the AMOVA analyses (Fig 2, 3), agreeing with earlier genetic studies on the species that suggested long term geographic isolation and gene flow barriers between the two taxa (Gómez-Díaz et al., 2006). Besides, molecular results generally agree with morphometric and behavioural differences between the two subspecies (Bretagnolle et al., 1990; Gómez-Díaz et al., 2006; Granadeiro, 1993; Thibault et al., 1997). Moreover, a recent study showed the Mediterranean and the Atlantic Cory's shearwaters are closely related to the Cape Verde shearwater, but genetic divergence between the Cape Verde shearwater and the Mediterranean Cory's was lower than the divergence with the Atlantic Cory's shearwater (Gómez-Díaz et al., 2006). In consequence, the current classification of the *Calonectris* species complex is paraphyletic and we therefore suggest all three taxa should be recognized as three distinct species.

Despite almost all haplotypes were clearly segregated in two groups corresponding to the Atlantic and the Mediterranean Cory's shearwaters (Fig. 3), we identified two possible immigrants from the Atlantic breeding in the Mediterranean (H3 and H61 occurring in Hyeres and Chafarinas colonies) and two cases of introgression (H63 and H13 occurring in Tenerife and Mallorca colonies). That is, two genetically Atlantic birds breeding in the Mediterranean showed an Atlantic phenotype, as indicated by their larger body measurements, suggesting two migration events rather than an introgression process (Fig. 4). On the contrary, there was one

genetically Atlantic bird breeding in the Mediterranean showing a Mediterranean phenotype (H13), and one genetically Mediterranean bird breeding in the Atlantic showing an Atlantic phenotype (H63). That is, there were two cases in which phenotype and genotype did not match, suggesting in this case introgression rather than migration events. Despite we found significant genetic structure between the two subspecies, they may not be entirely reproductively isolated. Ecological observations at the breeding colonies further support these hypotheses. That is, there are various reports of Atlantic pairs breeding in the Mediterranean (Lo Valvo et al., 1988; Martínez-Abraín et al., 2002; Sánchez, 1997; Thibault et al., 1998), and at least one case of hybridization between the two subspecies has been reported (Martínez-Abraín et al., 2002). Dispersal could take place when birds return from the wintering areas to the breeding colonies. Since Atlantic and Mediterranean Cory's populations mix at several wintering areas (González-Solís et al., 2007), it is likely that a few *C. d. borealis* enter the Mediterranean and a few *C. d. diomedea* remain and breed in the NE Atlantic. Moreover, gene flow estimates also indicate some exchange of individuals between the two taxa, but as less than 1 female per generation, suggesting that most dispersal movements do not result in effective gene flow. Many dispersers may not mate or successfully breed and are selected against, as also indicated by the existence of morphometric gradients through the geographic distribution of the species (Gómez-Díaz et al., 2007; Granadeiro, 1993).

Genetic differentiation within each taxon was lower than between taxa although a weak genetic substructure was also apparent within the Atlantic subspecies. Despite a high philopatry and site fidelity of Cory's shearwaters (Rabouam et al., 1998; Thibault et al., 1997), one migrant per generation would be enough to prevent genetic differentiation (Hedrick, 1999). In agreement, our analysis on recovery data suggests some short-range dispersal among neighbouring islands within each subspecies regions. This result is also supported by genetic analyses of dispersal movements among Cory's shearwaters from neighbour Sicily and Sardinia breeding colonies (Randi et al., 1989). Our results contrast with previous microsatellite analyses on the Mediterranean subspecies showing high levels of among and within population genetic structuring (Rabouam et al., 2000). These differences may arise from using distinct

molecular markers. That is, sex-biased dispersal has been suggested in Cory's shearwaters as females disperse in greater proportion than males (Rabouam et al., 1998; Thibault et al., 1997). If this is the case, our mtDNA analysis may overestimate gene flow levels compared to nuclear markers, resulting in the little genetic differentiation observed at local scales.

Spatial patterns of genetic variation

Both geographic distributions of populations and oceanographic habitat characteristics can influence patterns of genetic structure in marine organisms. We combined genetic and environmental data to evaluate whether patterns of genetic differentiation correlated with geographic distances and oceanographic differences across the breeding range of Cory's shearwaters. The genetic separation between the Mediterranean and the Atlantic subspecies is consistent with their mostly segregated distributions as well as with the oceanographic break that separates the two marine basins. As pointed out in a previous study, the Strait of Gibraltar may provide a physical boundary, but the oceanographic break between Atlantic and Mediterranean surface waters, the Almeria-Oran oceanographic front (AOOF), probably act as more realistic divide between the two Cory's shearwater subspecies (Gómez-Díaz et al., 2006). This oceanographic divide is well reflected in several marine species with Mediterranean-Atlantic distributions (Borsa et al., 1997; Naciri et al., 1999; Quesada et al., 1995), including other tetrapods such as marine mammals (Natoli et al., 2005).

Within each region, some evidences supported isolation by distance model in the Atlantic, whereas, within the Mediterranean, distance or oceanographic traits alone did not explain the observed patterns of genetic variation. Nevertheless, when combined, some influence of both oceanography and geography was apparent. Within the Mediterranean, there is a wide variety of different oceanographic environments (Zotier et al., 1999). Indeed, compared with the western Mediterranean, the east shows lower productivity, and relatively warmer waters and higher salinity (SeaWIFS, MODIS and NODC data). Furthermore, the comparison of oceanographic parameters and distribution maps suggest that larger colonies of Cory's shearwaters are established close to summer thermal fronts where summer winds are also more

regular (Zotier et al., 1999). Thus, specific marine habitat characteristics of different areas, such as differences in sea surface temperature, salinity, chlorophyll concentrations and winds, could have promoted divergence through local adaptation of populations.

In conclusion, considering ecological, morphological and genetic differences between the Atlantic and the Mediterranean Cory's shearwaters, we recommend that the two taxa be regarded as a different species. Besides, our results suggest that not only geographic distances among populations but also marine habitat characteristics act structuring Cory's shearwater populations. We suggest that more integrative approaches, combining genetic, oceanographic and ecological data can provide a better understanding on mechanisms of population differentiation in seabirds.

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Table 1. Number of birds sampled, localities and geographic coordinates from breeding colonies of Cory's shearwaters included in the present study.

Calonectris taxa	Localities		*No. Samples*	Coordinates Latitude	Longitude
C.d.diomedea (117)	Mallorca	Balearic Is.	11	39.5833	2.3667
	Menorca	Balearic Is.	10	39.8020	4.2878
	Ibiza	Balearic Is.	12	38.9618	1.1983
	Cabrera	Balearic Is.	6	39.2024	2.97514
	Columbretes	Spain	10	39.8500	0.6500
	Murcia	Spain	10	37.5833	-0.9833
	Tremiti	Italy	10	42.1256	15.4939
	Tuscany	Italy	6	42.4000	11.8667
	Sardinia	Italy	4	41.0756	8.2606
	Linosa	Italy	9	35.8667	12.8667
	Hyeres	France	10	43.0089	6.2106
	Chafarinas	Morocco coast.	10	35.1833	-2.4167
	Crete	Greece	9	36.4423	25.2272
C.d.borealis (124)	St.Maria	Azores Is.	9	36.9420	-25.1710
	Graciosa	Azores Is.	10	39.0557	-27.9549
	Corvo	Azores Is.	10	39.6745	-31.1060
	Faial	Azores Is.	7	38.5244	-28.7468
	Flores	Azores Is.	9	39.3749	-31.1974
	S.Miguel	Azores Is.	10	37.7064	-25.4433
	Madeira	Portugal	10	32.3445	-16.4857
	Selvagens	Portugal	7	30.1333	-15.8667
	Berlengas	Portugal	10	39.4089	-9.4939
	G.Canaria	Canary Is.	5	27.8456	-15.7887
	Lanzarote	Canary Is.	9	29.2930	-13.5372
	Tenerife	Canary Is.	10	28.4460	-16.2333
	La Palma	Canary Is.	8	28.7800	-17.7965
	Almería	Spain	10	37.3489	-1.6507

Table 2. Gene flow estimates (M, number of females per generation) among clades within the Mediterranean and the Atlantic subspecies.

C. d. diomedea

		Clade 1.1	Clade 1.2	Clade 1.3	Clade 1.4
Migration	Clade 1.1	-			
	Clade 1.2	2.04	-		
	Clade 1.3	7.76	*n.d	-	
	Clade 1.4	6.52	9.06	16.92	-
	Clade 2.1	5.84	-	-	*n.d

C. d. borealis

		Clade 1.1	Clade 1.2	Clade 1.3	Clade 1.4	Clade 1.5	Clade 2.1
Migration	Clade 1.1	-					
	Clade 1.2	6.32	-				
	Clade 1.3	11.12	11.76	-			
	Clade 1.4	2.20	9.32	*n.d	-		
	Clade 1.5	1.88	3.40	10.96	21.90	-	
	Clade 2.1		-	-	*n.d	19.86	-
	Clade 2.2	2.68	7.64	22.40	-	-	

**n.d* means no significant divergence between colony clades. Migration rates estimates in those cases result inaccurate and were not considered.

Figure legends

Figure 1. Map showing average sea surface temperature from April to September for four years and breeding colonies of *C. d. diomedea* (●) and *C. d. borealis* (O) Cory's shearwaters sampled across their geographic distribution.

Figure 2. Population network on Φ_{ST} pairwise genetic distances showing genetic relationships among Cory's shearwater breeding colonies (2A). Nesting of clades within the Mediterranean (2B) and the Atlantic (2C) subspecies is indicated by boxes (Gene flow estimates among colony clades are shown in Table 2). Geographic position of each locality is shown in Fig. 1 and Table 1.

Figure 3. Haplotype network showing genealogical relationships within Cory's shearwater. Haplotypes corresponding to Atlantic and Mediterranean breeders are indicated in black and white circles, respectively. The size of the circles is proportional to the number of birds sharing that haplotype.

Figure 4. Plot of first vs. second discriminant functions scores based on 4 biometric measurements of 158 Cory's shearwaters individuals included in the genetic analysis.

Figure 5. Distances and directions of 78 Cory's shearwaters recovered far away from the colonies where they were ringed. Points represent ringing and recovery records. Lines represent dispersal movements between colonies.

Fig. 1


Hyeres
Sardinia
Tremiti
Tuscany
Azores Is.
Berlengas
Columb
Murcia
Almeria
Balearic Is.
Linosa
Crete
Chafarinas
Mediterranean Sea
Madeira
Selvagens
Atlantic Ocean
Canary Is.

Fig 2A.

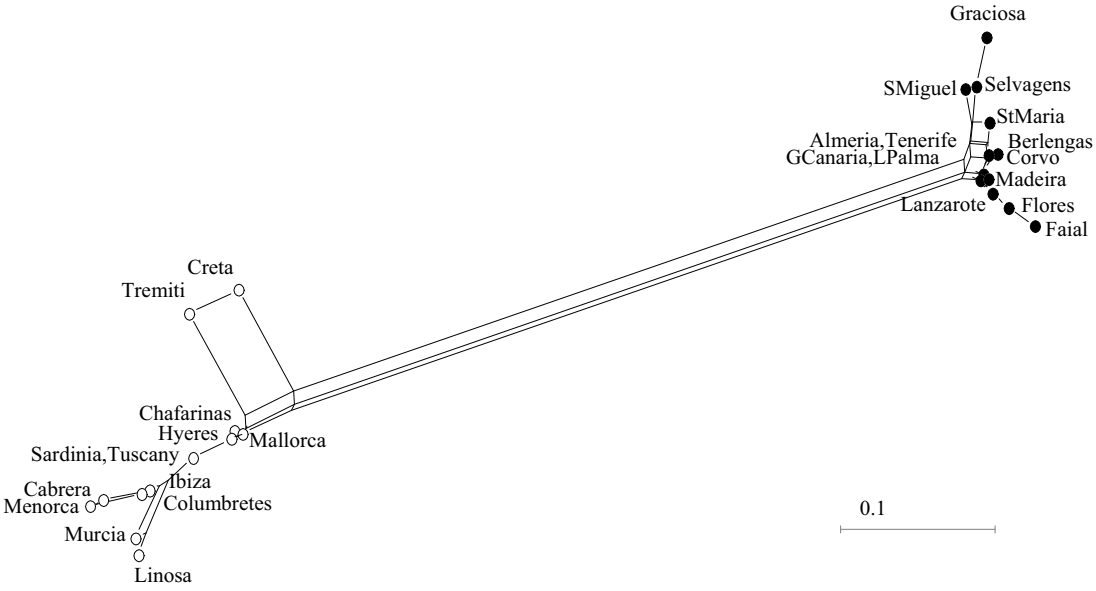


Fig 2B.

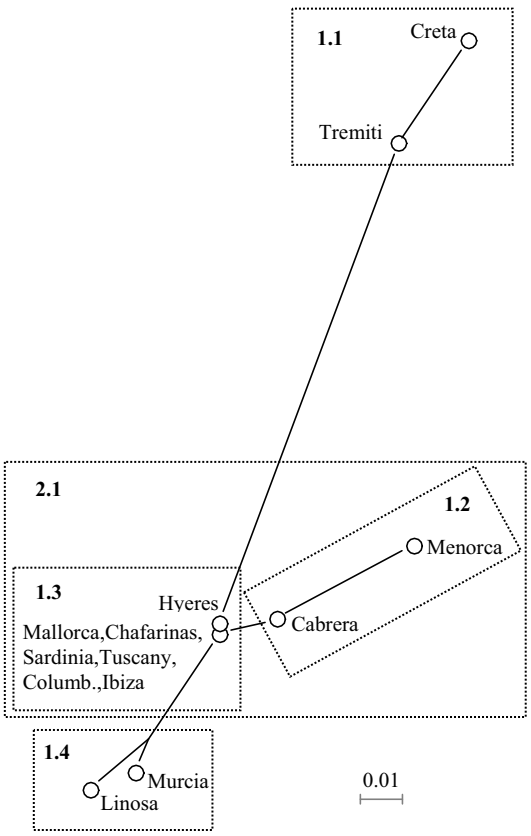


Fig 2C.

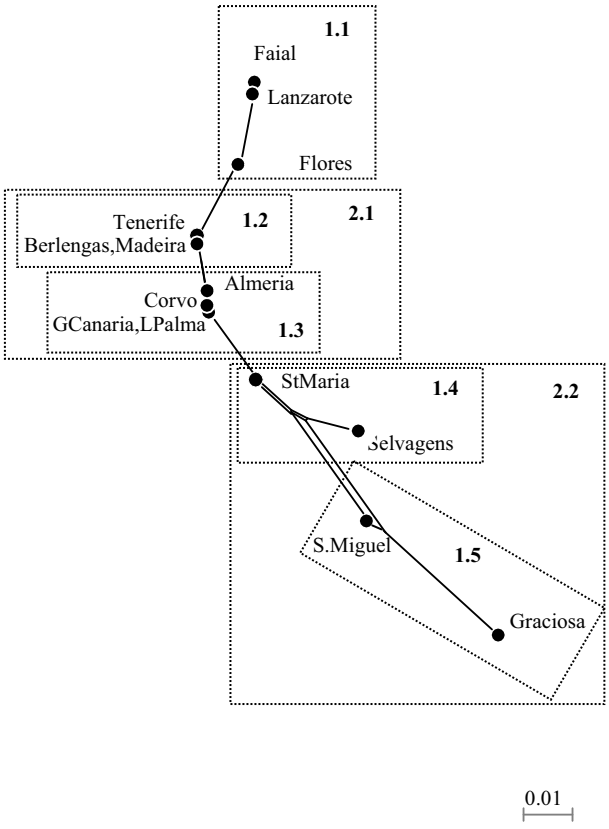
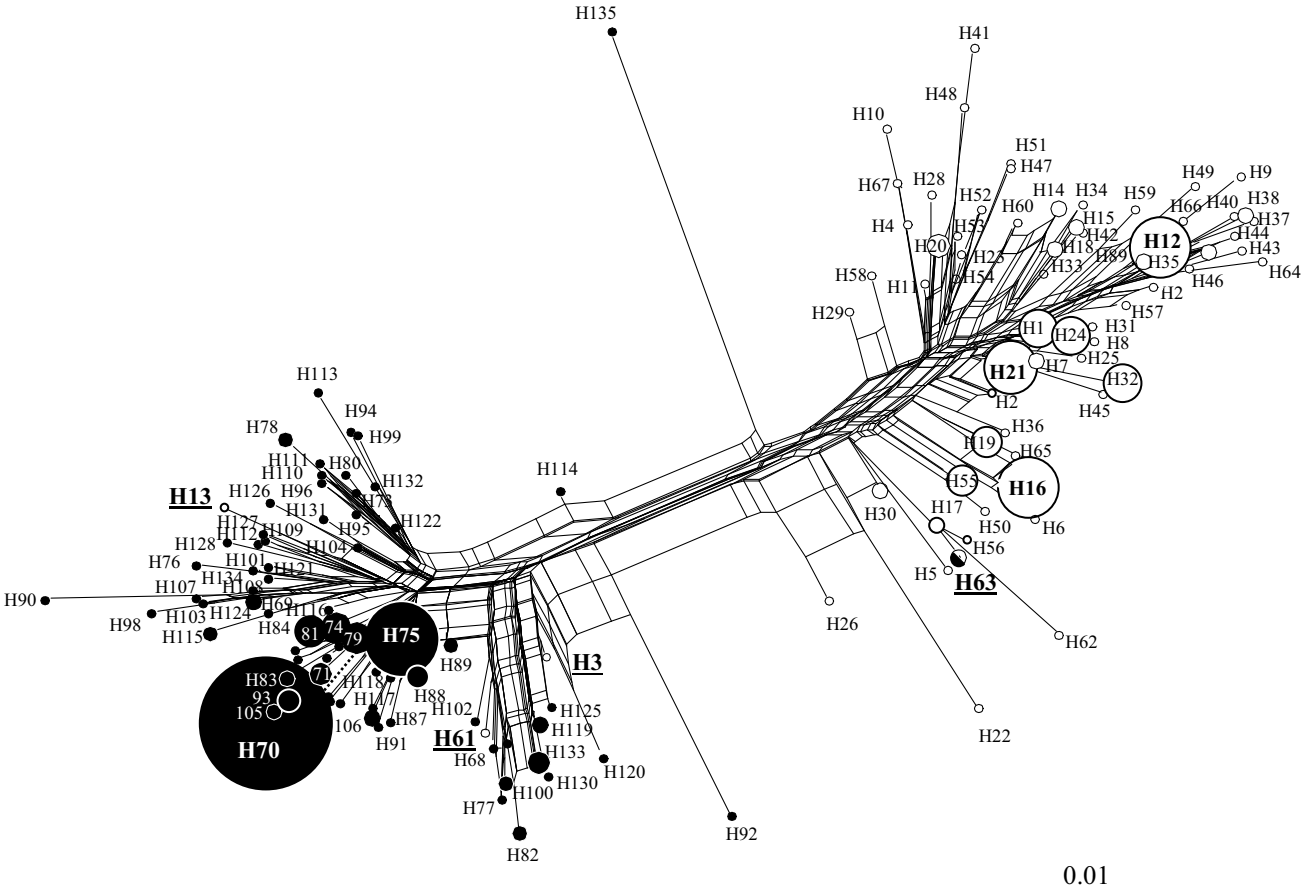


Fig. 3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H41
H48
H10
H51
H47
H67
H28
H52
H14
H34
H59
H49
H9
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H40
H38
H4
H15
H37
H20
H42
H12
H44
H18
H89
H35
H43
H58
H11
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H113
H94
H78
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H36
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H111
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H110
H132
H114
H55
H16
H13
H126
H96
H131
H17
H30
H50
H6
H127
H95
H122
H56
H128
H109
H104
H5
H63
H76
H101
H121
H107
H134
H90
H69
H103
H124
H84
H98
H115
H26
H62
81
74
79
H75
H89
H3
H83
93
105
H70
H88
H102
H125
106
H87
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H120
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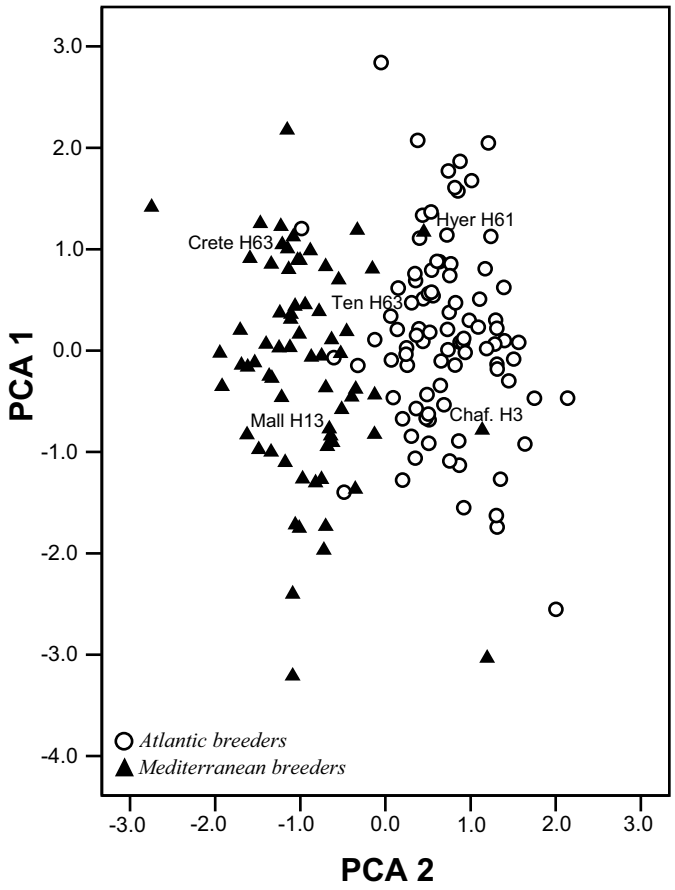
H100
H77
H82
H92
H22
0.01

Fig. 4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PCA 1
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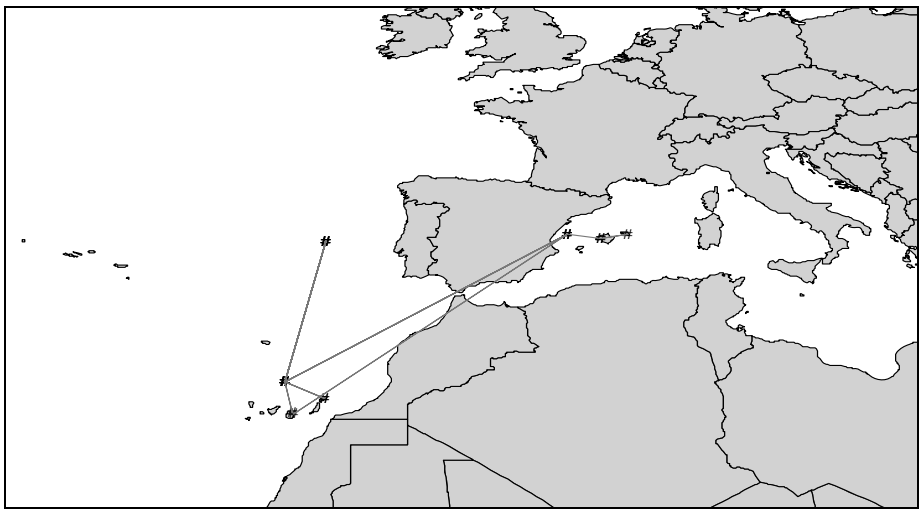
-1.0
0.0
1.0
2.0
3.0
PCA 2
Hyer H61
Crete H63
Ten H63
Mall H13
Chaf. H3
Atlantic breeders
Mediterranean breeders

Fig. 5



Ectoparasite community structure in three closely related seabird hosts: a multiscale approach

Elena Gómez-Díaz, Joan Navarro, Jacob González-Solís

(Submitted)

Capítol 3:

Com ja s'ha mencionat, factors ecològics que afectin a la distribució i l'abundància d'hostes i paràsits juguen un paper molt important en la dinàmica coevolutiva. Fins i tot, seria possible predir la congruència genètica d'hoste i paràsit a partir d'informació relativa a la seva ecologia. Així doncs, en aquest article examinem l'estructuració espacial i la dinàmica temporal de les comunitats d'ectoparàsits de les baldrigues a diferents nivells jeràrquics d'organització. Tanmateix, intentem identificar els factors ecològics que explicarien els patrons de distribució i d'abundància observats.

Estructura de la comunitat d'ectoparàsits en tres espècies d'ocells marins d'hoste properes: una aproximació multi- escala

Les comunitats de paràsits poden estar estructurades a diferents escales espacials depenent del nivell d'organització dels hostes, i per tant examinar aquesta estructura requereix d'una aproximació multi- escala. Varem investigar l'estructura de la comunitat d'ectoparàsits composada per tres polls de la ploma (*Halipeurus abnormis*, *Austromenopon echinatum* i *Saedmunssonia peusi)* i una espècie de puça (*Xenopsilla gratiosa*) al nivells de comunitat infra-, component i regional en tres espècies d'ocells marins properes, la baldriga cendrosa Mediterrània (*Calonectris d. diomedea*), la baldriga cendrosa Atlàntica (*C. d. borealis*) i la baldriga de Cap Verd (*C. edwardsii*). Varem examinar l'estructuració temporal i espacial de les infracomunitats, l'influencia de l'agregació espacial i la condició corporal de l'hoste en la comunitat component, i l'efecte de la connectivitat geogràfica i genètica en la comunitat regional. Les infracomunitats d'ectoparàsits mostraven un solapament substancial en els patrons temporals d'abundància però les espècies apareixien segregades dins del cos de l'hoste. Dintre de les comunitats components, totes les espècies d'ectoparàsits mostraven una distribució d'abundàncies agregada però els patrons d'agregació diferien significativament entre espècies, independentment de la distribució espacial dels hostes dintre de la colònia de cria. A una escala regional, la similitud en la comunitat d'ectoparàsits apareixia correlacionada amb les distàncies geogràfiques entre les colònies de l'hoste, però no amb les distàncies genètiques. Els nostres resultats destaquen la importància de la distribució geogràfica de l'hoste i la segregació espacial dintre de l'hoste com a factors claus per determinar l'estructura de la comunitat d'ectoparàsits en les espècies de baldrigues *Calonectris*.

Ectoparasite community structure in three closely related seabird hosts: a multiscale approach combining ecological and genetic data

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Running title

Ectoparasite community structure in Cory's shearwater

Abstract

Parasite communities can be structured at different spatial scales depending on the level of organization of the hosts, and hence examining this structure should be a multiscale process. We investigated ectoparasite community structure composed by three lice (*Halipeurus abnormis*, *Austromenopon echinatum* and *Saedmunssonia peusi)* and one flea species (*Xenopsilla gratiosa*) at the infra-, component and regional community levels in three closely related seabird hosts, the Mediterranean Cory's shearwater (*Calonectris d. diomedea*), the Atlantic Cory's shearwater (*C. d. borealis*) and the Cape Verde shearwater (*C. edwardsii*). We examined temporal and spatial structuring of infracommunities, the influence of host aggregation and body condition on the component community, and the effect of genetic and geographic connectivity among host populations on the regional community. Ectoparasite infracommunities showed a substantial species overlap in temporal patterns of abundance but species were spatially segregated within the host body. Within component communities, abundance of all ectoparasite species showed an aggregated distribution but aggregation patterns greatly differed among species, regardless of the spatial distribution of hosts within the breeding colony. At regional scale, similarity in ectoparasite communities correlated with geographic distances among host colonies, but not with genetic distances. Our results highlight the importance of host the geographic distribution of breeding colonies and the spatial segregation within the host body as key factors in determining ectoparasite community structure in *Calonectris* shearwaters.

Key words: community similarity, parasite assemblages, abundance, host connectivity, geographical distance, genetic distance, aggregation, space partioning, lice, fleas.

Introduction

Parasite assemblages interact with their hosts at different spatial scales. The structure of parasite communities may result from the interplay between the intrinsic characteristics of each parasite species, the extrinsic features of their patch of habitat, (i.e. the host, Poulin 2006) the off-host environment (i.e. host habitat, Krasnov *et al.* 2005b; Krasnov, Khokhlova & Shenbrot 2002), as well as the connectivity among host populations (Morand & Guégan 2000; Poulin & Morand 1999). However, the influence of each factor remains poorly understood and greatly depends on the level of organization of the parasite assemblage. Three hierarchies have been commonly defined for parasite assemblages, in which different spatial and temporal factors may act: infra-, component, and regional communities (Guégan, Morand & Poulin 2004). Infracommunities include all parasite species within an individual host. They can be temporally and spatially structured. Temporal variation in parasite infracommunities can be coupled to the breeding cycle of the host (Clayton & Walther 2001; Figuerola 2000). Spatial segregation is promoted by resource heterogeneity within the host body, favoring coexistence among parasite species (Mouillot, George-Nascimento & Poulin 2003).

Component communities include all infracommunities within the same host population. Their structure may result from differences in host susceptibility to infection or from differences in the exposure to parasites among individual hosts (Poulin 1998). Host traits, such as body size and condition, have been shown to correlate with susceptibility to infection (Whiteman & Parker 2004a). Spatial aggregation of host can affect probability of parasite transmission leading to differences in parasite infection (Rékási, Rózsa & Kiss 1997; Rózsa 1997; Rózsa, Rékási & Reiczigel 1996; Tripet, Christe & Moller 2003).

Regional community is composed by all component communities within a host species. At this level, structure is mainly determined by habitat characteristics (Krasnov *et al.* 1997; Krasnov *et al.* 2004a; Krasnov *et al.* 2004b; Krasnov *et al.* 2005b), host population size and density (Rékási *et al.* 1997; Rózsa 1997; Rózsa *et al.* 1996 Arneberg *et al.* 1998; Calvete *et al.* 2003; Lopez 2005) and host dispersal (Morand & Guégan 2000; Poulin & Morand 1999). For

example, climate has been shown to play an important role in shaping ectoparasite species distributions (Krasnov *et al.* 2005a). Host population size can influence infection rates as long as larger populations usually show a higher host aggregation favoring parasite transmission, but oppositely through their higher levels of genetic diversity which is related to a greater resistance to infection (Whiteman *et al.* 2006). Host dispersal among populations can favor parasite dispersal promoting homogenization of parasite communities (Poulin & Morand 1999; Proctor & Jones 2004). Geographic distance among host populations has often been used as a proxy for host dispersal (Goüy de Bellocq, Morand & Feliu 2002; Krasnov *et al.* 2005b). However, hosts do not necessarily disperse correspondingly to geographic distances. In this context, genetic similarity among host populations could better reflect host dispersal and thus help understanding the forces structuring parasite communities.

Seabirds, and especially petrels, offer a great opportunity to investigate factors influencing the structure and composition of parasite communities at different scales. Seabird feathers provide a heterogeneous environment, which many taxa of ectoparasites have colonized (Janovy Jr 1997). Indeed, ectoparasite fauna include highly specific species, such as feather lice (Phthiraptera) as well as more generalist, such as fleas (Siphonaptera). This provides opportunities to study factors influencing infection in relation to the parasite specificity. Moreover, petrels are particularly adequate to study effects of host isolation, since parasite dispersal is temporally limited to the reproductive periods and spatially structured in remote oceanic islands where they breed, grouped in archipelagos and oceans. In addition, monogamous breeding is widespread among petrels and most species show natal and breeding fidelity (Brooke 2004), which can limit opportunities for parasite transmission. In this context, dispersal patterns of seabird hosts are expected to influence structure of their ectoparasite communities at different scales.

In the present study we examine temporal and spatial structure of three lice species (*Halipeurus abnormis*, *Austromenopon echinatum* and *Saedmunssonia peusi*) and one flea species (*Xenopsylla gratiosa*) at three different levels of parasite organization on three closely related seabird taxa, the Mediterranean Cory's shearwater (*Calonectris d. diomedea*), the

Atlantic Cory's shearwater (*C. d. borealis*) and the Cape Verde shearwater (*C. edwardsii*). In particular, we aim; i) to evaluate the degree of temporal and spatial segregation among parasite species within parasite infracommunities ii) to assess the influence of host aggregation and body condition on spatial structure of the component community, and iii) to investigate the effect of population connectivity, as indicated by both, geographic distance and genetic similarity, on the regional ectoparasite community structure throughout the geographic range of the three seabird taxa.

Material and Methodology

Host-parasite system

Cory's shearwater *Calonectris diomedea* is a colonial and monogamous seabird breeding on islands along Mediterranean Sea and Macaronesic archipelagos (Azores, Canary islands, Madeira island and Cape Verde), showing two different subspecies that can be differentiated morphologically, the Atlantic (*C. d. borealis*) and the Mediterranean (*C.d. diomedea*) subspecies. Formerly, the species *C. edwardsii*, from Cape Verde islands, was also considered a subspecies but it is now regarded as a full species (see Gómez-Díaz *et al.* 2006). Cory's and Cape Verde shearwaters share 3 species of chewing lice being described as their primary hosts (Price *et al.* 2003): *Halipeurus abnormis* (Piaget, 1885), *Saedmunssonia peusi* (Eichler, 1949b), and *Austromenopon echinatum* (Edwards, 1960). Cory's and Cape Verde shearwaters also share 1 species of flea *Xenopsilla gratiosa* (Jordan et Rothschild, 1923), which is considered to be specific of seabird species from the genus *Calonectris*, *Puffinus,* and *Hydrobates* (Beaucournu, Degeilh & Guiguen 2005).

Chewing lice are permanent ectoparasites which complete their whole life cycle on the host where they feed mainly on feathers, dead skin, blood or secretions. As they are incapable of independent mobility, transmission occurs through passive dispersal during periods of direct contact between hosts (Clayton & Tompkins 1994 Lee & Clayton 1995). One species of louse often is confined to a single species of host, which suggests a long-term host-parasite

association (Emerson & Price 1981). On the contrary, fleas are obligatory blood feeders closely associated with the host's environment (nest). In most fleas, all stages of the breeding cycle occur out of the host body, except for the adults, which intermittently attack the host for feeding (Marshall 1981).

Study sites and field methods

We sampled breeding adult birds from 27 breeding colonies of the three *Calonectris* taxa across Mediterranean and NE Atlantic regions from 2001 to 2005 (Fig. 1). We estimated the total number of parasites on a bird from 1 minute visual counts of parasites on six body regions: left wing, tail, mantle, belly, head and breast. To assess whether visual counts reflected total abundance of parasites, we fully desparasitized a sample of 30 birds using the dust-ruffling method described by Clayton & Walther 1997, since this method has been proved to be an accurate predictor of total abundance (Clayton & Drown 2001). We compared dust-ruffling and visual counts using least square linear correlation.

Genetic analysis

Genetic analyses were performed on 38 Mediterranean Cory's, 75 Atlantic Cory's and 20 Cape Verde shearwaters from 11 breeding colonies. DNA was isolated from ethanol-preserved whole blood using the salting-out extraction protocol from Bruford *et al.* 1998. We amplified a 293 bp fragment of Domain I of the mitochondrial control region of all three *Calonectris* taxa using three specific primers previously designed for the species (Gómez-Díaz *et al.* 2006): either CAL2H (5'CATCCCATCCAACTTAAG3') or CAL4H (5'AGCCTATGTATGGATGTGCAT3') was used in conjunction with CAL1L (5'GGTCCTGAAGCTAGTAATAC3'). Reaction conditions and automated sequencing were those described by Gómez-Díaz et al. (2006).

We estimated F_{ST} genetic distances among *Calonectris* breeding colonies using ARLEQUIN 3.0 (Excoffier 2005). To visualize the genetic relationships among colonies, we generated a population similarity tree by neighbor-joining analysis on the F_{ST} pairwise distances

using NTSYSpc package version 2.1 (Rohlf 1997). In addition, we also estimated the effective population sizes for all breeding colonies using DnaSP 4.0 (Rozas & Rozas 1999) and calculated haplotypic diversity (H_s; Nei 1987) and nucleotide diversity (π; Nei 1987) to assess the level of genetic variation for each subspecies and breeding colony.

Spatial and temporal patterns of parasite infection

Prevalence was calculated as the proportion of birds infected by a given parasite species. Intensity, i.e. abundance of ectoparasites, was calculated as the number of ectoparasite individuals of a given species per individual host or body area (Bush *et al.* 1997). For subsequent analyses, intensity data were log transformed ($\log_{10}(x+1)$ to approach normality. Intensity values reported as mean number of parasites ± standard deviation and prevalence values reported as percentages of occurrence.

At the infracommunity level we examined temporal and spatial patterns of ectoparasite infection (all parasites in a host individual) throughout the breeding season on 109 Atlantic Cory's shearwaters from one breeding colony (Veneguera, Canary Is.) using MANOVA test. We analyzed patterns of spatial segregation and resource partioning among ectoparasites species on the host body following procedure described by Reed *et al.* 2000. We measured surface (in cm^2) of each of the six body areas considered in the visual counts of parasites Assuming a null model of uniform ectoparasite distribution, we compared the ectoparasite counts of each area against the expected number of ectoparasite individuals of each species for each area on the basis of the size of the area using a Chi-square test.

At the component community level, we tested for differences in intensity of infection for each ectoparasite species among shearwater individuals from one Atlantic breeding colony (90 birds from Vila, Azores Is.) using ANOVA. Then, we first examined the effect of host body condition on intensity of infection using ANCOVA (the model considered weigh and body size as covariables and sex as a factor). Body size covariable was calculated as the first axis of a principal component analysis (PC1 which accounted for 90% of the total variance) on 5 body measurements (tarsus, wing length, cranium-bill length, bill height and bill-height at nostril).

Secondly, we examined aggregation patterns for each ectoparasite species using the Green's coefficient of dispersion (Krebs 1989). Besides, we tested the effects of nest density on intensity of infection in 39 birds breeding at Vila Islet (Azores). That is, we correlated the intensity of each ectoparasite species with nest density, as measured by the number of nests within 1, 3, 5 and 10 m radius. Then, we tested for differences in intensity between breeding mates in 47 pairs from Vila Islet and 39 pairs from Veneguera (Gran Canaria, Canary Is.) using paired t-tests. Finally, we examined spatial patterns in ectoparasite prevalence and intensity within the component community. We investigated whether proximity among host breeding pairs would explain similarity in patterns of structure of their infracommunities. At one breeding colony (Vila, Azores Is.), we tested relationship between distance among nests and similarity in ectoparasite infracommunities of breeding birds using the Mantel test (Mantel 1967; Mantel & Valand 1970) implemented in the *zt* program (Bonnet & Van de Peer 2002). The significance test of the *r* statistic was determined by a nonparametric randomization procedure. Similarity in prevalence was measured using both the Jaccard and Morisita-Horn indices. Similarity in intensity was calculated as Euclidean distance.

At the regional level, we examined differences in infection parameters among component communities across the host geographic range. To avoid sampling bias owing to a temporal variation in ectoparasite intensity across the breeding season (see results), analyses on the spatial variation of the ectoparasite component communities were limited to 11 host breeding colonies sampled during incubation period. We calculated prevalence and intensity at two spatial levels: for each breeding colony and for each host taxa. To test whether ectoparasite intensities differ among species and across the breeding range of their host we conducted MANOVA tests at two spatial levels; among host taxa and among host breeding colonies. To account for inter-individual variability in body size, initial models included a body size covariable (PC1 calculated as described above). Since no significant effect was detected (see results), further models did not include the body size covariable.

To examine similarity in parasite communities among host breeding colonies, we calculated a dissimilarity matrix from the Chi-square distances based on the intensity of each

parasite species at each breeding colony for all pairwise combinations of breeding colonies. We constructed a cladogram from the dissimilarity matrix using the Neighbor-Joining clustering analysis implemented in the NTSYSpc package version 2.1 (Rohlf 1997). Cladograms based on Morisita similarity indices were also built and gave similar results (data not shown). Similarity of parasite communities were also calculated for prevalence data, using Euclidean distances among all pairwise breeding colonies and building a cladogram with UPGMA clustering analysis (NTSYSpc package version 2.1 (Rohlf 1997)). Neighbor-Joining cladograms based on Rekonen similarities were also built for prevalence data and gave similar results (data not shown).

To investigate whether genetic relationships among host colonies was related to similarity patterns of prevalence and intensity of the ectoparasite component communities, we used two proxies of population connectivity: (1) genetic distances among host breeding colonies; and (2) by sea geographic distances among host breeding sites. To examine correlation between the two indexes of population connectivity and patterns in intensity and prevalence of ectoparasite communities we applied both simple and partial Mantel test analyses using the program zt (Bonnet & Van de Peer 2002). First, we applied simple paired Mantel test to assess correlation between intensity or prevalence and either host genetic or geographic distances among breeding colonies. Then, to test the effect of all three variables together we applied the partial Mantel test. The goal is to test the correlation between matrices A and B while controlling the effect of a third matrix C, in order to remove spurious correlations (Smouse, Long & Sokal 1986). First we performed a partial mantel test between either ectoparasite abundance or prevalence and by-sea geographic distances while controlling host genetic distances. Then, we tested correlation between abundance or prevalence and host genetic distances while controlling by-sea geographic distances among colonies. Permutation approach applied was that developed by Anderson & Legendre 1999).

Results

Ectoparasite species and visual vs. dust-ruffling counts

We collected parasites from 585 individual birds from 7 Atlantic Cory's, 5 Mediterranean Cory's and 2 Cape Verde shearwaters breeding colonies. We recorded 3 lice (*Halipeurus abnormis, Austromenopon echinatum* and *Saedmunssonia peusi)* and 1 flea species (*Xenopsilla gratiosa*). Correlation between visual counts of the four ectoparasite species and the number of parasites obtained after applying the dust-ruffling method on 33 shearwaters were highly significant (all $P < 0.001$ for the four ectoparasite species). R^2 ranged from 0.61 to 0.78, indicating that visual counts provide a reliable estimate of total intensity of ectoparasites.

Temporal and spatial ectoparasite infracommunity structure

We found significant changes in mean intensity of the ectoparasite species with the progress of the breeding season (Fig 2). *H. abnormis* and *A. echinatum* intensities significantly decreased from pre-laying to chick rearing breeding periods (Fig 2A, B, *H. abnormis*: $F_{4,25} = 21.71$, $P < 0.001$; *A. echinatum*: $F_{4,25} = 21.71$, $P < 0.001$). The intensity of the flea species *X. gratiosa* also decreased from the beginning to the end of the breeding season (*X. gratiosa*: $F_{4,25} = 7.90$, $P < 0.05$), but the peak of greatest intensity for this species corresponded to the egg-laying period (Fig 2D). On the contrary, *S. peusi* intensity showed no significant differences throughout the study period (Fig 2C, *S. peusi*: $F_{4, 25} = 0.49$, $P = 0.74$).

We found significant differences in distribution and intensity among ectoparasite species in relation to host body area (*H. abnormis*: $\chi^2 = 3567.97$, $P < 0.001$, *A. echinatum*: $\chi^2 = 82.86$, $P < 0.001$, *S. peusi*: $\chi^2 = 11.82$, $P < 0.001$, *X. gratiosa*: $\chi^2 = 862.35$, $P < 0.001$) Except for *H. abnormis*, which occurred in all body areas, both *A. echinatum* and *S. peusi* lice and the flea *X. gratiosa* occurred in specific areas within the host body (Fig 3). Fleas were mainly distributed on the belly, tail and breast; the louse *S. peusi* appeared to be head specific and *A. echinatum* mainly occurred on the head, mantle and breast.

Spatial structure in the ectoparasite component community

Within the component community we did not find significant differences in ectoparasite intensity among host individuals, while differences among ectoparasite species accounted for most of the variability observed (Host individual: $F_{89, 267} = 1.78$, P = 0.163, Species: $F_{3, 267} = 200.98$, P < 0.001). Body condition did not correlate with intensity of infection of any of the four ectoparasite species considered (*X. g*: $F_{1, 50} = 0.17$, P = 0.68; *H. a*: $F_{1, 50} = 1.03$, P = 0.32; *A. e.*: $F_{1, 50} = 1.64$, P = 0.21; *S. p.*: $F_{1, 50} = 0.93$, P = 0.34).

The distribution of ectoparasite intensities among Cory's shearwaters from Vila islet (Azores Is.) was aggregated (Green's index > 1). However, aggregation patterns greatly differed among ectoparasite species. Green's index of dispersion values for the flea *X. gratiosa* were the greatest (9.32), whereas among lice species, *S. peusi* and *A. echinatum* showed lower values than *H. abnormis* (*H. abnormis* = 4.76, *A. echinatum* = 1.77 and *S. peusi* = 1.85). Correlation between spatial distribution of nests, as measured by the distance among host nests, and similarity in ectoparasite communities of nesting hosts was not significant (Mantel: $r_{1,2} = 0.04$, P = 0.32). Nevertheless, host nest aggregation, as measured by number of neighboring nests within 3 meters, was correlated with intensity of infection of nesting birds only for the flea species (flea: $R^2 = 0.15$, P < 0.05; lice species all $R^2 < 0.01$, all P > 0.10).

Ectoparasite intensity between male and female Cory's shearwaters from Vila islet (Azores) or Veneguera (Canary Is.) did not differ for any species (t-student, all P > 0.05). Nevertheless, intensity of fleas (*X. gratiosa*) and the louse *A. echinatum* were significant correlated between breeding mates from Vila islet (Azores Is.) (*X. gratiosa*: $R^2 = 0.59$, P < 0.001; *A. equinatum*: $R^2 = 0.14$, P < 0.01; *H. abnormis* $R^2 = 0$, P = 0.96; *S. peusi* $R^2 = 0.03$, P = 0.34), and for the flea species and the louse *H. abnormis* between breeding mates from Veneguera (Canary Is.) (*X. g*: $R^2 = 0.37$, P < 0.001; *H. a*: $R^2 = 0.17$, P < 0.01; *A. e.*: $R^2 = 0.01$, P = 0.59; *S. p.*: $R^2 = 0.01$, P = 0.59).

Spatial structure in ectoparasite regional community

To test whether ectoparasite intensity differs among species depending on host taxa we conducted a multivariate analysis of variance (MANOVA). We found significant differences among ectoparasite species as well as among host taxa (Species: Wilks' $\lambda = 0.10$, $F_{2.8, 1101} = 1016.41$, $P < 0.001$; Host taxa: $F_{2, 395} = 15.84$, $P < 0.001$). However, we found a significant interaction among the two factors (Wilks' $\lambda = 0.44$, $F_{5.6, 1101} = 29.93$, $P < 0.001$), which indicates that intensity of each ectoparasite species differ depending on the host taxa. Therefore, we tested for differences in ectoparasite intensity among host colonies for each host taxa separately. We found significant differences in intensity among ectoparasite species for the three host taxa and among host colonies for the Mediterranean Cory's shearwater (Atlantic Cory's shearwater: Species Wilks' $\lambda = 0.16$, $F_{2.6, 443} = 162.70$, $P < 0.001$, host colony $F_{4, 168} = 1.75$ $P = 0.14$, species*host colony Wilks' $\lambda = 0.57$, $F_{10.5, 1078} = 7.76$, $P < 0.001$; Mediterranean Cory's shearwater: species Wilks' $\lambda = 0.05$, $F_{2.8, 391} = 705.62$, $P < 0.001$, host colony $F_{3, 139} = 7.60$, $P < 0.001$, species*host colony Wilks' $\lambda = 0.70$ $F_{8.4, 391} = 12.28$ $P < 0.001$; Cape Verde shearwater: species Wilks' $\lambda = 0.08$, $F_{2.7, 218} = 225.24$, $P < 0.001$, host colony $F_{1, 80} = 2.52$ $P = 0.12$, species*host colony Wilks' $\lambda = 0.84$, $F_{2.7, 218} = 5.37$, $P < 0.05$). However, in all cases we found a significant interaction between ectoparasite species and host breeding colony, suggesting that differences in intensities of each ectoparasite species are not homogeneous among host breeding colonies.

Geographic distances among host breeding colonies were significantly correlated with the similarity index of ectoparasite community. Conversely we did not find correlation between either ectoparasite intensity or prevalence and host genetic distances (Fig. 4, Mantel test on intensity data: Geographic distances $r_{1, 2} = 0.72$, $P < 0.001$ and; genetic distances, $r_{1, 2} = 0.16$, $P = 0.14$; Mantel test based on prevalence data: Geographic distances $r_{1, 2} = 0.42$, $P < 0.01$ and; genetic distances $r_{1, 2} = 0.17$, $P = 0.12$). The partial mantel test further confirmed previous results. Indeed, when controlling for geographic distances, correlation between either ectoparasite intensity or prevalence and host genetic distances decreased (*Intensity*: $r_{1, 2} = -0.022$, $P = 0.497$; *Prevalence*: $r_{1, 2} = 0.073$, $P = 0.297$).

Neither genetic diversity nor effective population size of host breeding colonies showed significant correlation with mean intensity and prevalence of any ectoparasite species (Intensity: gene diversity all $R^2 < 0.10$, all $P > 0.10$, population size; all $R^2 < 0.20$, all $P > 0.10$. Prevalence: gene diversity all $R^2 < 0.01$, all $P > 0.10$, population size; all $R^2 < 0.15$, all $P > 0.10$), except for *A. echinatum* louse which showed a negative correlation between prevalence and colony gene diversity ($R^2 = 0.32$, $P < 0.05$).

Discussion

Infracommunity structure

Ectoparasite infracommunities were temporally and spatially structured. Patterns of distribution and abundance of infracommunities varied throughout the breeding cycle of the host (Fig. 2), which is probably related to changes in host behavior and opportunities for parasite dispersal (Proctor 2003). Indeed, most ectoparasite species show life-cycles synchronized with that of their hosts (Marshall 1981). During the prelaying period shearwaters mate on the breeding grounds. From laying to hatching, male and female shearwaters share incubation duties and spent approximately half of their time on the nest. In contrast, during chick rearing period shearwaters only visit the nest intermittently for a few hours to feed the chick and spent most of their time foraging at sea (Thibault, Bretagnolle & Rabouam 1997). This behavior may favor infestation of more opportunistic and periodic ectoparasites, such as fleas, at the commencement of the shearwater breeding period, when birds easier to reach on the ground. Likewise, physical contact between birds during host mating also provides opportunities for parasite exchange and reproduction of lice. Accordingly, greater abundance of adult and nymph lice during pre-laying and laying could reflect ectoparasite reproduction, dropping at the chick rearing period due to vertical transmission of ectoparasites from parents to the chick.

Spatially, the flea and the three lice species showed a noticeable segregation (Fig. 3). Fleas concentrated mainly on the belly area, probably because the brood patch offers a surface

free of feathers where fleas can easily bait. Regarding the two Ishnoceran species; *S.peusi* was nearly exclusively distributed on the head, whereas *H.abnormis* occupied the rest of the host body. This segregation probably results from the habitat heterogeneity provided by the different size and shape of the feathers, which vary predictably with the body region (Reed *et al.* 2000). For example, the small head feathers compared to the rest of feathers as well as the inability of birds to preen their head, may favor less mobile species, such as *S. peusi,* which would become specialized and restricted to this region.

Component community structure

Ectoparasite abundance commonly shows an aggregated distribution, with great parasite abundances occurring in a few host individuals (Anderson & May 1978 Lindell *et al.* 2002). In the present study, distribution of parasites among individual hosts matched a clumped pattern but the degree of aggregation differed among lice and the flea. In fleas, this pattern appeared more marked whereas lice species distributed less aggregately, especially the less abundant species (*S. peusi* and *A. echinatum*). The aggregated distribution of parasites can result from the spatial aggregation of hosts, i.e. host density (Whiteman & Parker 2004b). Parasite aggregation can be particularly important in colonial nesting species, such as most seabirds, due to the physical proximity of birds (Rózsa *et al.* 1996 Tripet *et al.* 2003). However, we did not find a significant correlation between distance among shearwater nests and similarity in infracommunity structure. Nevertheless, we found a positive effect of nest density on the abundance of fleas, as reported in previous studies (Krasnov *et al.* 2002). In addition, mean abundances of fleas, *X. gratiosa*, and one lice species, *A. echinatum*, were correlated between breeding mates. The observed differences in the effect of nest density and mating on the distribution and abundance patterns of the ectoparasite species are probably related to differences in parasite mobility (Clayton, Bush & Johnson 2004). That is, the species which showed similar values of abundance between mates (*X. gratiosa and A. echinatum*) and neighboring hosts (*X. gratiosa*) are the most mobile, increasing the likelihood of horizontal transmission.

Host body size and condition can explain differences of intensity within the component community since it may determine the resources and niches available for parasites (Clayton & Walther 2001). Further, host condition can notably influence the susceptibility of parasite infection (Whiteman & Parker 2004a). However, we did not detect a significant relationship between host size or condition and ectoparasite intensity, which contrasts with positive relationships reported in previous studies (Rózsa 1997 Morand *et al.* 1999 Clayton & Walther 2001). Nevertheless, these studies are based on interespecific comparisons or focused on fish species in which intraspecific differences in body size can be large, whereas within population variability of body size in Cory's shearwaters is very low.

Regional community structure

Ectoparasite communities differed significantly among host taxa and among host colonies within each taxa. These differences are often geographically structured (Goüy de Bellocq *et al.* 2002; Krasnov *et al.* 2005b; Poulin 2003; Poulin & Morand 1999). In agreement, our results show that similarity in parasite communities decreased with geographic distances among host colonies. Typically, this similarity can be mediated by host-dependent parasite dispersal (Poulin & Morand 1999 Poulin 2003). However, host dispersal may also be influenced by other factors than geographic distance. For example, shearwater dispersal is influenced by the distribution of land masses, prevailing winds and oceanographic features such as productivity and sea surface temperature (Gómez-Díaz & González-Solís 2007, González-Solís *et al.* 2007). In this context, if similarity in parasite community structure is mediated by host connectivity, genetic similarity among host populations should even better associate with structure of parasite communities. However, we did no find such association. Alternatively, in cases where host and parasite dispersal are not linked, other factors such as climate and differences in habitat characteristics among host localities, can also explain the spatial pattern in parasite community structure (Cumming 2002; Krasnov *et al.* 1997; Krasnov *et al.* 2004a; Krasnov *et al.* 2005a). For instance, latitudinal trends in ectoparasite richness among different hosts have been previously reported (see González & Poulin 2005; Poulin 2004). In the present

study correlation between both abundance and prevalence with latitude was poorly supported. Nevertheless, observed patters of parasite community structure match distinct climatic regimes across the host geographic range. Similarity cladogram of parasite community shows three main clusters, corresponding to a temperate Atlantic, Mediterranean and a Subtropical-tropical regime (Fig 4B). That is, Almeria population, although genetically belonging to the Atlantic subspecies of Cory's shearwaters is located within the Mediterranean and grouped with the rest of Mediterranean localities; Lanzarote population, although geographically belonging to the subtropical template Atlantic region of the Canary archipelago, it is much drier than Tenerife due to the absence of mountains, and thus climatically closer to the tropical Islands from the Cape Verde archipelago. Nevertheless, testing this hypothesis would need further examination of climatic variables (i.e. temperature and precipitation) as well as detailed habitat characteristics (i.e. vegetation and altitude) of each breeding colony.

Other factors, such as host population size and population density could also contribute to the observed patterns of regional community structure (Arneberg *et al.* 1998; Poulin & Valtonen 2001; Stanko, Krasnov & Morand 2006). If host population size is related with differences in genetic diversity among colonies, genetic susceptibility to infection could explain differences in abundance and prevalence among host colonies (Whiteman *et al.* 2006). However, we found patterns of geographic variation in ectoparasite community structure did not correlate either with effective size or with genetic diversity of host populations. This result may be related to the low levels of pathogenesis of lice, since we did not detect negative effects on body condition and physiological state of the host (unpublished data). Indeed, it has been suggested that most lice may establish mutualistic relationships with birds (Clayton & Tompkins 1994; Clayton & Tompkins 1995), and thus genetic resistance to parasitism may have not evolved for this host-parasite system.

In summary, we could detect the influence of several factors on the structure of parasite communities at different community levels. Ectoparasite species showed a significant spatial segregation within the host body. Host density and mating influenced patterns of abundance of the most mobile ectoparasite, i.e. the flea. Regional community structure was influenced by

geographic distances among host breeding colonies, but similarity was probably mediated by climatic resemblance rather than by host dependent parasite dispersal. Our results highlight the role of spatial factors as main determinants in shaping ectoparasite communities at different community levels.

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S

Table 1. Abundance and prevalence of three lice and one flea species for *Calonectris* hosts in relation to the breeding colony. Values are means ± standard deviations. Numbers of shearwaters sampled in each breeding colony are shown in brackets.

					Lice						*Flea*	
			Geographic Coordinates		*Halipeurus abnormis*		*Austromenopon echinatum*		*Saedmundssonia peusi*		*Xenopsylla gratiosa*	
Host Taxa	Island population	Archipelago/area	Latitude	Longitude	*Intensity*	*Prevalence*	*Intensity*	*Prevalence*	*Intensity*	*Prevalence*	*Intensity*	*Prevalence*
C .d. borealis	St. Maria (Vila) (90)	Azores Is.	36.94	-25.17	27.20 ± 12.52	100.0	2.92 ± 2.85	82.2 ± 0.4	3.36 ± 3.09	88.9 ± 0.3	2.38 ± 4.96	46.5 ± 1.4
	S. Miguel (8)	Azores Is.	37.71	-25.44	14.38 ± 7.60	100.0	3.38 ± 2.39	87.5 ± 0.4	4.25 ± 4.20	75.0 ± 0.5	2.13 ± 1.96	62.5 ± 1.2
	G.Canaria (107)	Canary Is.	27.85	-15.79	10.07 ± 7.74	97.2 ± 0.2	0.34 ± 0.76	22.9 ± 0.4	0.33 ± 0.75	20.2 ± 0.4	3.39 ± 6.67	56.9 ± 0.5
	Lanzarote (35)	Canary Is.	29.29	-13.54	31.49 ± 21.17	100.0	7.31 ± 7.90	88.6 ± 0.3	0.77 ± 1.46	37.1 ± 0.4	2.94 ± 4.44	74.3 ± 2.4
	Tenerife (9)	Canary Is.	28.45	-16.23	20.22 ± 7.90	100.0	3.67 ± 3.00	88.9 ± 0.4	3.11 ± 3.30	88.9 ± 0.3	2.00 ± 1.58	88.9 ± 0.7
	Almeria (31)	Mediterr. coast	37.35	-1.65	39.26 ± 20.80	100.0	4.19 ± 5.13	71.0 ± 0.5	1.65 ± 2.51	45.2 ± 0.5	0.48 ± 1.00	25.8 ± 0.5
N = 6	Total				23.77 ± 10.95	100.0	3.64 ± 2.25	81.4 ± 7.3	2.25 ± 1.56	67.0 ± 24.5	2.22 ± 1.00	59.4 ± 24.6
C .d. diomedea	Mallorca (29)	Balearic Is.	39.58	2.37	16.66 ± 9.97	100.0	1.17 ± 1.20	65.5 ± 0.5	0.41 ± 0.73	31.0 ± 0.5	0.41 ± 0.78	27.6 ± 0.5
	Ibiza (47)	Balearic Is.	38.96	1.20	26.38 ± 11.87	100.0	0.45 ± 0.80	29.8 ± 0.5	0.97 ± 2.17	36.2 ± 0.5	1.34 ± 1.85	51.1 ± 0.5
	Cabrera (20)	Balearic Is.	39.20	2.98	25.20 ± 12.28	100.0	0.50 ± 1.10	25.0 ± 0.4	0.95 ± 1.43	50.0 ± 0.5	0	0
	Menorca (47)	Balearic Is.	39.80	4.29	19.74 ± 11.38	100.0	0.32 ± 0.73	21.3 ± 0.4	0.66 ± 1.09	36.2 ± 0.5	0.15 ± 0.63	8.5 ± 0.3
N = 4	Total				22.00 ± 4.58	100.0	0.56 ± 0.38	35.4 ± 20.4	0.75 ± 0.27	38.3 ± 8.1	0.48 ± 0.60	21.8 ± 22.7
C. edwardsii	Raso (47)	Cape Verde	16.61	-24.59	17.89 ± 10.47	100.0	5.57 ± 4.37	91.4 ± 0.3	1.74 ± 2.31	62.9 ± 0.5	0.26 ± 0.61	20.0 ± 0.4
	Boavista (35)	Cape Verde	15.98	-22.78	28.57 ± 21.26	100.0	8.23 ± 7.75	93.6 ± 0.3	0.74 ± 1.13	46.8 ± 0.5	1.11 ± 1.82	40.4 ± 0.5
N = 2	Total				23.23 ± 7.55	100.0	6.90 ± 1.88	92.5 ± 1.6	1.24 ± 0.71	54.8 ± 11.4	0.69 ± 0.60	30.2 ± 14.4

Figure legends

Figure 1. Breeding colonies of Mediterranean (●) and Atlantic (O) Cory's shearwaters and Cape Verde shearwaters (Δ) sampled across their geographic distribution.

Figure 2. Ectoparasite abundance in Cory's shearwaters from Gran Canaria (Canary Is.) throughout the breeding season: (A) *Halipeurus abnormis*, (B) *Austromenopon echinatum*, (C) *Saedmunssonia peusi* and (D) *Xenopsilla gratiosa*. PL: pre-laying, EL: egg-laying, MI: mid-incubation, H: hatching, and CR: chick-rearing stages. Lines indicate mean values for adult ectoparasites (continuous and grey line), nymphs (discontinuous lines) and for total abundance (continuous and black line). Intervals indicate standard error.

Figure 3. Mean percentatge of ectoparasite species on each of 5 host body regions in 109 Cory's shearwaters from Gran Canaria (Canary Island) sampled during the incubation period. *X.g.* = *Xenopsilla gratiosa, A.e.* = *Austromenopon echinatum*, *S.p*. = *Saedmunssonia peusi* and *H.a.* =*Halipeurus abnormis*.

Figure 4. Neighbour- Joining cladogram showing relationships among Cory's and Cape Verde shearwaters breeding colonies based on genetic data (A) and ectoparasite intensity data of their component communities (B). Groups corresponding to Atlantic and Mediterranean Cory's shearwater and Cape Verde shearwaters are indicated in bold, italics and normal font, respectively. Genetic cladogram is based on F_{ST} pairwise genetic distances among colonies. Ectoparasite intensity cladogram is based on Chi-squared pairwise distances among host breeding colonies.

Fig. 1

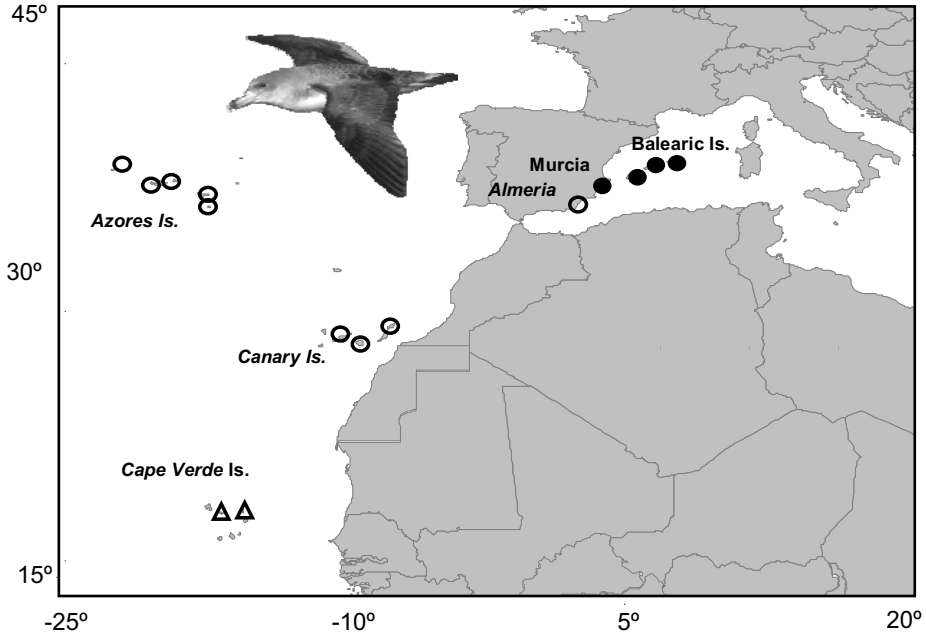

45º
30º
15º
-25º
-10º
5º
20º
Azores Is.
Canary Is.
Cape Verde Is.
Murcia
Almeria
Balearic Is.

Fig. 2

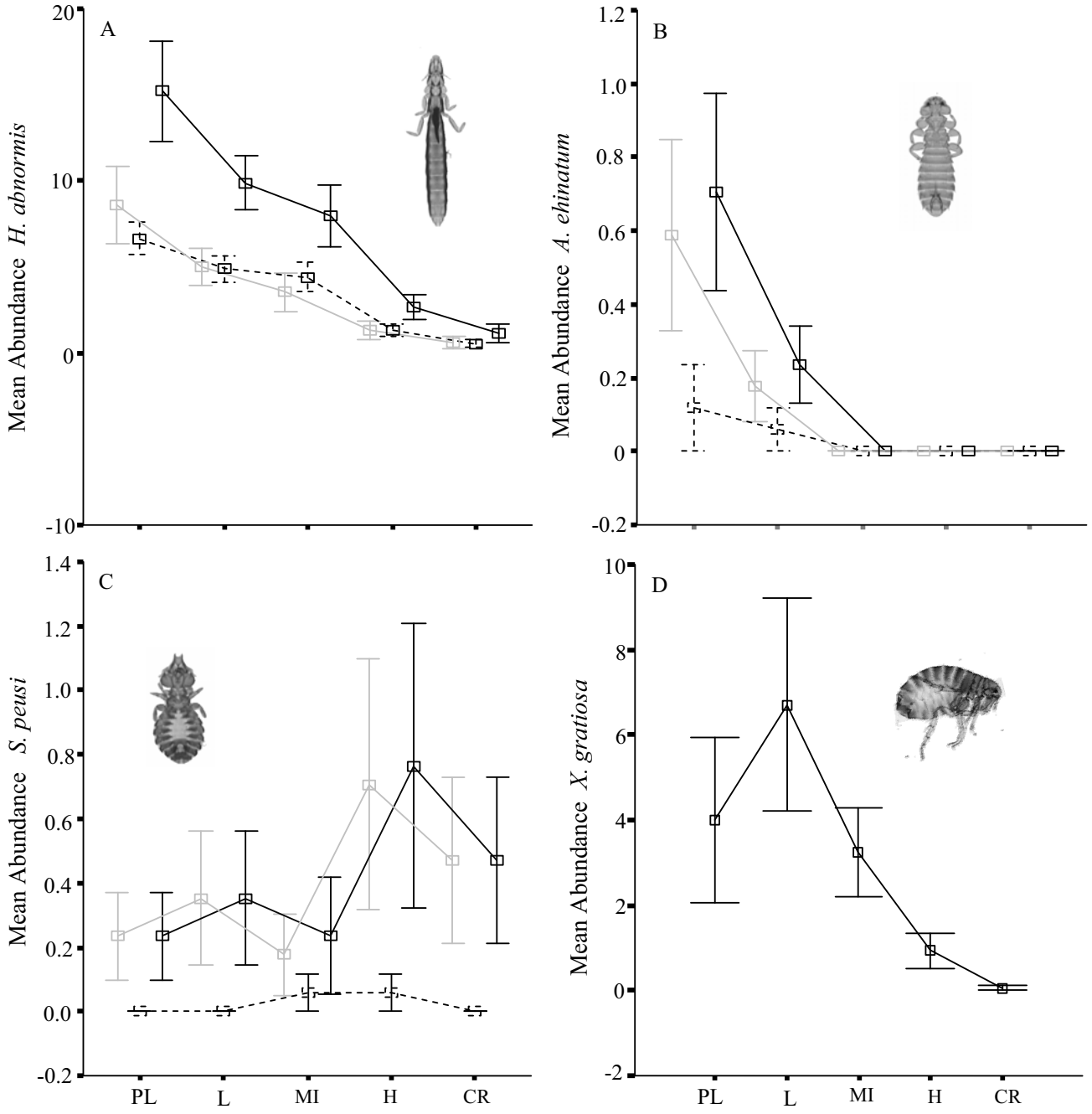

A
B
C
D
Mean Abundance H. abnormis
Mean Abundance A. ehinatum
Mean Abundance S. peusi
Mean Abundance X. gratiosa
20
10
0
-10
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
10
8
6
4
2
0
-2
PL
L
MI
H
CR

Fig. 3

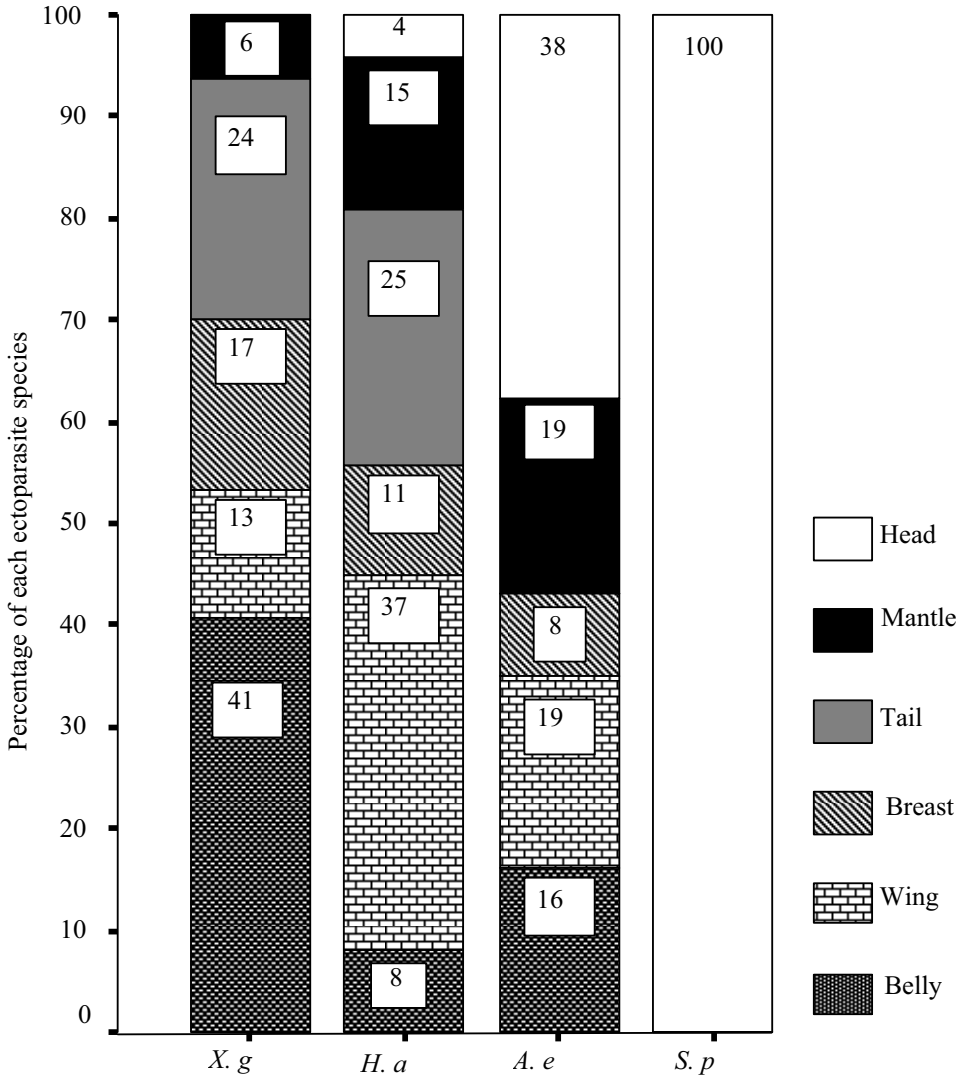
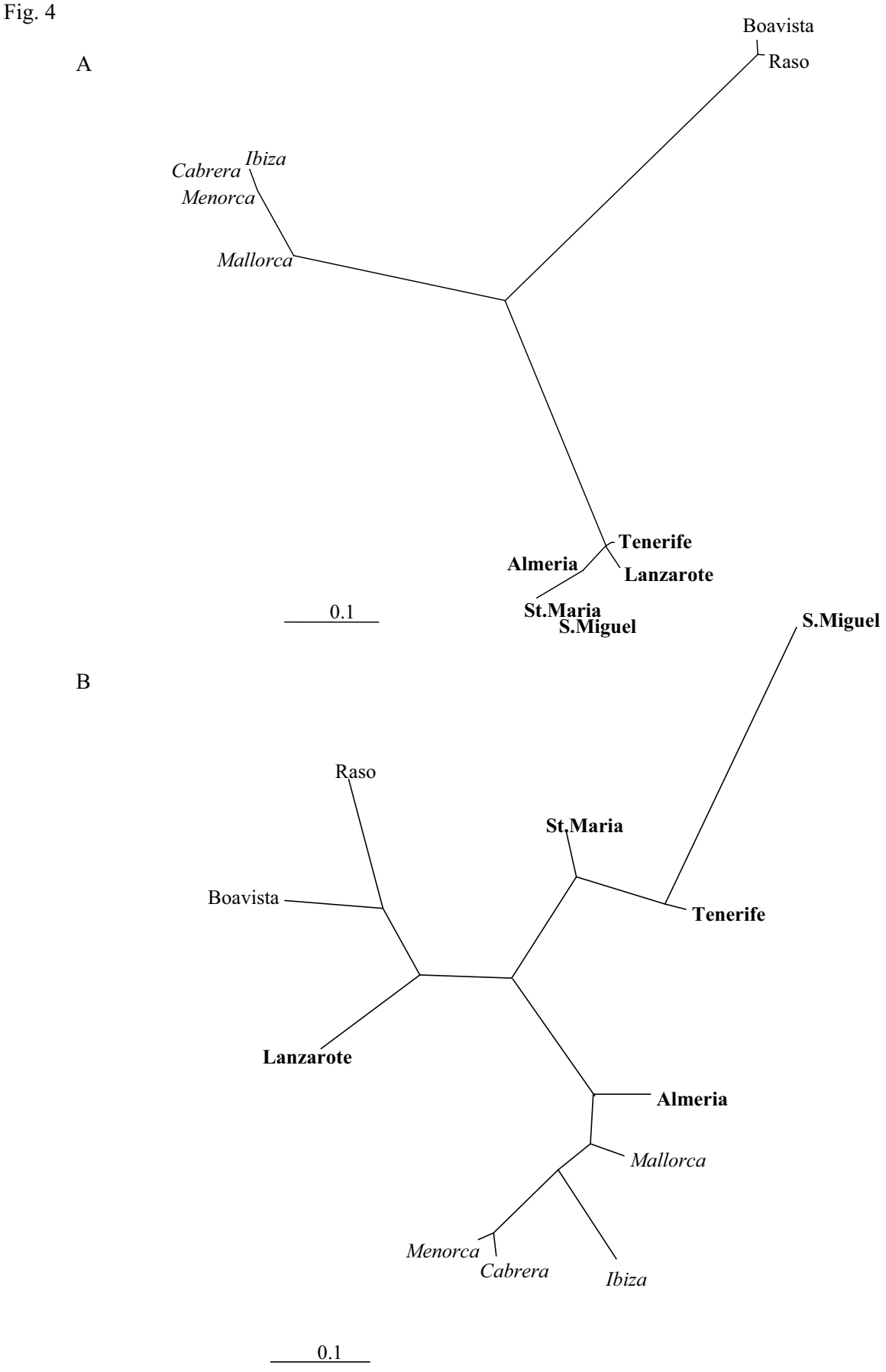

Percentage of each ectoparasite species
100
90
80
70
60
50
40
30
20
10
0
6
24
17
13
41
4
15
25
11
37
8
38
19
8
19
16
100
X. g
H. a
A. e
S. p
Head
Mantle
Tail
Breast
Wing
Belly

Fig. 4


A
Boavista
Raso
Ibiza
Cabrera
Menorca
Mallorca
Tenerife
Almeria
Lanzarote
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S.Miguel
0.1
B
S.Miguel
Raso
St.Maria
Boavista
Tenerife
Lanzarote
Almeria
Mallorca
Menorca
Cabrera
Ibiza
0.1

Lack of host-dependent genetic structure in ectoparasites of *Calonectris* shearwaters

Elena Gómez-Díaz, Jacob González-Solís, Miguel Ángel Peinado, Roderic D. M. Page

Molecular Ecology (provisionally accepted and pending final approval)

Capítol 4

Una vegada coneixíem l'estructuració filogeogràfica i l'estructuració genètica de poblacions de l'hoste, el següent pas era fer el mateix per cadascuna de les especies d'ectoparàsits i comparar els patrons obtinguts. Amb aquest objectiu, en aquest article varem escollir una regió del genoma mitocondrial homòloga en hoste i ectoparàsits per investigar de forma paral·lela les relacions genètiques entre les poblacions de cadascuna de les tres espècies de polls de la ploma i de l'espècie de puça. Això ens va permetre comparar els patrons de diferenciació i les taxes de divergència en hoste i ectoparàsits i avaluar la influencia de l'especificitat del paràsit en el grau de congruència amb l'hoste.

Manca d'estructuració genètica hoste- depenent en els ectoparàsits de les baldrigues del gènere *Calonectris*

Varem comparar els patrons de diferenciació del DNA mitocondrial en tres espècies de polls de la ploma hoste- específics (*Halipeurus abnormis*, *Austromenopon echinatum* and *Saemundssonia peusi*) i una puça generalista (*Xenopsylla gratiosa*), de 22 colònies de *Calonectris.* Els tres hostes: la baldriga cendrosa Atlàntica i la Mediterrània (*Calonectris d. diomedea* i *C. d. borealis*) i la baldriga de Cap Verd (*C. edwardsii*) mostren una estructuració filogeogràfica distinta. Els polls de la ploma hoste- específics apareixien indiferenciats entre les tres espècies de *Calonectris*, mentre que la puça, més generalista, mostrava uns nivells de diferenciació genètica significatius. Ni les distàncies genètiques entre les poblacions d'hoste, ni la seva distribució geogràfica explicava els patrons de variabilitat genètica observats en els ectoparàsits. La manca de diferenciació entre els polls de la ploma es inesperada, donat que els polls dels ocells marins mostren nivells de coespeciació notables amb els seus hostes, i tenen una taxa d'evolució en el DNA mitocondrial elevada. Discutim les implicacions d'aquestes troballes.

Lack of host-dependent genetic structure in ectoparasites of *Calonectris* shearwaters

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Running title

Host-ectoparasite genetic structure in *Calonectris*

Abstract

We compared patterns of mitochondrial DNA differentiation in three host-specific lice (*Halipeurus abnormis*, *Austromenopon echinatum* and *Saemundssonia peusi*) and one generalist flea (*Xenopsylla gratiosa*), parasitizing 22 colonies of *Calonectris.* The three closely related hosts: the Atlantic and the Mediterranean Cory's (*Calonectris d. diomedea* and *C. d. borealis*) and the Cape Verde (*C. edwardsii*) shearwaters show distinct phylogeographic structure. The host-specific lice appeared undifferentiated among the three *Calonectris* taxa, whereas the more generalist flea displayed significant levels of population differentiation. Neither genetic distances among host populations, nor their spatial distribution explained the patterns of genetic variability observed in the ectoparasites. The lack of differentiation among lice is unexpected, given that seabird lice show high levels of cospeciation with their hosts, and have an elevated rate of mitochondrial DNA evolution. We discuss the implications of these findings.

Keywords: lice, fleas, coevolution, population differentiation, host specificity, congruence.

Introduction

Host-parasite cospeciation has mainly been investigated at inter-specific level (e.g. Paterson *et al.* 1993; Hafner *et al.* 1994; Page 1994; Johnson *et al.* 2003a), whereas factors and processes acting at microevolutionary scale have received little attention. At the population level, the dynamics and coevolution of host-parasite interactions would ultimately depend on the genetic variation and its structure in both interacting species (Thompson *et al.* 1994). Therefore, factors acting at this level can play an important role as the causal factors driving coevolution (Clayton and Johnson 2003; Clayton *et al.* 2004). Under a cospeciation scenario, any factor promoting the isolation of hosts will result in the isolation of the parasite thus favouring host and parasites to codiverge. In this context, congruent patterns of genetic structuring for both host and parasites at different levels would be expected.

Several factors such as the relative rates of host-parasite dispersal, host specificity and host geographic distribution, can influence congruence between host and parasite population genetic structures (Blouin 1995; Dybdahl and Lively 1996; Johnson *et al.* 2002; McCoy *et al.* 2003; Weckstein 2005). Firstly, only in cases where host and parasite dispersal are linked is genetic structure expected to be correlated (McCoy *et al.* 2003). Besides, the degree of congruence will also depend to some extent on host specificity which in turn is influenced by the ecology and the dispersal ability of the parasite (Whiteman *et al.* 2004, Clayton *et al.* 1992, Hahn *et al.* 2000; Tompkins and Clayton 1999). Parasites that differ in their ability to disperse are expected to differ in their degree of host specificity, and ultimately in the degree of congruence observed (Johnson *et al.* 2002). Where parasites lack host specificity, the geographic distribution of the hosts can also play an important role in structuring parasite populations (Weckstein 2005). Finally, ecological factors that affect the distribution and abundance of host and parasites, i.e. patchily distribution of parasites among hosts as well as spatial patterns of distribution of their hosts, can also affect congruence (Clayton *et al.* 2004; Rannala and Michalakis 2003).

Lice (Insecta: Phthiraptera) have long be seen as useful model organisms for understanding coevolutionary relationships between hosts and parasites because of their high host specificity (Hafner and Nadler 1988; Paterson *et al.* 1993; Page 1994; Hafner *et al.* 1994; Page 1996; Paterson *et al.* 2000; Paterson *et al.* 1995). Louse species are often restricted to a single species or genus of host, and spend all their life cycle on that host (Marshall 1981). Thus opportunities for vertical transmission down through a host lineage are much more likely than horizontal transmission between different host lineages (Page 1996). Nonetheless, differences in the degree of host specificity among lice genera on the same hosts have been previously reported. For example, pigeon body lice are be more host specific than wing lice (Johnson *et al.* 2002). Conversely, fleas are obligatory blood feeders closely associated with the host's environment (nest). Fleas spend only one stage of their life on the host during the breeding season, and tend to be less host specific than lice. Whereas similarity in the degree of genetic structure is expected among highly specific species, it may decrease as more generalist the parasite (Clayton *et al.* 2004). Although several studies investigated congruence among evolutionary trees for lice and their vertebrate hosts, few studies have investigated the degree of congruence among different species of lice on the same host species (Johnson *et al.* 2002), and no study to date and no study to date explores those patterns including other ectoparasite species simultaneously. In this context, fleas offer a great opportunity to investigate the influence of dispersal ability of parasites in host-specificity which would explain differences in the degree of congruence among different ectoparasite species.

Seabirds, particularly petrels and shearwaters, provide an interesting model to investigate the ecological effects of host isolation and geographic distance on the genetic structure of parasite communities. Since they breed on oceanic islands and spend most of their life in the open ocean, dispersal of parasites is spatially and temporally limited. Furthermore most procellariform species show strong philopatry to natal and breeding sites which would limit dispersal promoting genetic isolation and differentiation among populations (Brooke 2004). Nevertheless, some seabirds studies can travel enormous distances (Croxall *et al.* 2005),

although the extent of population genetic and phylogeographic structure can vary extensively among species (Friesen *et al.* 2007).

Here we chose the Atlantic, the Mediterranean Cory's (*Calonectris diomedea*) and the Cape Verde (*C. edwardsii*) shearwaters, two closely related seabirds that share three lice (*Halipeurus abnormis*, *Austromenopon echinatum* and *Saemundssonia peusi*) and one flea species (*Xenopsylla gratiosa*), to assess the influence of the host geography and phylogeographic structure on the genetic structure of ectoparasites. We aim i) to compare patterns of genetic structure and levels of genetic differentiation among the three host taxa (the Mediterranean, the Atlantic Cory's and the Cape Verde shearwaters) and ectoparasites (the three lice and the flea species), and ii) to examine if host specificity might reflect differences in the degree of similarity between host and ectoparasite population genetic relationships.

Material and Methodology

Study species and sampling

Cory's shearwater is a colonial and monogamous seabird breeding on islands distributed across the Mediterranean Sea and the NE Atlantic archipelagos, and is differentiated into two subspecies. *Calonectris edwardsii*, from Cape Verde islands, was previously considered a subspecies of *C. diomedea*, but it is now regarded as a full species (see Gómez-Díaz *et al.* 2006).

The Atlantic, the Mediterranean Cory's and Cape Verde shearwaters share 3 species of chewing lice for which have been described as a primary hosts (Price *et al.* 2003): *Halipeurus abnormis* (Piaget, 1885) (Ischnocera: Philopteridae), *Saemundssonia peusi* (Eichler, 1949b) (Ischnocera: Philopteridae), and *Austromenopon echinatum* (Edwards, 1960) (Amblycera: Menoponidae) . Cory's and Cape Verde shearwaters also share 1 species of flea (Siphonaptera: Pulicidae) - *Xenopsylla gratiosa* (Jordan and Rothschild, 1923), which is considered to be specific of three seabird genus: *Calonectris*, *Puffinus,* and *Hydrobates*, and comprising a total of

8 species mainly distributed over the Atlantic and the Mediterranean regions (Beaucournu *et al.* 2005).

From 2001 to 2005 we collected blood samples and ectoparasites from adult birds on 25 breeding colonies of *Calonectris diomedea* across the Mediterranean and Atlantic regions, and two breeding colonies of *C. edwardsii* at Cape Verde islands (Fig. 1). We collected ectoparasites from their hosts by visual examination or by using the dust-ruffling method described by Clayton and Walther (1997). Ectoparasites from individual hosts were kept separated and care was taken to clean all working surfaces between host fumigation. Ectoparasites were stored in absolute ethanol at -20 ºC for subsequent genetic analyses. Louse and flea specimens from each locality were taxonomically identified by Ricardo Palma based on morphological characters (representative lice specimens of each *Calonectris* taxa were deposited at the Museum of New Zealand *Te Papa Tongarewa*).

Amplification and Sequencing

Host DNA was isolated from ethanol-preserved whole blood using the salting-out extraction protocol from Bruford *et al.* (1998). We amplified the mitochondrial cytochrome b gene in two fragments of approximate lengths of 420 and 680 bp using the two primer pairs L14987/H15685 and L15562/H16025. We amplified a 293 bp fragment of Domain I of the mitochondrial control region of all three *Calonectris* species using three specific primers previously designed for the species: CAL2H, CAL4H and CAL1L. Reaction conditions and automated sequencing for both, mitochondrial control region and cytochrome b gene, were those described by Gómez-Díaz et al. (2006).

For fleas and lice we extracted DNA from individual specimens using a salting out protocol for insects (Sunnucks and Hales 1996) and DNeasy Tissue Kit (Quiagen). To assure our species identifications were correct; from individual lice, we extracted DNA by removing the head from the body of the louse and placing both in the digestion buffer. After extraction, the head and body of each louse were stored for further examination.

For all three louse species we amplified 360 base pairs (bp) of the cytochrome oxidase I gene (COI) using the primers L6625 and H7005 as described by Hafner *et al*. (1994). We amplified 665 bp of the mitochondrial cytochrome b gene of *A. echinatum* using previously published primers for *Dennyus* sp. L11120 and H11823 (Page *et al.* 1998). In both *S. peusi* and *H. abnormis* we amplified 563 and 600 bp respectively, of the cytochrome b using two specific primer pairs that we designed based on a few published sequences of various louse species; (CYB-146-L (5' CGAGAATCTTTCTTTCCTTCCATTA 3') and CYB-825-H (5' AAAGTATCATTCTGGTTGAATGTG 3') for *S. peusi*, and P3-HaL (5' TGGGTCTTTGCTGGGAGTAT 3') and P3-HaH (5' ATCAGGGTCCATGACCACAT 3') for *H. abnormis*. For fleas we amplified COII gene by using the primers AtLeu and BtLys (Maekawa *et al.* 1999) following Dittmar et al. (2003) and 359 bp of the cytochrome b gene using primers A5 and B 1.1 (Dittmar de la Cruz and Whiting 2003). PCR reactions were carried out in a total volume of 25 μL containing 40 mM Tris (pH 8.0), 200 mM KCl, 6 mM $MgCl_2$, 0.01% gelatin, 0.4 mM of each primer, 0.15 mM of each dNTP, 2 mM of $MgCl_2$, 0.5u BioTaq DNA polymerase (BioRad Laboratories) and 10-20 ng of DNA template. For both COI and COII amplification procedures followed those outlined in Hafner et al. (1994) and Dittmar et al. (2003), respectively. Amplification conditions for the cytochrome b gene were adjusted in each louse and flea species separately. Each reaction started with 4 min at 94º C, then the amplification was carried out for 40 cycles of denaturation at 94º C for 45 s, annealing at 52ºC (for L11120/H11823, *A. echinatum*; and CYBL/CYBH, *S. peusi*), 56ºC (for A5/B1.1, *X. gratiosa*) or 58ºC (for P3-HaL/P3-HaH, *H. abnormis*) for 45 s, and extension at 72º C for 1 min 30 s. A final extension step at 72º C for 5 min was performed.

Amplification products were separated by electrophoresis in 6% polyacrylamide stained using ethidium bromide and visualized under UV. PCR products were purified using the JETquick PCR Product Purification Spin Kit (Genomed Inc., St.Louis, U.S.A). PCR products were sequenced with the same amplification primers on an automated ABI-301 DNA Sequencher (Applied Biosystems, Foster city, U.S.A) using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems). We used Bioedit version 5.0.1 (Hall 1999) to

assemble, edit and align sequences and all variable sites were confirmed by visual inspections of the chromatograms. To assess the reliability of the data we compared COI and CYB sequences with previously published data on various seabird louse species. Sequences reported in this paper have been placed in GenBank under accession numbers ().

Genetic analyses

To compare genetic differentiation of host and ectoparasites, analyses were first carried out separately for each species. We then contrasted results and tested whether genetic relationships were correlated and related to geographical distributions.

Genetic analyses of the *Calonectris* species (Fig. 3a) were derived from the Domain I of the mitochondrial control region and the cytochrome b gene as described by Gómez-Díaz et al. (2006). Analyses of louse and flea species were derived from the mitochondrial COI (louse) or COII (fleas) and the cytochrome b gene (*Calonectris* and ectoparasite individuals analyzed and geographic locations of populations sampled are included in Table 1).

We used the partition homogeneity test (Farris *et al.* 1994; Swofford 2003) to examine whether there was evidence for different phylogenetic signals between cytochrome b and the cytochrome oxidase I and II genes. No significant differences were found between mitochondrial markers for all species except for *X.gratiosa* (P=0.001). Thus, we performed all the analysis for a composite sequence of 959 bp in *H. abnormis*, 1025 bp in *A. echinatum*, and 898 bp in *S. peusi*; except for the flea species in which we analyzed data for each gene partition separately as well as for the composite sequence (1061 bp). We tested for neutrality for each ectoparasite species using the Tajima's test included in the DNAsp package (Rozas and Rozas 1999). We calculated genetic statistics at the intraspecific level as the gene diversity index, the number of haplotypes and the number of polymorphic sites using DNAsp. Genetic distances at the intraspecific level were calculated using MEGA version 3.0 (Kumar *et al.* 2005). To visualize genealogical relationships of host and ectoparasite species, we constructed a haplotype tree using the split decomposition algorithm implemented in SplitsTree v4.6 (Huson and Bryant 2006).

Both host spatial structure and geographic distribution of populations can be responsible for the observed patterns of genetic differentiation among ectoparasite species populations. We first tested isolation by distance by measuring correlation between ectoparasite genetic distances, measured as Φ_{ST} / (1- Φ_{ST}), and geographic distances, calculated as ln (by-sea geographic distance) of colony pairs (Rousset 1997). Then, we tested correlation between ectoparasite and host genetic distances, both measured as Φ_{ST} / (1- Φ_{ST}). In both cases, we applied Mantel test analysis using *zt* (Bonnet and Van de Peer 2002). The significance test of the *r* statistic was determined by employing a randomization procedure in which the original value of the statistic is compared with the distribution found by randomly reallocating the order of the elements in one of the matrices. To control for the effect of a third matrix C testing the correlation between matrices A and B, we applied partial Mantel test (Smouse *et al.* 1986). First we performed the partial Mantel test between ectoparasites genetic and by-sea geographic distances while controlling host genetic distances. Then, we tested correlation between ectoparasite and host genetic distances while controlling by-sea geographic distances among colonies. In this case, permutation approach applied was that developed by Anderson and Legendre (1999).

Results

For *Calonectris* sequences, the results of the Tajima's test were not significant considering both cytochrome b and the control region (Tajima's D = -1.02; P > 0.10 and D = -0.89; P>0.10, respectively). Regarding ectoparasite species, results of the neutrality test were not significant for all three genes analyzed (COI, COII and CYB) except for cytochrome b data of *H. abnormis* that deviate from neutrality expectations.

Genetic statistics (polymorphic sites, number of haplotypes, haplotype diversity and nucleotide diversity indices) of *Calonectris* and the four ectoparasites species are shown in Table 2.

As regards all three *Calonectris* taxa, in the control region, the average pairwise sequence divergence (uncorrected pairwise distance) of all *Calonectris* populations was 4.4% (S.E. 0.007; Bootstrap 1000 replicates). The sequence divergence estimated between the Atlantic and Mediterranean shearwaters was 2.7% (S.E. 0.8%); similar to the divergence from the Mediterranean subspecies to the Cape Verde shearwater (3.3%; S.E. 1.0%), whereas the divergence between the Atlantic subspecies and the Cape Verde shearwater was slightly greater (4.3%; S.E. 0.9%). In the cytochrome b gene (CYB), uncorrected per cent sequence divergence of all *Calonectris* populations was 0.8% (S.E. 0.002; Bootstrap 10000 replicates). For the CYB, sequence divergence estimated between the Atlantic and Mediterranean shearwaters was 0.8% (S.E. 0.3%) similar to the divergence from the two Cory's shearwater subspecies to the Cape Verde shearwater (0.8%; S.E. 0.3%).

Intraspecific diversity (levels of polymorphism, haplotype and nucleotide diversity) was smaller in lice than for *Calonectris* shearwaters and most louse populations shared identical haplotypes. Genetic divergence among populations in all three lice species for both gene partitions analyzed, cytochrome oxidase I (COI) and cytochrome b (CYB) genes, was low (Table 2). In the COI gene, uncorrected per cent sequence divergence for all species was nearly 0% (0.1%, S.E. 0.001; Bootstrap 10000 replicates). In the CYB gene, average pairwise sequence divergences of the body lice *S. peusi* was slightly greater (0.3%, S.E. 0.001; Bootstrap 10000 replicates), whereas values for either *A. echinatum* or *H. abnormis* did not significantly differ from 0% (0.1%, S.E. 0.001; Bootstrap 10000 replicates). In contrast, flea's genetic structure appears more diverse and complex and displayed great levels of genetic variability in both gene partitions. In the CYB gene, the average pairwise sequence divergence was 7.6% (S.E. 0.008; Bootstrap 10000 replicates), whereas it was 5.5% in the COII (cytochrome oxidase II) gene (S.E. 0.006; Bootstrap 10000 replicates).

Network analysis on *Calonectris* mtDNA, control region and cytochrome b combined data set, indicated a clear geographical pattern as three groups of haplotypes corresponding to each of the two geographically isolated Cory's shearwater subspecies and the Cape Verde species (Fig. 2). In contrast, phylogenetic networks of lice (*A. echinatum*, *S. peusi* and *H.*

abnormis) suggested no genetic structure within each louse species and confirmed the low levels of intraspecific genetic variability observed for lice. Although differentiation appeared slightly greater in *S. peusi* (Fig. 3a), in any case no spatial pattern was apparent (Fig. 3a-c).

The network of the flea *X. gratiosa* obtained for either the two gene partitions separately (COII and CYB genes) or the combined data set, supported intraspecific genetic structure in fleas but genealogical relationships among haplotypes did not agree with their geographic distributions and different flea host races corresponding to each of the three *Calonectris* taxa cannot be distinguished (Fig. 3d). Split decomposition analyses also suggested conflicting phylogenetic signals for the flea. Nevertheless, the network showed great intraspecific divergence, contrary to the results for the other three ectoparasite species.

We applied simple and partial Mantel test analyses to examine correlation among genetic distances of host and ectoparasite species as well as to test the existence of spatial patterns of variation in the genetic structure of populations. For all four ectoparasite species analyzed, simple Mantel test analyses revealed no significant correlation between genetic and geographic distances among populations except for the louse species *S. peusi* (*X. g*: r = 0.117, P = 0.105; *A. e*: r = -0.146, P = 0.132; *H. a*: r = -0.133, P = 0.177; *S. p*: r = 0.302, P = 0.045). We did not find any correlation in genetic distances between *Calonectris* and its ectoparasites (*X. g*: r = 0.007, P = 0.197; *A. e*: r = -0.189, P < 0.05; *H. a*: r = -0.105, P = 0.202; *S. p*: r = 0.109, P = 0.205). In contrast, a spatial pattern of variation in genetic structure was apparent for *Calonectris* as genetic and geographic distances were significantly correlated (r = 0.607, P < 0.001). Partial Mantel test analyses showed similar results. But in this case, in all four ectoparasite species genetic and geographic distances among populations were not correlated (*X. g*: r = 0.097, P = 0.141; *A. e*: r = 0.062, P = 0.367; *H. a*: r = -0.091, P = 0.290; *S. p*: r = 0.286, P = 0.069) neither were ectoparasite and host genetic distances (*X. g*: r = 0.0065, P = 0.4060; *A. e*: r = -0.136, P = 0.138; *H. a*: r = -0.041, P = 0.394; *S. p*: r = -0.043, P = 0.384).

Discussion

Lice have long been seen as useful models in coevolutionary studies and cospeciation between seabirds and feather lice has previously been tested (Paterson *et al.* 1993; Page *et al.* 2004; Paterson and Gray 1997; Paterson *et al.* 2000; Banks *et al.* 2006; Page 1994). Several ecological characteristics of seabirds such as a strong phylopatry, nesting fidelity as well as the fact of living on islands suggest that seabirds and their feather lice represent a host-parasite model in which codivergence can be favoured. Indeed, recent genetic studies on the genus *Calonectris* found deep patterns of phylogeographic structure among the two Cory's subspecies and the Cape Verde species (Rabouam *et al.* 2000; Gómez-Díaz *et al.* 2006). In this context, congruent patterns of genetic structuring among host and parasites are expected, at least a two levels; either between the two Cory's subspecies and the Cape Verde species as well as among populations within each subspecies. However, all three lice species analyzed here exhibited no significant population genetic structure and appeared genetically undifferentiated compared to their seabird host taxa.

Previous work has found an increase rate of evolution in louse mitochondrial DNA, both with respect to other insects (Johnson *et al.* 2003b), a with respect to vertebrate hosts (Hafner *et al.* 1994; Page *et al.* 1998), including in seabirds (Paterson *et al.* 2000; Page *et al.* 2004). On the contrary, here mitochondrial rates of sequence divergence obtained indicated *Calonectris* lice would go far behind host differentiation. Our results also contrast with previous studies that suggested a close association between gene flow in pocket gophers and their lice (Nadler *et al.* 1990). There are several examples of louse species showing very little differentiation compared to their seabird hosts, i.e. *Paraclisis hyalina* on albatrosses. However, all those cases corresponded to louse species parasitizing more than one host, and thus low host specificity of lice may explain incongruence (see Page *et al.* 2004).

Under a cospeciation scenario, synchronous genetic chance and equivalent rates of molecular evolution between host and parasites are expected (Page 1996). Thus in this case, lice may undergo "*failure to speciate"* in response to their host (Paterson and Banks 2001; Johnson

et al. 2003a) ever since all three *Calonectris* taxa have already begun to speciate (Gómez-Díaz *et al.* 2006). Insufficient time of isolation for lice could explain the low levels of genetic divergence observed (Rannala and Michalakis 2003). Otherwise, failure to speciate is likely to occur when gene flow among parasite populations is much higher that of their hosts (Johnson *et al.* 2003a). Since *Calonectris* speciation is allopatric (Gómez-Díaz *et al.* 2006) and lice are relatively immobile, ongoing gene flow among lice from all three *Calonectris* taxa is virtually impossible. Furthermore, for a seabird species living on islands and spending most of its life in the open ocean, lice dispersal is both spatially and temporally limited. However, in some locations up to four species of seabirds may be sympatric with *Calonectris* which could promote parasite dispersal among different localities. Nevertheless, no records of straggling *Calonectris* lice parasitizing other seabird species have been previously documented (see Price *et al.* 2003). In cases where parasite gene flow is independent of host dispersal, geographic distances among localities would be a better proxy examining spatial genetic structure of parasite populations (Blouin 1995; Weckstein 2005; McCoy *et al.* 2005). But in the present study, genetic variation in lice was not structured according neither to host nor to geographic distances among localities. Apart from host vagility, alternative hypothesis such as frequent metapopulation extinction and recolonization events could also act reducing parasite genetic structure (Nadler 1995). Nevertheless, empirical data supporting this hypothesis has not been documented.

Owing to differential levels of host specificity among ectoparasite species (lice and fleas), we would expect to find differences in the degree of congruence observed (Clayton *et al.* 2004). Furthermore, previous evidences suggested that body lice would be more host specific than wing lice and differences in the degree of congruence in the two groups have been documented (Johnson *et al.* 2002). That is, host phylopatry and nest fidelity would limit patterns of dispersal of high specific parasites that spent their whole life cycle on the host and show vertical transmission (i.e. the three lice species here considered) (Clayton *et al.* 2004). On the contrary, the more generalist fleas with greater ability to dispersal could be transmitted horizontally between hosts in the same nest and nearby ones as well as between related seabird host species that share breeding grounds with *Calonectris* shearwaters. Hence, whereas for lice

host and parasite genetic structure might be congruent, in fleas patterns of population structure would be less than the observed for *Calonectris*. In both cases, our results disagree with previous expectations. First, we did not detect significant differences in patterns of genetic differentiation between wing (*Halipeurus* and *Saedmundssonia*) and body (*Austromenopon*) lice. Even so, the degree of genetic divergence was slightly greater in the less mobile lice *S. peusi*. This result agrees with previous findings suggesting this species to be highly specialized being restricted to a particular region in the host body (Gómez-Díaz et al., submitted). Second, regarding differences in congruence between lice and fleas, interestingly whereas in lice all three species displayed low levels population differentiation and phylogeographic structure was lacking, in fleas intraspecific genetic structure is pronounced. Previous genetic studies on lice showed that mitochondrial DNA differentiation for conspecific lice living on different host species was strong (10% of sequence divergence) compared with lice specific of only one host species (1%) (Johnson *et al.* 2002). For all three lice species analyzed here, *Calonectris* shearwaters are primary hosts, whereas the flea species *X. gratiosa* is specific of three different seabird's genus (*Calonectris*, *Puffinus,* and *Hydrobates*) (Beaucournu *et al.* 2005). In this context, our results are in line with previous findings since genetic differentiation for fleas was about ten times greater than in lice. Hence, the genetic structure of flea populations may result from local adaptation to different host species. That is, for such a generalist parasite, great levels of genetic variability can provide evolutionary potential for local host race formation. Previous examples have been reported for ticks and lice parasitizing sympatric hosts (McCoy *et al.* 2001; Johnson *et al.* 2002; McCoy *et al.* 2002; McCoy *et al.* 2003). Besides, empirical evidences confirmed *X. gratiosa* parasitizes sympatric (sharing habitat) and syntopic (sharing nest) seabird species of *Calonectris* shearwaters (Beaucournu *et al.* 2005; personal observation). In this case and according to the local adaptation hypothesis, we would expect a greater genetic similarity between individuals parasitizing different host species in the same breeding grounds, habitat specific species, than between individuals of different host populations of the same host species, host specific species. But whether fleas would be considered as habitat specific rather than host specific parasites, remains poorly understood. Subsequently, further genetic studies in fleas

parasitizing different sympatric seabird hosts across the distribution range of *Calonectris* shearwaters (i.e. *Puffinus*, *Hydrobates*, *Oceanodroma* and *Bulweria* sp.) are needed.

In conclusion, despite of the genetic differentiation of the three seabird taxa, mitochondrial genetic differentiation among lice populations was weak and showed no congruence with the host genetic structure. In contrast, the flea species displayed high levels of intraspecific variability, but neither host genetic relationships nor geographic distributions of populations explained patterns of genetic structuring.

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Table 1. Host species and geographic locations of the three lice and the flea species individuals included in the genetic analyses.

							Coordinates	
Calonectris taxa	Location		*H.a*	*A.e*	*S.p*	*X.g*	Latitude	Longitude
C.d.diomedea	Mallorca	Balearic Is.	*	*	*	*	39.5833	2.3667
	Menorca	Balearic Is.	*	*	*		39.8020	4.2878
	Eivissa	Balearic Is.	*	*	*		38.9618	1.1983
	Murcia	Spain	*	*	*	*		
	Tremiti	Italy	*			*	42.1256	15.4939
	Tuscany	Italy				*		
	Linosa	Italy	*				35.8667	12.8667
	Hyeres	France	*	*	*	*	43.0089	6.2106
	Chafarinas	Morocco coast.				*	35.1833	-2.4167
	Crete	Greece	*	*	*	*	36.4423	25.2272
C.d.borealis	St.Maria	Azores Is.	*	*	*	*	36.9420	-25.1710
	Graciosa	Azores Is.	*	*	*		39.0557	-27.9549
	Corvo	Azores Is.	*	*	*		39.6745	-31.1060
	Madeira	Portugal	*	*	*	*	32.3445	-16.4857
	Selvagens	Portugal	*	*			30.1333	-15.8667
	Berlengas	Portugal	*	*	*	*	39.4089	-9.4939
	G.Canaria	Canary Is.	*	*	*		27.8456	-15.7887
	Lanzarote	Canary Is.	*	*	*	*	29.2930	-13.5372
	La Palma	Canary Is.	*	*		*	28.7800	-17.7965
	Almeria	Spain	*	*	*	*	37.3489	-1.6507
C.edwardsii	Raso	Cape Verde Is.	*	*	*	*	16.6091	-24.5947
	Boavista	Cape Verde Is.	*	*	*	*	15.9833	-22.7833

Resultats

Table 2. Genetic statistics for each ectoparasite species and host taxa. Number of sequence, percentage of variable sites, number of haplotypes and both haplotypic and nucleotide diversity are shown.

Species	n sequences	variable sites		n haplotypes		Haplotypic diversity		Nucleotide diversity	
Lice		*COI*	*CYB*	*COI*	*CYB*	*COI*	*CYB*	*COI*	*CYB*
H. abnormis	20	2/359	5/600	3	5	0.195 ± 0.115	0.368 ± 0.135	0.0056 ± 0.0034	0.0083 ± 0.0024
A. echinatum	18	1/360	3/665	2	3	0.471 ± 0.082	0.307 ± 0.132	0.0013 ± 0.0002	0.0013 ± 0.0003
S. peusi	16	3/332	8/563	4	7	0.442 ± 0.145	0.817 ± 0.033	0.0014 ± 0.0005	0.0028 ± 0.0006
Flea		*COII*	*CYB*	*COII*	*CYB*	*COII*	*CYB*	*COII*	*CYB*
X. gratiosa	15	108/702	75/359	14	13	0.971 ± 0.032	0.963 ± 0.033	0.055 ± 0.003	0.076 ± 0.005
Host		*CR*	*CYB*	*CR*	*CYB*	*CR*	*CYB*	*CR*	*CYB*
C. d. diomedea	24	29/293	14/957	20	9	0.975 ± 0.024	0.812 ± 0.058	0.024 ± 0.002	0.0037 ± 0.006
C. d. borealis	21	30/293	13/957	19	9	0.986 ± 0.022	0.824 ± 0.060	0.024 ± 0.002	0.0023 ± 0.004
C. edwardsii	11	16/293	8/957	7	5	0.909 ± 0.066	0.836 ± 0.080	0.016 ± 0.003	0.0030 ± 0.001

Figure legends

Figure 1. Breeding colonies of Mediterranean (●) and Atlantic (◍) Cory's shearwaters and Cape Verde shearwaters (○) sampled across their geographic distribution.

Figure 2. Haplotype network showing genealogical relationships within *Calonectris*. Groups of haplotypes corresponding to the Atlantic and the Mediterranean Cory's and Cape Verde shearwaters are indicated in black, grey and white circles, respectively. The size of the circles is proportional to the number of birds sharing that haplotype.

Figure 3. Haplotype networks showing genealogical relationships within each ectoparasite species *S. peusi* (A), *A. echinatum* (B), *H. abnormis* (C) and *X. gratiosa* (D). Haplotypes corresponding to the Atlantic and the Mediterranean Cory's and Cape Verde shearwaters are indicated in black, grey and white circles, respectively. The size of the circles is proportional to the numbers of ectoparasites sharing that haplotype.

Figure 1.

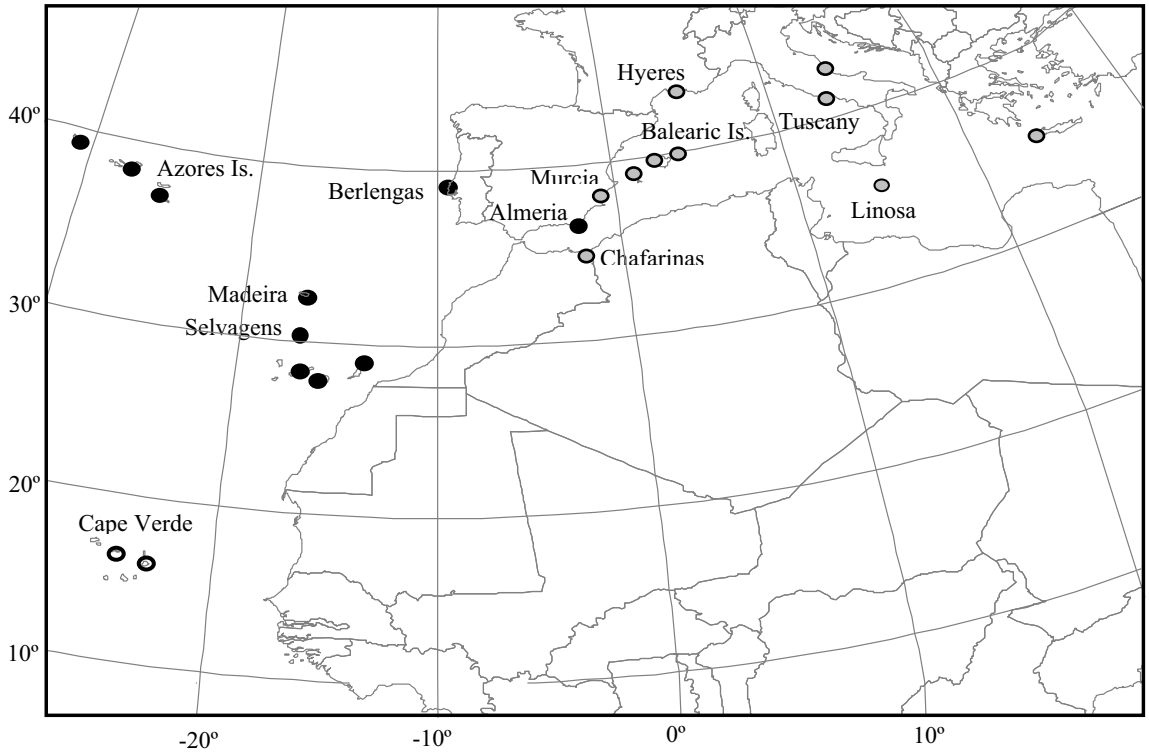

Hyeres
Tuscany
Balearic Is.
Azores Is.
Berlengas
Murcia
Almeria
Linosa
Chafarinas
Madeira
Selvagens
Cape Verde
40º
30º
20º
10º
-20º
-10º
0º
10º

Figure 2.

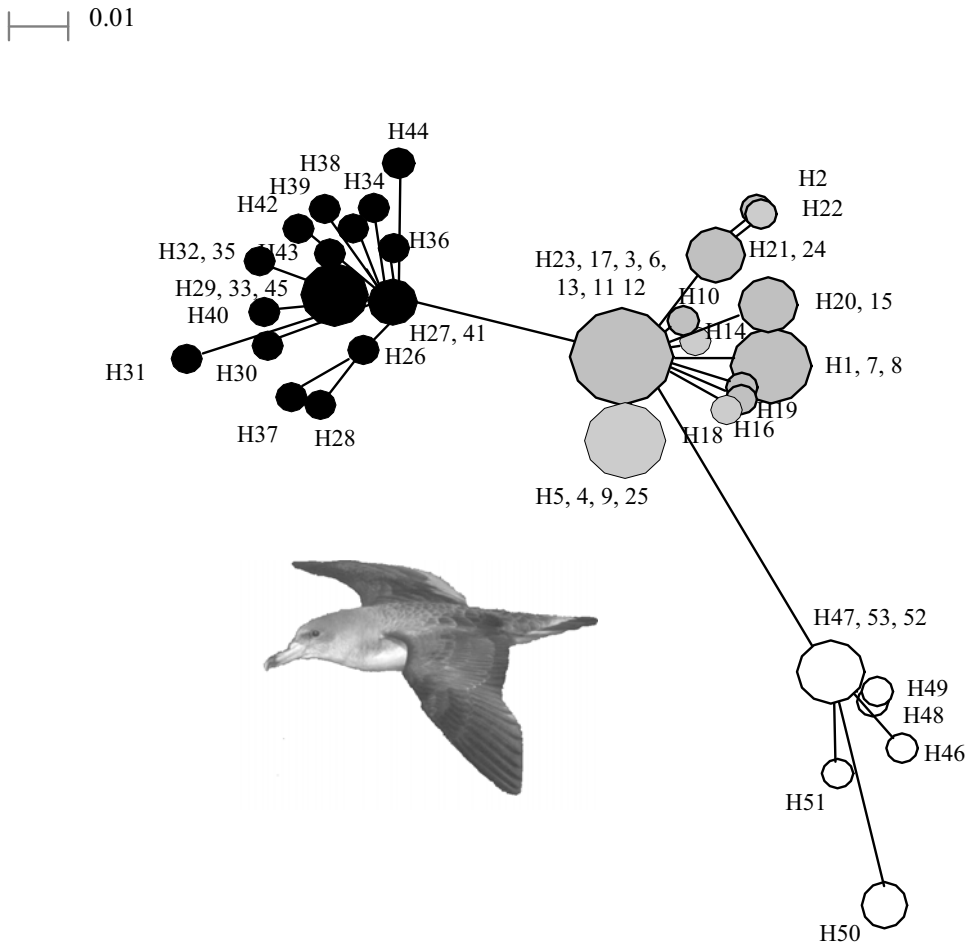
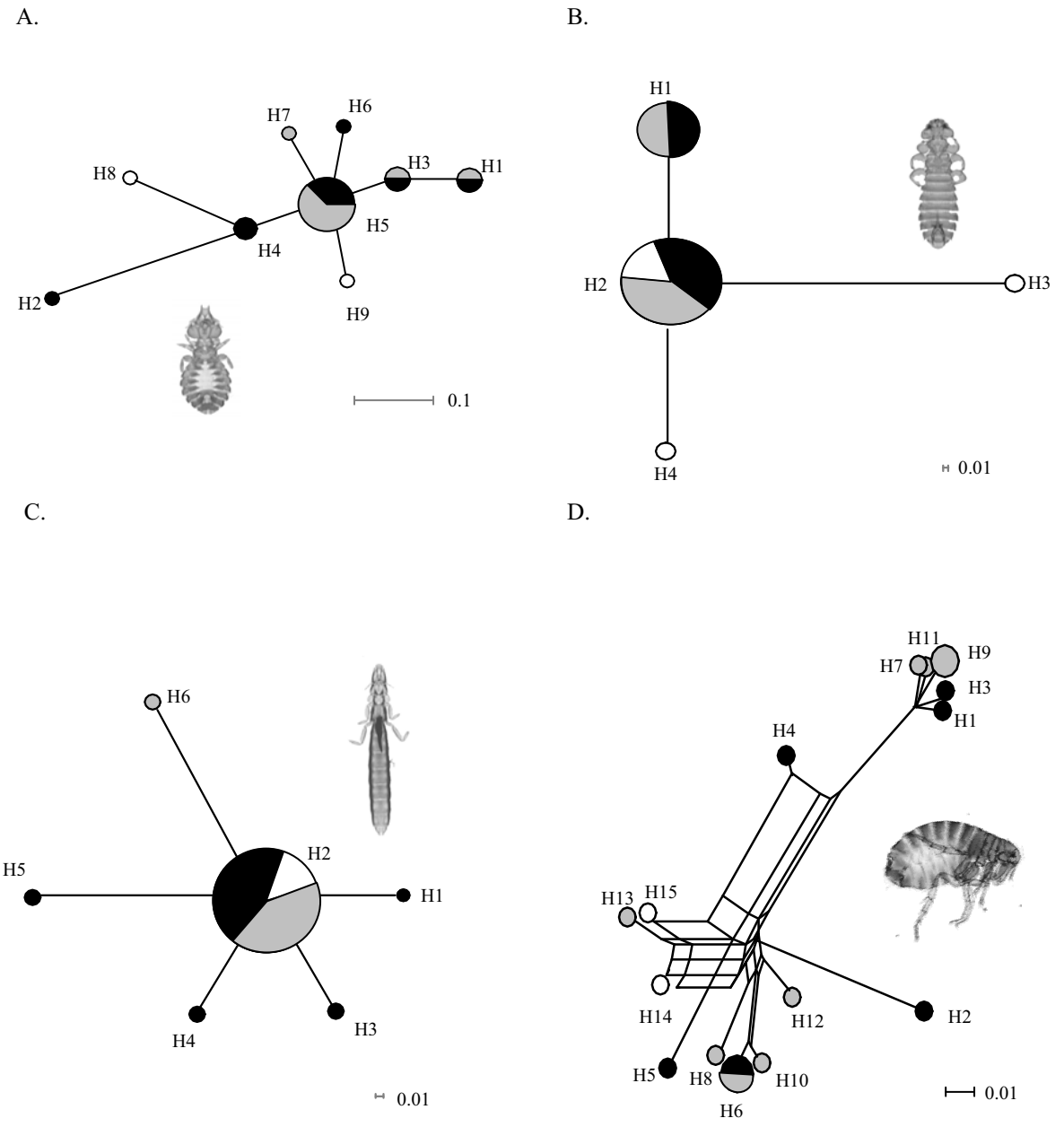

0.01
H44
H38
H39
H34
H42
H36
H32, 35
H43
H29, 33, 45
H40
H27, 41
H31
H30
H26
H37
H28
H2
H22
H21, 24
H23, 17, 3, 6,
13, 11 12
H10
H20, 15
H14
H1, 7, 8
H19
H18
H16
H5, 4, 9, 25
H47, 53, 52
H49
H48
H46
H51
H50

Figure 3.


A.
H7
H6
H8
H3
H1
H5
H4
H2
H9
0.1
B.
H1
H2
H3
H4
0.01
C.
H6
H5
H2
H1
H4
H3
0.01
D.
H11
H9
H7
H3
H1
H4
H13
H15
H14
H12
H2
H5
H8
H10
H6
0.01

Appendix 1. The site matrix shows variable positions on a composite sequence of 1250 bp of the cytochrome b gene (1-956 bp) and the control region (957-1250 bp) in the 53 haplotypes found in all three *Calonectris* taxa. Dots indicate identity with the most common genotypes.

Nucleotide positions

Haplotype	1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 99 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 12
Hap_1	TGAGATACAG GTGGTAAAGA ATTATTGGAT ATAGATAAAT GAGAAGGGGA AGGAGGAGAG GTGGTCGAGG TGCTTTAGAA GAGAAGGTGA AG
Hap_2	.......T.A ..A.G..GT. G.......G. .....C...C ..AG...... ..A....A.. ..A....... ....C..A.. A.....A... G.
Hap_3	.........A ..A.....T. G.......G. .....C...C ...G.A.... ...G...A.. ..A....... ......GA.. ......A... ..
Hap_4	...A.....A ..A.....T. G.......G. .....C...C ...G...... ....A.. ..A.C..... ....A.. ......A... ..
Hap_5	...A.....A ..A.....T. G.......G. .....C...C ...G...... ....A.. ..A.C..... ....A.. ......A... G.
Hap_6	.........A ..A.....T. G.......G. .....C...C ...G.A.... ...G...A.. ..A....... ......GA.. ......A... G.
Hap_7	.... G.
Hap_8	....G...... ....
Hap_9	...A.....A ..A.....T. G.......G. .....C...C ...G...... ....A.. ..A.C....A ....A.. ....
Hap_10	.........A ..A..G..T. G.......G. .....C...C ....G.A.... ..A....... ......GA.. ......AC.. G.
Hap_11	.........A ..A.....T. G.......G. .....C...C ..AG...... ...G...A.. ..A....... .A....GA.. ......A... G.
Hap_12	...A.....A ..A.....T. G.......G. .....C...C ...G...... ....A.. ..A....... ....A... G.
Hap_13	.........A ..A.....T. G.......G. .....C...C ...G....A. .A...AGA.. ..A....... ....A.. ......AC.. G.
Hap_14	C........A ..A.....T. G.......G. .....C...C ...G....A. G..G.A.A.. ..A.....A. ....C.GA.. .... G.
Hap_15	.........A ..A.....T. G.......G. .....C...C A..G...... ...G...A.. ..A....... ......GA.. .G....A... G.
Hap_16	......G..A ..A....... ....G....A. .A...A.A.. ..A....... .....C.A.. ......A... G.
Hap_17	.........A ..A.....T. G.......G. .....C...C ...G.A..A. .....A.A.. ..A....... ....A.. ......A... G.
Hap_18	....G...A.. GA........ ..A....... ......G... .... G.
Hap_19	.........A ..A.....T. G.......G. .....C...C .....A..AG ...G...AG. ..A....... ......GA.. ......A... G.
Hap_20	....A.....T. G.......G. .....C...C A..G...... ...G...A.. ..A....... ......GA.. .G....A... G.
Hap_21	.........A ..A....GT. G.......G. .....C...C ..AG...... ....A.. ..A....... ....C..A.. A.....A... G.
Hap_22	.........A ..A....GT. G.......G. .C...C...C ...G....A. ....A.. ..A....... ....C..A.. A.....A... G.
Hap_23	.........A ..A.....T. G.......G. .....C...C ...G...... ...G...A.. ..A....... ......GA.. ......A... G.
Hap_24	.........A ..A....GT. G.......G. .....C...C ..AG...... ..A....A.. ..A....... ....C..A.. A.....A... G.
Hap_25	...A.....A ..A.....T. G.......G. .....C...C ...G...... ....A.. ..A.C..... ....A... G.
Hap_26	........G. A.A......G G.CG..A.G. .....C..GC ...G...T.. .....AG... ..CA.....A .A....G... .....T.C.. GA
Hap_27	........G. A.A......G G.CG....G. .....C..GC ...G...T.. .A.G...... ..CA...... ......G... .....T.C.. G.
Hap_28	........G. A.A......G G.CG....G. .....C..GC ...G...T.. .....AG... ..CA.....A .A.C..G... ..A.GT.C.. GA
Hap_29	.A......G. A.A......G G.CG....G. ..G..C..GC ..AG...T.. .....AG... ..CA.....A ......GA.G .....T.CA. G.
Hap_30	........G. A.A.G....G G.CG..A.G. .....C..GC ....T.G .....AG... ..CA.....A .A..C.G... .....T.C.. G.
Hap_31	........G. A.A......G G.CG....G. .....C..GC A.AG...T.. .A.G...AG. ..CA.....A .AT...GA.. .....C.C.G G.
Hap_32	.A......G. A.A......G G.CG....G. ..G..C..GC .G.G...T.. .....AG... ..CA.....A ......GA.G .....T.CA. G.
Hap_33	.A......G. A.A......G G.CG....G. ..G..C..GC ....T.. .....AG... ..CA.....A ......GA.G .....T.CA. G.
Hap_34	........G. A.A......G G.CG..A.G. .....C..GC ..AG...T.. .....AG... ..CA.....A .A....G.G. .....T.C.. G.
Hap_35	.A......G. A.A......G G.CG....G. ..G..C..GC .G.G...T.. .....AG... ..CA.....A ......GA.G .....T.C.. G.
Hap_36	........G. A.A......G G.CG....G. ..C..C..GC ....T.. .A.GA.GA.. ..CA.....A .A....G... .....T.C.. G.
Hap_37	........G. A.A......G G.CG....G. .....C..GC ...G...T.. .....AG... ..CA.....A .A.C..G... ...G.T.C.. GA
Hap_38	........G. A.A......G G.CG.C.... .....C..G. ....T.. .A.GA.G... ..CA.....A .A....G... .....T.C.. G.
Hap_39	.... A.A......G G.CGC..TG. .....C..GC ...G...T.. .A.GA.GA.. ..CA.....A .A....G... .....T.C.. G.
Hap_40	.A......G. A.A......G GCCG....G. ..G..C..GC ...G...T.. .....AG... ..CA.....A ......GA.G .....T.CA. G.
Hap_41	........G. A.A......G G.CG....G. .....C..GC ...G...T.. .A.G...... ..CA.....A ......GA.G .....T.CA. G.
Hap_42	........G. A.A......G G.CG....G. ...A.C.GGC ....T.. .....AG... ..CA.....A ......G... .....T.C.. G.
Hap_43	........G. A.A......G G.CG....G. .....C..GC .....A.T.. .A.G..GA.. ..CA.....A .A....G... ....GT.C.. G.
Hap_44	........G. A.A......G G.CG....G. .....C..GC ..AG...T.. .....AG..A ..CA.....A CA....G... .....T.C.. G.
Hap_45	.A......G. A.A......G G.CG....G. ..G..C..GC ...G...T.. .....AG... ..CA.....A ......GA.G .....T.CA. G.
Hap_46	..G.....G. ACAA..G... G.......G. ....GC...C ...GG..T.. ...G.A.... AC....AGA. ......GA.. .....A.... G.
Hap_47	..G.....G. A.A...G... G.......GG ....GC...C ...GG..T.. ...G.A.... AC...TAGA. ......GA.. .....A.... G.
Hap_48	..G.....G. A.A...G... G.......GG ....GCG..C ...GG..T.. ...G...A.. AC...TAGA. ......GA.. .....A.... G.
Hap_49	..G.....G. A.A...G... G.......GG ....GC...C ...GG.AT.. .....A.... AC....AGA. ......GA.. .....A.... G.
Hap_50	..G.GC..G. A.A...G... G.......G. G...G....C ..AG...T.G ..AG.A.... AC.....GA. ..TC..GA.. .....A.... G.
Hap_51	..G.GC..G. A.A...G... G.......G. ....GC...C ...GG..TAG .A.G...... AC....AGA. ....C.GA.. .....A.... G.
Hap_52	..G.GC..G. A.A...G... G.......G. G...G....C ...GG..T.. ...G...A.. AC...TAGA. ......GA.. .....A.... G.
Hap_53	..G.....G. A.A...G... G.......GG ....GC...C ...GG..T.. ...G...... AC....AGA. .A....GA.. .....A.... G.

Appendix 2A. The site matrix shows variable positions on a composite sequence of 1025 bp of the cytochrome b (1-665 bp) and the cytochrome oxidasa I gene (666-1024 bp) in the 4 haplotypes found in *A. echinatum*. Dots indicate identity with the most common genotypes.

	Nucleotide positions
Haplotype	1234
Hap_1	TACA
Hap_2	...G
Hap_3	C.TG
Hap_4	.G.G

Appendix 2B. The site matrix shows variable positions on a composite sequence of 959 bp of the cytochrome b gene (1-600 bp) and the cytochrome oxidasa I gene (601-959 bp) in the 6 haplotypes found in *H.abnormis*. Dots indicate identity with the most common genotypes.

	Nucleotide positions
Haplotype	1234567
Hap_1	CAGTATT
Hap_2	...G...
Hap_3	T..G...
Hap_4	...G..C
Hap_5	.G.GG..
Hap_6	..AG.C.

Appendix 2C. The site matrix shows variable positions on a composite sequence of 898 bp of the cytochrome b gene (1-563 bp) and the cytochrome oxidasa I gene (564-898 bp) in the 9 haplotypes found in *S. peusi*. Dots indicate identity with the most common genotypes.

	Nucleotide positions
	1 1
Haplotype	1234567890 1
Hap_1	TTGGGTGAGA A
Hap_2	G.A...AGA. G
Hap_3	........A. .
Hap_4	G.A.....A. .
Hap_5	G.......A. .
Hap_6	GC......A. .
Hap_7	G..A....A. .
Hap_8	G.A.AC..A. .
Hap_9	G.......AG .

Resultats

Appendix 2D. The site matrix shows variable positions on a composite sequence of 1061 bp of the cytochrome b gene (1-359 bp) and the cytochrome oxidasa II gene (360-1061 bp) in the 53 haplotypes found in *X. gratiosa*. Dots indicate identity with the most common genotypes.

	Nucleotide positions										
	1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 0000000001 1111111112										
Haplotype	1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890										
Hap_1	AAAAAAAAAT CTATTAGGTA TTAATACTTA TAATTCTATT ATTAAATCTA ACTATCCATT TTATATGTTA GATTCCAATT TCTTAATACG ATACCACAAG TCCAACTTTT TGTACAAATA
Hap_2	G.TGG.T... T..C..TA.. AGG...T.CT C..CCTA..C GC.T..CT.. T..T.TT.CA C..AG.ACCC T....T...C ATGCG.G.TA ...T..TG.C ..T.C.AGCC ....T.....
Hap_3	....G..... ....G...... ....
Hap_4	.....G.... .... C......... ....TTG... ......C... ..C....... ...G...... CA..TG..A.
Hap_5	..T...T... TC...GAA.. AG...GT.C. CC..CTG... ...T.GC... T..TCTT.CA C..A..A.C. T...TT..A. AT...GA.T. G...TGT..C A.T.TTAA.. CA..T.G.AG
Hap_6	.GT..GT..C T.G.C.AAC. A.G.C.TC.. ..GC.TG.C. ..CT..C... .T.T..T..A C.GA..A... T..CTT.G.. AT...GGGTA .C....T.G. ..T.T.AG.. ..C.T..GA.
Hap_7	.... C....C.... ....C....... ...G...... ....T.....
Hap_8	.GT..GT..C T.G.C.AA.. G.G.C.T... ..GC.TG.C. ..CT..C... .TCT..T..A C.GA..A... T..CTT.G.. AT...GGGTC .C....T.G. ..T.T.AA.. ..C.T..GA.
Hap_9	.... C....C.... ....C....... ...G...... ....T.....
Hap_10	.GT..GT..C T.G...AAC. G.G.C.T... ..GC.TG.C. ..CTG..... .TCT..T..A C.GA..A... TGC.TT.G.. AT...GGGTA .C....T.G. ..T.T.AGC. .ACGTG..A.
Hap_11	.T........ ....G...... .... C....C.... ....C....... ...G...... ....T.....
Hap_12	..T...T... T.....AA.. G.....T... C..C..A.C. ..C...C..G .T.T..TG.A C..A..A... T.C.TT.G.. AT...GA.TA .C....T... ..T.T.AAC. .A..TG..A.
Hap_13	..T..GTGG. .A..C.AA.G AA..C.T.C. CC...TA... ...T..C... T..T.TT.CA C..A..AC.. T...TT.... AT.C.GG.TA ...TT.TG.C .TT.T.AA.. CA..TG..A.
Hap_14	..T..GTGG. .A..C.AA.G AA..C.T.C. CC...TA.C. ...T..C.C. T..T.TT.CA CC.A..AC.. T...TT.... AT.C.GA.TA ...TT.TG.C .TT.T.AG.. ....T..GA.
Hap_15	..T..GTGG. .A..C.AA.G AA..C.T.C. CC...TAGC. ...T..C.C. T..T.TT.CA CC.A..AC.. T...TT.... AT.C.GA.TA ...TT.TG.C .TT.T.AG.. CA..TG..A.

Appendix 2D (continued).

	Nucleotide positions						
	1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 111						
	2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 888						
Haplotype	1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 123						
Hap_1	CTTTTATCTA TTTATATTTT AACTCAATTA TCCATTATTA TCTTATTTTC CCATTCACAG CAT
Hap_2	T.AC...TA. ......C.CC C...T.T... CTTT..T.C. .AC.TA..CA .TCC.T.T.A TCC
Hap_3	....TT.. ...
Hap_4	TAA...CTA. CC..... C.T.T.T... CTTT..T..T .A..TA.CC. TT..C..TGA TT.
Hap_5	.AA...CTAG .CCG.....C CTT.T.T... CTTTAAT... .AC.TA..CT T...CT.T.A TTC
Hap_6	..G....TA. A......C.C C.TCT.TC.T CTTT.ATA.. .T.ATA...T TTT.CT.T.A TT.
Hap_7	....CG.... ....G..CT ....T .... T..
Hap_8	..G....TA. A......C.C C.TCT.TC.T CTTT.ATA.. .T.ATA...T TTT.CT.T.A TT.
Hap_9	.....G.... ....G..CT ....T .... T..
Hap_10	..A....TA. A........C C.TCT.TC.T CTTT.ATA.. .T.ATAC... TTT.CT.T.A TT.
Hap_11	.....G.... ....G..CT ....T .....A.... T..
Hap_12	..A....TAG A...CG...C C.T.T.TC.T CTTT.CTA.. CT.ATA.... TTT....T.A TT.
Hap_13	TAA...CTA. CC..... C.T.T.T... CTTT..T..T .A..TA.CC. TT..C..T.A TT.
Hap_14	..G....TA. A........C C.TCT.TC.T CTTT.ATA.. .T.ATA...T TTT.CT.T.A TT.
Hap_15	TAA...CTA. CC..... C.T.T.T... CTTT..T..T .A..TA.CC. TT..C..TGA TT.

Geographic assignment of seabirds to their breeding origin: combining morphology, genetics, stable isotopes and trace elements in Cory's shearwater.

Elena Gómez-Díaz, Jacob González-Solís

Ecological Applications (In press)

Capítol 5

Les amenaces per als ocells marins s'han multiplicat al llarg de les últimes dècades de la mà d'un increment en la pressió antropogènica. L'estudi de la filogeografia i de l'estructuració genètica de les poblacions d'ocells marins és essencial per a mesurar el impacte d'aquestes activitats i per a delinear unitats de conservació apropiades. Però les eines moleculars per si mateixes a vegades no són suficients per assolir alguns d'aquets objectius. Així, doncs aquest article va molt més enllà quant a les implicacions d'aquesta tesi per a la conservació dels ocells marins. El que proposem aquí és una nova aproximació combinant diversos marcadors intrínsecs (moleculars, biomètrics i biogeoquímics) per l'assignació geogràfica i que obre noves expectatives per a l'estudi de la mortalitat d'ocells marins al mar.

Assignació geogràfica d'ocells marins a les seves colònies de cria: combinant morfologia, genètica, isòtops estables i elements traça en la baldriga cendrosa.

Les pesqueries del palangre, els vessaments de petroli i els parcs eòlics en alta mar; son algunes de les amenaces que estàn incrementant la mortalitat d'ocells marins al mar, però el impacte d'aquestes activitats en poblacions específiques ha estat difícil de determinar fins ara. Varem testar la utilització de marcadors moleculars, mesures morfomètriques, així com isòtops estables ($\delta^{15}N$ i $\delta^{13}C$) i elements traça en la primera ploma primària (crescuda al final del període reproductor) per assignar el origen geogràfic de baldrigues *Calonectris*. En conjunt, varem mostrejar individus de tres taxons: 13 llocs de cria de la baldriga cendrosa Mediterrània (*Calonectris d. diomedea*), 10 de la baldriga cendrosa Atlàntica (*Calonectris d. borealis*) i un de la baldriga de Cap Verd (*C. edwardsii*). Varem avaluar les taxes d'assignació a tres escales espacials diferents: colònia de cria, arxipèlag de cria i taxa. Els anàlisis genètics basats en la regió de control del genoma mitocondrial (198 individus de 21 colònies) van assignar correctament el 100% dels ocells als tres taxa, però fracassaven a l'hora de detectar una estructuració geogràfica a menor escala. L'anàlisi discriminant basats en la composició d'elements traça va obtenir la millor taxa d'assignació correcta a la colònia (77.5%). Les mesures corporals o els isòtops estables van tenir èxit assignant individus entre taxa (87.9 and 89.9%, respectivament) però van fracassar entre colònies (27.1 i 38.0%, respectivament). La combinació de totes tres aproximacions (morfometria, isòtops, i elements traça en 186 ocells de 15 colònies de cria), va millorar substancialment la taxa de classificacions correctes (86.0, 90.7 i 100% entre colònies, arxipèlags i taxa, respectivament). La validació del model utilitzant dos sets de dades independents i la validació creuada Jackknife van confirmar la robustesa de l'aproximació combinada en l'assignació de colònies (62.5, 58.8 i 69.8% per cada test de validació, respectivament). L'aplicació preliminar del model discriminant basat en els valors d'isòtops estables $\delta^{15}N$ i $\delta^{13}C$ i elements traça (219 ocells de 17 colònies de cria) mostrava que 41 baldrigues mortes en palangrers del Mediterrani occidental provenien principalment de les colònies de cria de Menorca (48.8%), Eivissa (14.6%) i Creta (31.7%). Les nostres troballes

assenyalen que els anàlisis combinats d'isòtops estables i elements traça en ploma pot assolir elevades taxes d'assignació d'ocells en el medi marí, obrint noves expectatives a l'estudi de la mortalitat d'ocells marins al mar.

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GEOGRAPHIC ASSIGNMENT OF SEABIRDS TO THEIR ORIGIN: COMBINING MORPHOLOGIC, GENETIC, AND BIOGEOCHEMICAL ANALYSES

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Abstract. Longline fisheries, oil spills, and offshore wind farms are some of the major threats increasing seabird mortality at sea, but the impact of these threats on specific populations has been difficult to determine so far. We tested the use of molecular markers, morphometric measures, and stable isotope ($\delta^{15}N$ and $\delta^{13}C$) and trace element concentrations in the first primary feather (grown at the end of the breeding period) to assign the geographic origin of *Calonectris* shearwaters. Overall, we sampled birds from three taxa: 13 Mediterranean Cory's Shearwater (*Calonectris diomedea diomedea*), 10 Atlantic Cory's Shearwater (*Calonectris diomedea borealis*) and one Cape Verde Shearwater (*C. edwardsii*) breeding sites. Assignments rates were investigated at three spatial scales: breeding colony, breeding archipelago, and taxa levels. Genetic analyses based on the mitochondrial control region (198 birds from 21 breeding colonies) correctly assigned 100% of birds to the three main taxa, but failed in detecting geographic structuring at lower scales. Discriminant analyses based on trace elements composition achieved the best rate of correct assignment to colony (77.5%). Body measurements or stable isotopes mainly succeeded in assigning individuals among taxa (87.9 and 89.9%, respectively) but failed at the colony level (27.1 and 38.0%, respectively). Combining all three approaches (morphometrics, isotopes, and trace elements on 186 birds from 15 breeding colonies), substantially improved correct classifications (86.0, 90.7, and 100% among colonies, archipelagos and taxa, respectively). Validations using two independent data sets and jackknife cross-validation confirmed the robustness of the combined approach in the colony assignment (62.5, 58.8, and 69.8% for each validation test, respectively). A preliminary application of the discriminant model based on stable isotope $\delta^{15}N$ and $\delta^{13}C$ values and trace elements (219 birds from 17 breeding sites) showed that 41 Cory's Shearwaters caught by western Mediterranean long-liners came mainly from breeding colonies in Menorca (48.8%), Ibiza (14.6%), and Crete (31.7%). Our findings show that combining analyses of trace elements and stable isotopes on feathers can achieve high rates of correct geographic assignment of birds in the marine environment, opening new prospects for the study of seabird mortality at sea.

Key words: assignment methods; biogeochemical markers; bird mortality at sea; longline fisheries; mitochrondrial DNA; spatial structure.

Introduction

Interactions between seabirds and anthropogenic activities frequently result in the death of many individuals. Threats posed by human activities at sea, such as long-lining, offshore wind farms, and oil spills, have increased dramatically over the last decades. Globally, long-lining is one of the world's most serious threats for pelagic seabirds (Lewison et al. 2005). Seabird interactions with longline fisheries often results in the accidental mortality of many seabirds that are hooked when stealing baits (Brothers et al. 1999). Besides, recent major oil spills have been directly responsible for the deaths of several hundred thousands of birds (Cadiou et al. 2003, Carter 2003, García et al. 2003). In addition, the construction of wind facilities offshore may become Europe's most extensive technical development in marine habitats, but relevant data on the impact on seabirds are only just starting to become available (Garthe and Hüppop 2004, Hüppop et al. 2006).

Since seabirds are particularly vulnerable to any factor increasing adult mortality, chronic and accidental impacts of human activities at sea can jeopardize the viability of populations (Lebreton and Clobert 1991). Although conservation concerns have mainly focused on the impact of threats at the breeding colonies (Boersma et al. 2002), the exposure of mixing populations to the same threats in specific wintering areas can compromise the viability of multiple populations (González-Solís et al. 2007). Thus, understanding the impact of seabird mortality at sea requires not only quantifying the number of seabirds killed, but also determining their

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origin. Ultimately, knowing the provenance will allow reassessment of the threats faced at sea on the corresponding breeding grounds.

Assignment methods to analyze seabird mortality at sea are only recently starting to be used. ("Assignment" refers to the assignment of individual birds to a breeding colony, breeding archipelago, or to the correct taxon [species or subspecies].) Banded birds can give information about their origin, but recoveries are typically very few, even when some large-scale ringing programs have expended enormous efforts (Browne et al. 2004). Nevertheless, intrinsic markers, either biological (e.g., genetic and morphometric variation) or biogeochemical (stable isotope signatures and trace elements concentrations), open new venues in the assignment of dead birds to their population of origin.

Morphological variation can be used to assess the geographic origin whenever morphotypes are geographically segregated across the breeding range (Webster et al. 2002). Although several studies have shown that the spatial resolution of this approach is often limited (Stratford and Partridge 1996, Cadiou et al. 2003, 2004), examining morphological differences is easy, fast, and inexpensive, and whenever possible should always be considered as a potential tool in assignment studies.

Individual assignment based on genetic data has been extensively used in forensic applications, and their value in the assignment mostly demonstrated (Manel et al. 2005). Most seabird species show strong philopatry and site fidelity (Austin et al. 1994, Bried and Jouventin 2002), which should promote genetic differentiation between populations and thus enhance the potential value of molecular markers for assignment studies. However, recent studies on several seabird species have shown that dispersal plays a major role as a homogenizing force preventing genetic differentiation (see review by Friesen et al. 2007), and thus molecular markers are rendered ineffective (Edwards et al. 2001).

The existence of habitat-specific isotopic signatures also allows the assignment of individuals to their geographic origin (Hobson 2005, Wunder et al. 2005). As tissues incorporate the isotopic content of the prey consumed in a predictable manner, which in turn show spatial structure and pattern in their biogeochemical attributes, these patterns may be used to identify the geographic origin of individuals from any given population. Feather analyses are being extensively used for this purpose, particularly when molting patterns are known, because once formed, feathers become inert, reflecting the elements and the isotopic forms assimilated through the diet when and where they were grown (Chamberlain et al. 1997, Cherel et al. 2000, Becker et al. 2002). Isotopic signatures among different populations can differ for several reasons. Natural isotopic gradients in baseline $\delta^{13}C$ have been described in the marine environment (Mehl et al. 2004, Forero et al. 2005, Quillfeldt et al. 2005). In addition, differences in diet or foraging behavior among geographically disjunct seabird populations should result in spatial differences in N and C isotope values of feathers grown under distinct oceanic conditions (Cherel et al. 2006).

A relatively new approach in the assignment of individuals of unknown origin is the use of trace elements. Recent studies suggest their value as intrinsic markers for assignment studies (Szép et al. 2003, Hobson 2005). Whereas isotope composition mainly differs on a continental or a regional scale (>1000 km) (Marra et al. 1998), elemental composition of some animal tissues can vary microgeographically (10–1000 km) among populations at the local scale (Szép et al. 2003). However, whether trace element concentrations vary in accordance with biogeographical gradients is unclear, and data regarding patterns of variation in the marine environment is still lacking.

Overall, despite the potential value of morphology, molecular markers, trace elements, and stable isotopes for the assignment of individuals of unknown origin, several studies showed little or no geographic structure among populations over regional to local scales (Edwards et al. 2001, Wassenaar and Hobson 2001, Cadiou et al. 2004, Riffaut et al. 2005). Therefore, a thorough examination of the potential strengths and weaknesses of the different types of markers is needed. Differences in the accuracy among intrinsic markers and the potential benefit of combining different approaches have been reported (Royle and Rubenstein 2004, Kelly et al. 2005), but comparative studies and further applications are still scarce, particularly in the marine environment.

In the present study we use the Mediterranean Cory's Shearwater (*Calonectris diomedea diomedea*), the Atlantic Cory's Shearwater (*Calonectris diomedea borealis*), and the Cape Verde Shearwater (*C. edwardsii*) as a case study in the analysis of different assignment methods to identify the population of origin of oceanic birds. In this paper we aim first to assess the existence of spatial patterns of variation in the three taxa by using genetic, morphometric, and biogeochemical markers. Second, we evaluate the utility of all four markers as effective tools in assigning *Calonectris* shearwaters to source populations. Finally, we carry out a preliminary application of the results obtained to assign 41 Cory's Shearwaters caught by longliners from Catalonian harbors (northeast Spain) operating on the Mediterranean coast.

Materials and Methodology

Study species, study area, and sampling design

Cory's Shearwaters breed on islands across the Mediterranean from the Iberian coast to the Adriatic and Aegean (mainly *C. diomedea diomedea*), and in the northeast Atlantic islands, from the Canary to Azores archipelagos (mainly *C. diomedea borealis*), whereas the Cape Verde Shearwater is an endemic of the Cape Verde Archipelago (*C. edwardsii*) (Appendix A). Although strong patterns of phylogeographic structure have been described within the genus separating the two Cory's

subspecies and the Cape Verde Shearwater species (Gómez-Díaz et al. 2006), most genetic studies have failed to detect spatial structuring among populations (Randi et al. 1989, Wink et al. 1993, Heidrich et al. 1996, Carneiro da Silva and Granadeiro 1999), but see Rabouam et al. (2000). According to previous genetic studies on the species (Gómez-Díaz et al. 2006), we considered Almeria as an Atlantic Cory's Shearwater colony within the Mediterranean. The Almeria–Oran Oceanographic Front represents a major oceanographic discontinuity in the Mediterranean, and is the real boundary between Atlantic and Mediterranean surface waters (Beckers et al. 1997). The influence of Atlantic waters entering the Mediterranean would explain the pattern observed for Almeria.

From 2001 to 2005 we collected blood samples, the first (innermost) primary feather, and morphometric measurements from adult birds from up to 21 breeding colonies of the Cory's Shearwater across the Mediterranean and the northeastern Atlantic, and one breeding colony of the Cape Verde Shearwater (Fig. 1). In addition, we collected tissue samples, the first primary feather and morphometric measurements from 41 Cory's Shearwaters caught by Mediterranean longliners in 2003 and 2004. Birds were caught during prelaying exodus (7), incubation (3), chick rearing (3), at the end of the breeding period (October) (26), or the postbreeding period (November) (2). In Cory's Shearwater the first primary feather is known to be grown at the end of the breeding period, when parents are still feeding their chicks (Monteiro and Furness 1996; J. González-Solís and E. Gómez-Díaz, *personal observation*).

We considered three spatial scales in the analyses. First, at a local scale, we considered a colony level corresponding to single breeding colonies at a specific island. At a regional scale we considered an archipelago level, grouping several colonies breeding on geographically neighboring islands (i.e., the Balearic, Azores, and Canary archipelagos). Finally, we grouped colonies into the three main taxa involved: the Atlantic and Mediterranean Cory's Shearwater, and the Cape Verde Shearwater.

Genetic analyses

Genetic analyses were performed on 188 Cory's and 10 Cape Verde Shearwaters from 21 breeding colonies. We chose the mitochondrial control region as a genetic marker to investigate the genetic structure of the species. As no genetic structure was found below the subspecies level (see *Results*), we also employed ISSR (Inter Simple Sequence Repeats) multilocus fingerprinting to examine separately closer genetic relationships among Atlantic and Mediterranean Cory's Shearwater breeding colonies.

DNA isolation, amplification, and sequencing

DNA was isolated from ethanol-preserved whole blood using the salting-out extraction protocol from Bruford et al. 1998). We amplified a 293 bp fragment of Domain I of the mitochondrial control region of all three *Calonectris* species using three specific primers that had been designed previously for the species (Gómez-Díaz et al. 2006): either CAL2H (5′CATCCCATCCAACTTAAG3′) or CAL4H (5′AGCCTATGTATGGATGTGCAT3′) was used in conjunction with CAL1L (5′GGTCCTGAAGCTAGTAATAC3′). Reaction conditions and automated sequencing were those described by Gómez-Díaz et al. (2006). Representative sequences for each breeding colony are available in GenBank (accession nos. DQ371968-DQ372018).

Population structure

We evaluated population genetic structure based on the F_{ST} estimates or Wright's fixation index of population differentiation using the nested analysis of molecular variance (AMOVA) included in ARLEQUIN (Schneider et al. 2000). The AMOVA treatment reflects the correlation of the haplotypic diversity in a hierarchical analysis (Excoffier et al. 1992). In addition, we performed a neighbor-joining clustering analysis, NTsysPC (Version 1.60; Rohlf 1997), based on the F_{ST} pairwise genetic distances showing genetic relationships among the Cory's Shearwater and the Cape Verde Shearwater colonies. To assess the existence of a spatial pattern in the genetic structure of the species, we tested the correlation between genetic distances, measured as $F_{ST}/(1 - F_{ST})$, and geographic distances, calculated as ln(geographic distance by sea) of population pairs (Rousset 1997). We applied Procrustes analysis (least-squares orthogonal mapping) a nonparametric approach for the comparison of the two kinds of nonlinearized data sets, using SPSS 12.0 (SPSS 2003). The method is based on matching corresponding points (landmarks) from each of the two data sets, and provides a measure of fit (m^2). The significance test of the m^2 statistic was determined by employing a randomization approach to one of the data sets (Protest).

ISSR fingerprint

We screened a subset of samples with six RAPD (Random Amplified Polymorphic DNA) and two ISSR (Inter Simple Sequence Repeats) makers. Based on clarity and resolution of fingerprint profiles from this screening, we chose the two ISSR markers ((ACTG $(AC)_7$) and (GTCAAGG $(CT)_6$), which produced scoreable and highly polymorphic PCR products. To avoid intra-assay variability, the amplification protocol was performed below high-stringency conditions and PCR reactions were established according to a standardized protocol (Arribas et al. 1997) with annealing at 65°/50°C (AC/CT primer). We electrophoresed PCR (Polymerase Chain Reaction) products on an 8 mol/L urea/6% polyacrylamide sequencing gel. ISSR markers were detected by silver staining (Bassam et al. 1991). First, we performed duplicate PCR reactions in a control set of individuals to evaluate the reproducibility of ISSR

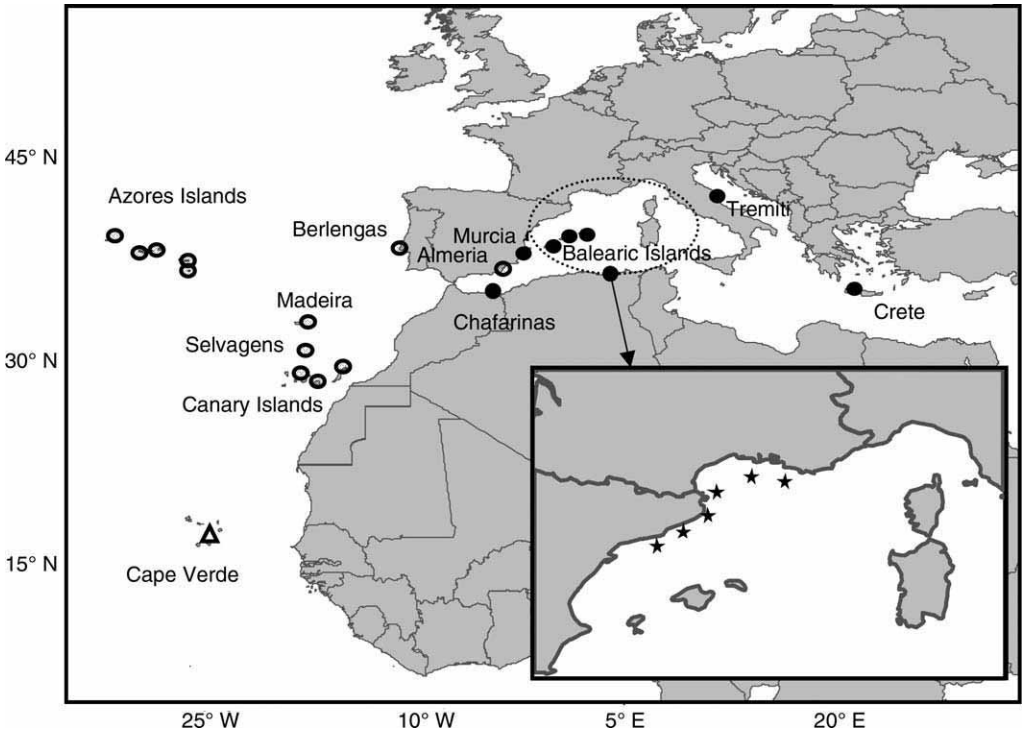

FIG. 1. Breeding colonies of Cory's Shearwater and the Cape Verde Shearwater sampled across the Mediterranean and the Atlantic. The Mediterranean (solid circles), the Atlantic (open circles), and Cape Verde Shearwater (open triangle) breeding colonies (*Calonectris diomedea diomedea*, *C. diomedea borealis*, and *C. edwardsii*, respectively) and locations where bycatch Cory's Shearwaters were caught (stars), are indicated.

fragments. We checked repeatability for independent DNA extractions, different PCR reactions, and different PCR runs to explore all possible sources of error, and we excluded for subsequent statistical analyses nonreproducible bands as well as faint bands (Pérez et al. 1998). All the scoring was repeated twice, and to further avoid biased results, analyses were restricted to breeding colonies with a sample size >10 birds (Nybom 2004). For the data analysis, assumptions following analytical procedures by Lynch and Milligan 1994 were applied.

Biometric analysis

We performed a clustering analysis on the biometric data to assess the population structure and the degree of morphologic differentiation among Cory's Shearwater colonies and the Cape Verde Shearwater across its geographical distribution. Birds were sexed using the molecular method described by Fridolfsson and Ellegren 1999. We included six biometric measures: tarsus, wing, maximum head (cranium length plus the bill length) and bill length, bill depth, and bill depth at nostril, of 703 sexed individuals from 19 distinct colonies. To remove the effect of sexual size dimorphism on the assessment of morphometric differences among breeding colonies, morphometric variables of males and females were standardized by subtracting the species mean of each sex. We calculated the Euclidean distance for all pairwise combinations of populations and constructed a cladogram from the similarity matrix using the Neighbor-Joining clustering analysis implemented in the NTSYSpc package version 2.1 (Rohlf 1997). To investigate the existence of spatial gradients in morphology, we tested the relationship between each of the six morphometric measures and the corresponding latitude and longitude coordinates of the sampled colonies by least square and curvilinear regressions using the SPSS 12.0 package (SPSS 2003).

Isotopic and trace elements analysis

We analyzed C and N stable isotope signatures and trace element concentration on the first primary feather of 219 individuals from 17 breeding colonies. First, feathers were cleaned from surface contaminants using NaOH and ground to powder using a SPEX 6750 Freezer/Mill (SPEX CertiPrep, Metuchen, New Jersey, USA). To analyze stable isotopes, 0.3 mg of this powder was weighed to the nearest microgram and placed into a Sn (tin) capsule. Samples were oxidized with CuO and CO_3O_4/Ag at about 900°C in a Flash EA 1112 Elemental Analyser (CE Elantech, Lakewood, New Jersey, USA) coupled to a pirolizator TC-EA and a breath bench, through an interface Conflo III (Finnigan MAT, San Jose, California, USA). NO_X was reduced with Cu at 680°C. The combustion products, N_2 and CO_2, were dried using $MgClO_4$ and transported to a Delta C Finnigan MAT mass spectrometer (Isotopic

ratio mass spectrometry, Serveis Científico-Tècnics of University of Barcelona, Barcelona, Spain). International standards were run with each of 12 samples; IAEA CH_7 (87% of C), IAEA CH_6 (42% of C) and USGS 24 (100% of C) for ^{13}C and IAEA N1 and IAEA N2 (with 21% of N) and IAEA NO_3 (13.8% of N) for ^{15}N.

For the analysis of trace elements, 0.1 g of feather powder was digested in 1 mL of nitric acid and 0.5 mL of hydrogen peroxide using teflon bombs for 12 hours at 60°C. The result of the digestion was diluted in 7 mL of distilled water. Analyses were performed using the Atomic Adsorption Spectrophotometry (AAS) technique with a mass Perkin Elmer Optima 6000 Elan Spectrophotometer (Isotopic ratio mass spectrometry, Serveis Científico-Tècnics of University of Barcelona, Barcelona, Spain). Accuracy of analysis was checked by measuring certified reference material (Human Hair CRM 397). To check the reproducibility of the procedure, we included sample replicates as well as negative controls in each set of samples analyzed. In addition, we replicated the analysis in 50 of the samples selected at random. We compared mean breeding colony values of trace element composition between replicates using t tests for two related samples (SPSS 2003). Only those elements showing significant correlations, $R^2 > 0.5$ and no significant differences in mean breeding colony values between the two analyses, were considered for the assignment analyses.

First, to evaluate the degree of structuring among Cory's and the Cape Verde Shearwater colonies, for both C and N stable isotope and trace elements, we calculated the Euclidean distance for all pairwise comparisons of mean colony values and constructed a cladogram from the similarity matrix using the Neighbor-Joining clustering analysis implemented in the NTSYSpc package version 2.1 (Rohlf 1997). Second, to investigate the existence of spatial gradients, we tested the relationships of carbon and nitrogen isotopic signatures and trace element concentrations with the corresponding latitude and longitude coordinates of the sampled colonies by least-square and curvilinear regression using the SPSS 12.0 package (SPSS 2003).

Assignment analyses

Assignment tests on multilocus ISSR data were performed using DOH (Paetkau et al. 1995), a frequency-based program (*available online*).[4] We split our data set into both baseline data, to calculate population frequency likelihoods, and test data, for the frequency-based assignment, randomly grouping 80% and 20% of the individuals, respectively.

We employed classificatory discriminant analysis (SPSS 2003) to evaluate the ability of morphometrics, stable isotopes, and trace elements in the assignment of birds to their geographical origin. Trace element concentrations were transformed logarithmically to approach normality. We carried out the analysis separately and in combination using the same subset of birds (185 individuals from 15 breeding colonies). For all the analyses, we split the original data set into training data, to build models, and an independent data set, to test the classificatory method, randomly grouping 80% and 20% of the individuals, respectively. In addition, we tested models by jackknife cross-validation. Models were built step by step, including independent variables according to the Wilks' lambda criterion, and colonies were weighted according to the sample size. Discriminant functions were used to classify the individuals previously excluded in testing for the accuracy of the model. Validation of the model was further examined using samples from three distinct Cory's Shearwater colonies sampled in previous years and analyzed separately.

After comparing all three assignment approaches (see *Results*), we built a final model using trace elements and stable isotopes on a larger data set, covering almost the entire breeding range of the species, and applied this model to assign 41 bycatch Cory's Shearwaters.

Results

Genetics

Clustering analysis on mtDNA data indicated a clear geographical pattern; three groups of colonies corresponded to each of the two geographically isolated Cory's Shearwater subspecies and the Cape Verde species (Fig. 2A).

AMOVA results suggested strong genetic structuring among the three main taxa ($F_{CT} = 0.61$, $P < 0.001$). The largest variance component was due to differences among subspecies (60.5%); whereas within each subspecies, among breeding colonies, the variance component was lower (37.2 %). However, a second AMOVA, at a local scale, failed to reveal genetic structuring among breeding colonies within either the Atlantic or the Mediterranean subspecies region ($F_{ST} = 0.040$, $P = 0.350$; $F_{ST} = 0.00$, $P = 0.450$, respectively).

Procrustes analyses revealed a significant correlation between genetic and geographic distances among colonies considering both the Atlantic and the Mediterranean Cory's Shearwater subspecies regions ($m^{12} = 0.63$, $P < 0.001$). However, at a local scale among colonies within each subspecies region, the correlation was lower. Whereas spatial correlation was still significant within the Mediterranean ($m^{12} = 0.63$, $P = 0.020$), significance did not hold within the Atlantic ($m^{12} = 0.93$, $P = 0.716$).

Biometrics

The cladogram represents the similarity pattern of the 19 breeding colonies included in the analysis, considering six biometric measures (Fig. 2B). Three groups were clearly defined, corresponding to each of the three taxa. A first group included populations corresponding to the

[4] ⟨www.biology.ulberta.ca/jbrzusto/Doh.php⟩

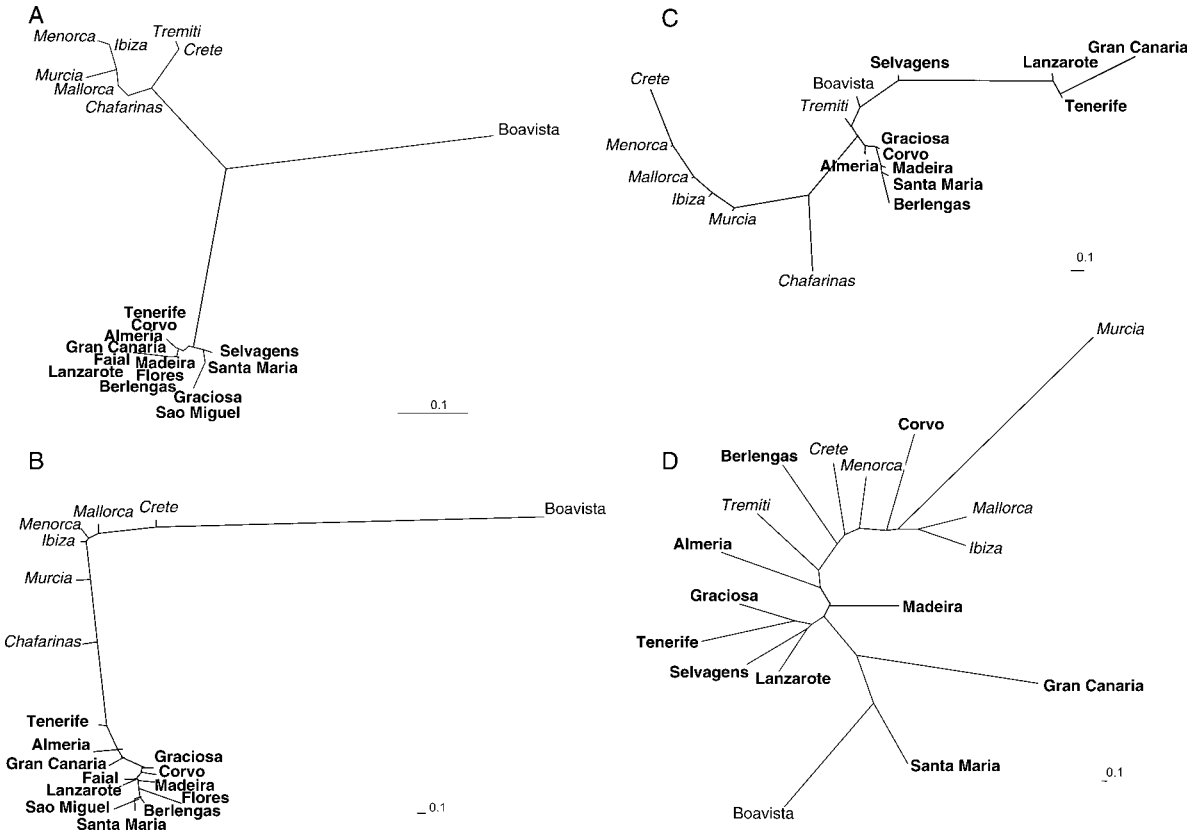

FIG. 2. Neighbor-joining cladogram showing relationships among Cory's and Cape Verde Shearwaters breeding colonies based on (A) genetic, (B) biometric, (C) δ^{13} C and δ^{15} N signatures, and (D) trace elements composition of first primary feathers. Groups corresponding to the Atlantic Cory's, Mediterranean Cory's, and Cape Verde Shearwater breeding colonies are indicated by boldface type, italics, and lightface type, respectively. Biometric, trace elements, and isotope cladograms are based on Euclidean pairwise distances among colonies. The genetic cladogram is based on F_{ST} pairwise genetic distances among colonies; the length of the scale bar in each graph represents 0.1 units of genetic distance.

largest in size, the Atlantic Cory's Shearwater. All Mediterranean breeding colonies grouped separately corresponding to the intermediate in body size, the Mediterranean Cory's Shearwater. The smallest in size, the Cape Verde Shearwater breeding colony, was grouped separately.

Linear regression analyses revealed a significant positive longitudinal pattern within the Mediterranean subspecies in three of six biometric measures for males and females (tarsus: $R^2 = 0.16$, $n = 108$, $P < 0.001$ and $R^2 = 0.10$, $n = 94$, $P = 0.002$; bill depth at nostril: $R^2 = 0.24$, $n = 108$, $P < 0.001$ and $R^2 = 0.13$, $n = 96$, $P < 0.001$; bill depth: $R^2 = 0.13$, $n = 108$, $P < 0.001$ and $R^2 = 0.14$, $n = 96$, $P < 0.001$, for males and females, respectively). For the Atlantic subspecies no significant association was found between any of the six measures and either longitude or latitude (all measures: $R^2 < 0.15$, $n = 224$ and $R^2 < 0.05$, $n = 228$, for males and females, respectively). Both linear and quadratic regression coefficients and F statistics are shown in Appendix D. In a few cases quadratic models fit substantially better than linear models.

Isotopes and trace elements

The cladogram based on carbon and nitrogen signatures grouped most breeding colonies according to their geographical distributions. All Mediterranean breeding colonies grouped together except Tremiti, ungrouped, and Almeria, grouped within the Atlantic cluster. In the Atlantic, northeast and southeast Atlantic breeding colonies grouped separately (Fig. 2C). The similarity tree based on trace elements composition showed no geographical structure among breeding colonies, nor among the three main taxa. Nevertheless, breeding colonies appeared well differentiated and large distances separated each node (Fig. 2D).

Regression models based on stable isotope composition of primary feathers showed significant spatial gradients for both δ^{15}N and δ^{13}C values across the breeding range of the study species (Fig. 3; Appendix C). Regression coefficients and F statistics are shown in Appendix D. Least squares regression models, although significant, did not explain much variability, whereas curvilinear models, in particular cubic regressions, better fit the spatial gradients observed (Appendix D). Carbon

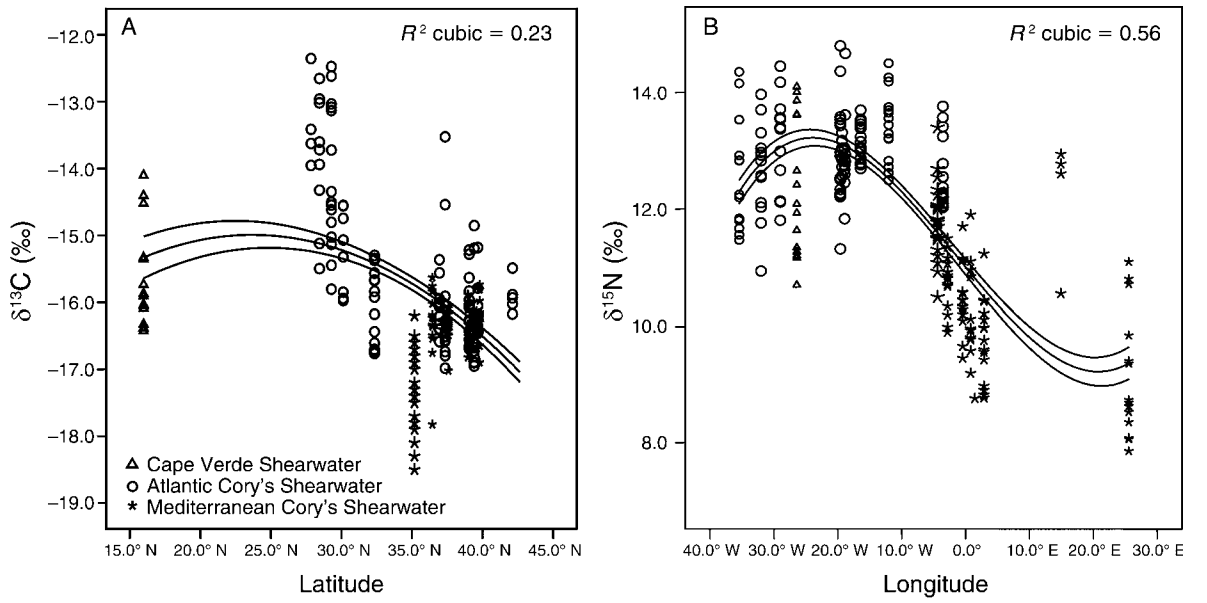

FIG. 3. Relationships between (A) δ^{13}C feather signatures and latitude, and (B) δ^{15}N feather signatures and longitude. Note the decreasing trend from west to east in δ^{15}N, whereas δ^{13}C values tend to decrease from north to south across the breeding range of the species. "R^2 cubic" means R^2 for cubic regression.

isotope ratios were associated with latitude ($R^2 = 0.17$ and 0.24; least squares and cubic regression, respectively) (Fig. 3A), decreasing from south (Gran Canaria Island, $\delta^{13}C = -13.34 \pm 0.69$) to north (Menorca Island, $\delta^{13}C = -16.23 \pm 0.32$), except for the most southern Cape Verde breeding colony, which showed unexpected low values ($\delta^{13}C = -15.66 \pm 0.76$). No significant association was found between carbon isotopic signatures and longitude ($R^2 = 0.06$). At a local scale, within each Cory's subspecies region, the latitudinal decrease in carbon values was further apparent within the Atlantic ($R^2 = 0.49$ and 0.61; least squares and cubic regression) (Fig. 4A), but also within the Mediterranean ($R^2 = 0.35$ and 0.39; linear and cubic regression).

Globally, nitrogen isotope values in feathers tended to show an inverse relationship with longitude, decreasing from west to east ($R^2 = 0.45$ and 0.56; least squares and cubic regression) (Fig. 3B). Indeed, significant differences in mean nitrogen isotopic signatures split the eastern *C. diomedea diomedea* ($\delta^{15}N = 10.57 \pm 1.34$) from the western *C. diomedea borealis* breeding colonies ($\delta^{15}N = 12.97 \pm 0.74$; $t = -15.36$, $P < 0.001$). However, the colony from Tremiti showed greater nitrogen isotope values ($\delta^{15}N = 12.28 \pm 1.03$) than those reported within the entire Mediterranean ($\delta^{15}N = 10.57 \pm 1.34$). No significant association was found between nitrogen isotope values and latitude ($R^2 = 0.07$). Considering each subspecies range separately, nitrogen isotope values tended to decrease with longitude within the Mediterranean ($R^2 = 0.23$ and 0.64; least squares and cubic regression) (Fig. 4B), whereas no pattern was apparent within the Atlantic ($R^2 = 0.07$ and 0.08; linear and cubic regression; Appendix D).

Results from ANOVA showed significant differences in trace element composition (Na, Mg, Mn, Ba, S, Zn, Al, P, Ca, Cr, Rb, Sr, Sb, La, Ce, Pr, Nd, Hg, and U) of primary feathers across the study species range (all $P < 0.001$) (Appendix B). Most elements showed weak associations with either latitude or longitude (least squares and cubic regression coefficients, R^2, ranging from 0 to 0.20). Only a few elements showed $R^2 > 0.20$ (Appendix D). That is, Mg, Sr, and U concentrations varied with longitude across the breeding range (Mg, $R^2 = 0.20$, Sr, $R^2 = 0.23$, and U, $R^2 = 0.34$; for cubic regression), and Hg was mainly associated with latitude ($R^2 = 0.28$ and 0.37; least squares and cubic regression). No significant spatial correlations were found in Hg levels within the Atlantic or the Mediterranean. In contrast, Mg, Sr, and U were notably associated with longitude within the Mediterranean subspecies range (Mg, $R^2 = 0.27$ and 0.42, Sr, $R^2 = 0.25$ and 0.45, and U, $R^2 = 0.35$ and 0.44, for least squares and cubic regression, respectively.) (Regression coefficients and F statistics are shown in Appendix D.)

Geographic assignment

Genetic assignment based on ISSR data failed to assign individuals to breeding colonies. In the randomization procedure only 62 out of 313 Cory's Shearwaters were assigned to the putative colony. The rate of correct assignment was 22.4% among breeding colonies for the Mediterranean Cory's Shearwater subspecies, whereas in the Atlantic only 16.8% of individuals were correctly classified. For the independent data set 11.5% and 11.9% of individuals were correctly assigned among the Atlantic and the Mediterranean subspecies, respectively.

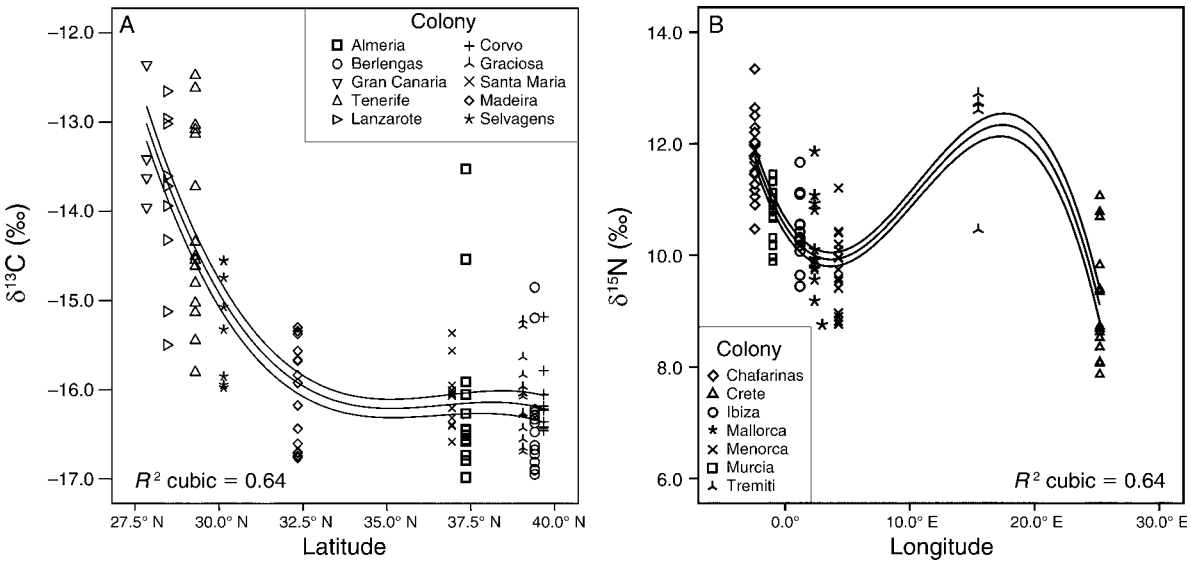

FIG. 4. Significant associations between geographic coordinates and stable isotopes within each subspecies region are also shown; either (A) between δ^{13}C signatures and latitude among Atlantic Cory's Shearwater breeding colonies, or (B) between δ^{15}N and longitude among Mediterranean Cory's Shearwater breeding colonies. "R^2 cubic" means R^2 for cubic regression.

The discriminant analysis based only on trace elements composition achieved the best rate of correct assignment at both colony and archipelago levels, whereas discriminant models based only on either morphometric measures or stable isotopes just showed high rates of correct assignment among taxa (Table 1). In both independent data set and independent analysis, the ability to correctly classify individuals dropped noticeably in all three assignment approaches when used separately; the more detailed the scale of the analysis, the larger the drop (Table 1). Combining different approaches substantially improved the rate of correct assignments at both colony and archipelago levels in most cases. Models based on isotopes and trace elements, as well as models including those with morphology, achieved the best assignment results. Cross-validations, independent data, and independent analysis further confirmed the ability of the combined

TABLE 1. Classification rates obtained for each assignment method at each scale of analysis when used alone and in conjunction.

Scale	Biometrics	Isotopes	Elements	Biometrics and isotopes	Biometrics and elements	Isotopes and elements	Biometrics and isotopes and elements
Training data (n = 130)							
Colony	27.1	38.0	77.5	46.5	82.9	82.2	86.0
Archipelago	41.9	58.9	75.2	65.1	76.0	83.7	90.7
Taxa	87.6	89.9	89.9	99.2	98.4	96.9	100.0
Test data (n = 56)							
Colony	14.3	33.9	62.5	41.1	48.2	66.1	62.5
Archipelago	48.2	53.6	69.6	64.3	75.0	80.4	82.1
Taxa	83.9	89.3	89.3	98.2	98.2	96.4	98.2
Cross-validation							
Colony	22.5	28.7	56.6	31.0	65.1	62.8	69.8
Archipelago	41.1	52.7	70.5	61.2	69.0	76.0	79.8
Taxa	84.5	89.9	86.0	98.4	96.9	92.2	100.0
Independent analysis (n = 17)							
Colony	17.6	11.8	23.5	17.6	29.4	41.2	58.8
Archipelago	52.9	70.6	41.2	64.7	47.1	88.2	94.1
Taxa	64.7	94.1	58.8	88.2	70.6	70.6	88.2

Notes: Correct assignments (percentages) are shown for training data, test data, jackknife cross-validation, and independent analysis. Independent test data were the 20% of the data randomly excluded prior to building the discriminant model. Independent test analysis corresponded to samples collected in previous years and analyzed separately.

TABLE 2. Assignment rates, validations, and misclassifications (training data) of the final discriminant model, based on stable isotope signatures (C and N) and trace elements composition of the first primary feather of 226 Cory's Shearwaters from 17 breeding colonies.

Taxa	Archipelago	Breeding colony	*N*	Training data		Misassignments
				No. assigned	Assigned (%)	
Atlantic Cory's Shearwater	Azores Is.	St. Maria	13	12	92.3	Cape Verde
Atlantic Cory's Shearwater	Azores Is.	Graciosa	17	15	88.2	Berlengas, Madeira
Atlantic Cory's Shearwater	Azores Is.	Corvo	13	10	76.9	Ibiza, Berlengas, Madeira
Atlantic Cory's Shearwater	Canary Is.	Gran Canaria	4	4	100.0	
Atlantic Cory's Shearwater	Canary Is.	Lanzarote	18	15	83.3	Selvagens, Madeira, Graciosa
Atlantic Cory's Shearwater	Canary Is.	Tenerife	9	8	88.9	Graciosa
Atlantic Cory's Shearwater	Madeira	Madeira	18	16	88.9	Graciosa, Ibiza
Atlantic Cory's Shearwater	Selvagens	Selvagens	8	7	87.5	Graciosa
Atlantic Cory's Shearwater	Berlengas	Berlengas	18	18	100.0	Almeria
Atlantic Cory's Shearwater	Almeria	Almeria	15	14	93.3	Madeira
Mediterranean Cory's Shearwater	Balearic Is.	Mallorca	12	7	58.3	Menorca, Ibiza
Mediterranean Cory's Shearwater	Balearic Is.	Menorca	15	11	73.3	Mallorca, Ibiza
Mediterranean Cory's Shearwater	Balearic Is.	Ibiza	15	12	80.0	Mallorca, Crete
Mediterranean Cory's Shearwater	Murcia	Murcia	15	15	100.0	
Mediterranean Cory's Shearwater	Tremiti	Tremiti	5	5	100.0	
Mediterranean Cory's Shearwater	Crete	Crete	15	9	60.0	Menorca, Ibiza, Berlengas
Cape Verde Shearwater	Cape Verde	Boavista	15	14	93.3	Gran Canaria

Notes: Assignments for 41 bycatch Cory's Shearwaters of unknown origin and dates when the birds were caught are also shown. Misassignments correspond to training data.

models, based only on isotopes and trace elements or together with morphology, to discriminate among geographic origins (Table 1).

A final model built on 219 individuals from 17 breeding colonies combined C and N isotopic signatures and trace element concentrations, to assign bycatch birds whose origin was unknown. Discriminant analysis achieved rates of correct assignment >80% for all the scales of the analysis (80.2% among colonies, 87.4% considering archipelagos, and 98.1% among taxa) (Table 2). Cross-validations, independent data sets and independent analysis confirmed the utility of the model to discriminate among geographic origins (73.5, 75, and 64.7% of correct assignments among colonies). The discriminant model for the whole data set (100% of cases) included $\delta^{15}N$ ($F_{10,215} = 71.58$, $P < 0.001$), Sr ($F_{20,428} = 53.86$, $P < 0.001$), $\delta^{13}C$ ($F_{30,625} = 46.50$, $P < 0.001$), Ca ($F_{40,805} = 39.17$, $P < 0.001$), Sb $F_{50,965} = 33.10$, $P < 0.001$), Hg ($F_{60,1105} = 28.64$, $P < 0.001$), Mn ($F_{70,1225} = 25.70$, $P < 0.001$), Ce ($F_{80,1327} = 23.70$, $P < 0.001$), Ba ($F_{90,1414} = 21.99$, $P < 0.001$), P ($F_{100,1486} = 20.34$, $P < 0.001$), U ($F_{110,1547} = 18.90$, $P < 0.001$), and Na ($F_{120,1597} = 17.72$, $P < 0.001$). The preliminary application of the discriminant model combining isotopes and trace elements on 41 Cory's Shearwaters caught by Mediterranean longliners assigned individuals to Menorca (48.8%), Ibiza (14.6%), Crete (31.7%), and one Atlantic breeding colony (Graciosa, 4.8%). Locations where bycatch birds were caught are shown in Fig. 1, and the corresponding bycatch dates are indicated in Table 2.

DISCUSSION

Genetics

Mitochondrial DNA analyses revealed a strong genetic structuring among the three main taxa, agreeing with their geographically segregated distributions. However, the Mediterranean colony of Almeria was placed within the Atlantic cluster. This result agrees with previous genetic studies on the species, pointing out the Atlantic subspecies identity of Almeria (Gómez-Díaz et al. 2006).

The mitochondrial control region is a rapidly evolving locus extensively used for population-level studies (see Pearce 2006), and its potential utility to discriminate among geographic origins has been previously shown in a number of species (Lovette et al. 2004). However, this gene appeared ineffective for the geographic assessment of Cory's Shearwaters beyond the taxon level. Similarly, although ISSR multilocus fingerprints revealed substantial genetic variability, assignment tests failed to classify individuals to the breeding colony. The low level of genetic structuring observed for both molecular markers explains the weak spatial resolution and the low rates of correct assignments. Lack of genetic structure has been repeatedly reported for the species using different genetic markers (Randi et al. 1989, Wink et al. 1993, Heidrich et al. 1996, Carneiro da Silva and Granadeiro 1999, Gómez-Díaz et al. 2006). Analyses based on microsatellite fingerprints found genetic structuring at lower levels, both among and within breeding sites, but failed to detect a correlation between spatial and genetic patterns of variation (Rabouam et al. 2000).

TABLE 2. Extended.

Cross-validation		Application data (bycatch birds)	
No. assigned	Assigned (%)	Assigned (%)	Collection dates (no. birds)
10	76.9		
13	76.5	4.9	May (1), August (1)
9	69.2		
3	75.0		
14	77.8		
7	77.8		
13	72.2		
4	50.0		
17	94.4		
13	86.7		
6	50.0		
9	60.0	48.8	May (3), August (1), October (16)
9	56.3	14.6	June (1), October (5)
14	93.3		
3	60.0		
9	60.0	31.7	May (3), July (3), October (7)
13	86.7		

Biometrics

The biometric pattern of variation matched the geographical distributions of the three taxa separating the two Cory's Shearwater subspecies and the Cape Verde Shearwater. But in agreement with genetic analyses, the Mediterranean colony of Almeria intermingled with the Atlantic cluster. Concerning the spatial pattern of variation in morphology, we found a longitudinal geographic gradient in body measurements within the Mediterranean subspecies as a slight increase in body size from east to west Mediterranean subspecies, a biometric pattern previously recognized by other authors (Massa and Lo Valvo 1986, Granadeiro 1993). This pattern is probably related to the complex longitudinal oceanographic subzonation within the Mediterranean basin. Specific marine habitat characteristics of different areas within the Mediterranean, such as differences in sea surface temperature, wind speed, and primary production, probably promoted divergence in body size through local adaptation of populations (Zotier et al. 1999). On the contrary, no spatial pattern was apparent within the Atlantic, although some authors have recently reported slight latitudinal differences from the Canary to Azores Islands (Thibault et al. 1997, Gómez-Díaz et al. 2006).

Morphological measures could be used to reliably assign most birds to the taxon (species or subspecies), provided that the sex of the birds was previously determined, since Cory's Shearwaters show an appreciable sexual size dimorphism. However, assignment tests based on morphological data failed to identify the geographic origin of Cory's Shearwaters at their breeding colony. Recent studies on the seabird genus *Puffinus* sp. showed that only species with widespread distributions would exhibit noticeable geographic variation in their morphometrics (Bull et al. 2005). Consequently, data supplementary to the more traditional biometric approach, such as isotopes, trace elements, or genetic markers, are probably necessary for the geographic assignment of most seabird species.

Stable isotopes and trace elements

Stable isotopes signatures separated most breeding colonies according to their geographic distributions, although to a lesser extent than the biometric and genetic structuring. The influence of Atlantic waters entering the Mediterranean would explain the isotopic similarity of the three most western Mediterranean colonies (i.e., Murcia, Almeria, and Chafarinas) to those of the Atlantic. In contrast, although showing the largest distances compared to any other approach, no geographical structure among breeding colonies was evident for trace elements.

In the present study, both $\delta^{15}N$ and $\delta^{13}C$ values of the first primary feather varied across the breeding range of Cory's Shearwaters and differed from those for the Cape Verde Shearwater. Since the first primary feather is grown at the end of the breeding period, we can expect the isotopic signatures of this feather to reflect those from the prey consumed around the breeding area. However, evidence of biogeographic patterns of stable isotope values in marine ecosystems is still scarce, and several studies showed large differences in the degree of geographic variation of isotope biomarkers, depending on the spatial scale of analysis (Chamberlain et al. 1997, Marra et al. 1998, Wassenaar and Hobson 2001, Wunder et al. 2005). Whereas most studies successfully applied C and N isotope ratios to discern geographically distinct subspecies, most failed to discriminate among geographic origins at regional and local scales (see Hobson 1999 for a complete review). In line with results found in other studies in both terrestrial and marine ecosystems, we found feather isotope values of C and N to vary by latitude and longitude, respectively. However, the strength of these patterns depended on the geographic scale considered (see Fig. 2C, D). The geographic break in isotope values between Atlantic and Mediterranean regions effectively separated the two subspecies. However, isotope geographical gradients within each region, although eventually significant, were too weak to reliably assign the origin of birds at local scales. This inaccuracy probably resulted from variance associated with isotope values at a single location, or from similar isotopic baselines values at different areas (Royle and Rubenstein 2004). Alternatively, similarities in isotopic values among neighboring colonies may simply result from birds sharing the same foraging areas. During the chick-rearing period, when the first primary feather is grown, shearwaters use a flexible foraging strategy, i.e., they forage close to the colonies, mainly to

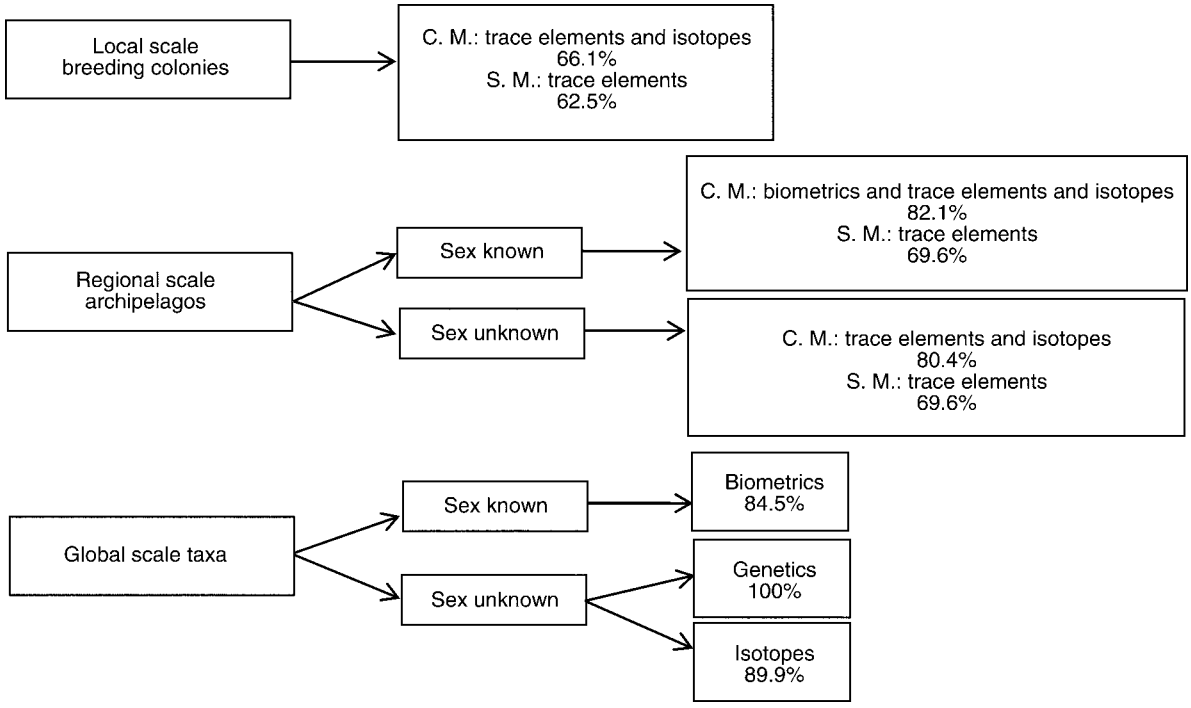

FIG. 5. Decision tree showing the recommended approach for the geographic assignment of seabird specimens, depending on the spatial scale of resolution desired and the possibility to determine the gender. Decisions have been made based on rates of correct assignment for test data validations. C. M. denotes the combined model, i.e., when using several markers, and S. M. denotes the single model, i.e., when using a single marker.

feed the chicks, but far offshore for self feeding (Granadeiro et al. 1998, Cherel et al. 2005), potentially overlapping with shearwaters breeding in other islands.

However, sharing foraging areas of neighboring islands cannot solely explain the lack of spatial resolution of isotopic values, because this should then hold true for the trace element composition of these feathers. Instead, we found specific trace element profiles associated with each breeding location, but in contrast with stable isotopes, we could not detect latitudinal or longitudinal patterns in feather composition. Differences in elemental composition of feathers allowed us to discriminate the origin of birds at different geographic scales, that is, from specific breeding colonies (77.5% of correct assignments), considering archipelagos (75.2%), and identifying taxa (89.9%). However, correct classification at the colony and archipelago levels decreased to 62.5% and 69.6%, respectively, when we classified 20% of the samples randomly excluded from the analyses prior to building the discriminant model. The model performed even worse for classifying an independent set of samples collected in previous years and analyzed separately, dropping to 23.5%, 41.2% correct classification. These results illustrate the limitations of these models to classify samples other than those used to build them. The drop in the classification rates of samples collected in previous years may reflect interannual variability in trace element composition. In addition, the lack of spatial gradients in trace elements composition across the breeding range of the species underlies the need for a geographically widespread sampling design. Sampling discontinuities and missing populations would lead to inaccuracies in the assignment of individuals to specific locations. In addition, missclassifications in the validation methods among, i.e., Cape Verde and Canary/Azores missassigned birds (Table 2), would be explained because baseline levels of the three areas are similar. In this case, population trees for any intrinsic marker (Fig. 2) can help us to infer whether the most likely reason for the missassignments is a close similarity among colonies. Still, trace element composition was the intrinsic marker showing the best assignment rates, suggesting that such composition strongly depends upon the microgeographical location of the breeding site. This probably reflects the importance of local conditions and the similarity in foraging areas of birds breeding in the same colony (Szép et al. 2003), although atmospheric deposition on feathers might also explain these differences to some extent (Breitburg and Riedel 2005).

The combination of several methods enhanced the reliability of the classificatory method both across the different spatial scales (86.0, 90.7, and 100.0%, from colony to taxon level), and for all validation methods (e.g., at the archipelago level, 82.1, 94.1, and 79.8% for independent data, independent analysis, and jackknife cross-validation, respectively). The benefits of using multiple intrinsic markers has been demonstrated

previously (Royle and Rubenstein 2004), and our results highlight that a combined approach should be used when possible.

The preliminary application of the discriminatory model on Cory's Shearwaters caught by Mediterranean longliners identified three source breeding sites in the Mediterranean (Menorca, Crete, and Ibiza islands) and one from the Atlantic (Graciosa Island). Ancillary information on the capture date, location, and numbers of dead birds recovered may help to contrast model predictions and to test ecological hypotheses. Interestingly, both Menorca and Ibiza breeding colonies are within the operating area of Catalonian longliners and represent major breeding colonies for the species in the central Mediterranean (Thibault et al. 1997). Furthermore, most of the birds assigned to Crete were caught out of the breeding period (86.0%), either during the prelaying period (i.e., April and May) or during the postnuptial migration (i.e., October and November), when birds have to cross the operating area of the Catalonian longliners returning from or going to the wintering grounds in the subSaharan coast (Ristow et al. 2000). Finally, although we could expect all individuals to belong to the Mediterranean subspecies, we cannot completely rule out the Atlantic identity of some specimens, since there is evidence of Atlantic Cory's Shearwaters entering the Mediterranean (Lo Valvo and Massa 1988, Sánchez 1997, Thibault and Bretagnolle 1998, Martínez-Abraín et al. 2002). However, those birds were caught in May and August, within the breeding period of the species, and their biometric measurements do not support their Atlantic identity. As mentioned above, ghost populations resulting from incomplete sampling designs would force the model to assign birds elsewhere. In the present study, although the sampling design covered almost the whole breeding range of the species, a few central Mediterranean populations were missed (mainly Zembra, Malta, Corsica, and Sardinia), which may partly explain assignment errors when applying the model.

Conclusions

Overall, the effectiveness of the four types of biomarkers evaluated, i.e., genetic, morphometric, trace element, and isotopic traces for the geographic assignment of Cory's Shearwaters, varied with the spatial scale of analysis. The application of intrinsic markers depends on there being enough differences among potential source breeding colonies to unequivocally assign individuals to their source. Which method should be applied also depends on the level of spatial resolution desired and the ancillary information we have, such as the sex of the bird (Fig. 5). Whereas the identification of Atlantic and Mediterranean Cory's Shearwater subspecies and the Cape Verde species was successfully achieved for all four types of markers, large differences arose in the correct assignment of the breeding colonies among these methods. Both morphometric and genetic approaches appeared ineffective at discriminating among geographically close breeding sites. With respect to biogeochemical markers, stable isotopes showed some latitudinal and longitudinal trends, but the predictive power of these trends to classify individuals among putative sources was rather weak. In contrast, trace elements appeared more sensitive to microgeographical differences than were stable isotopes, and allowed the best rate of correct assignment of feathers at local scales. However, trace elements did not show geographical gradients, and thus ghost populations, the result of incomplete sampling designs, can force the model to erroneously assign birds to very distant colonies rather than identifying their true origin. Adding isotopes to models based on trace elements did not enhance the rate of successful assignment of the training data, but substantially improved classification rates of the independent data sets, increasing the robustness and accuracy at all the spatial scales of analysis. Determining the origin of dead individuals is important for any attempt to assess the potential impact of human activities, such as oil spills, long-line fishing, or offshore wind farms, responsible for an increase in seabird mortality at sea. Our study shows that it is feasible to use biogeochemical markers of seabird feathers, such as trace elements and stable isotopes, to identify the impact of such activities on specific breeding seabird populations at a local scale.

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APPENDIX A

Geographic location of Cory's and Cape Verde Shearwaters and sample sizes for each analysis (*Ecological Archives* XXXXXX).

APPENDIX B

Morphological measurements for all *Calonectris* breeding colonies included in the biometric analysis (*Ecological Archives* XXXXXX).

APPENDIX C

Mean and standard deviation of stable isotopes signatures and trace elements concentrations of Cory's Shearwater and the Cape Verde Shearwater breeding colonies (*Ecological Archives* XXXXXX).

APPENDIX D

Regression models on the latitudinal and longitudinal gradients in stable isotopes signatures, trace elements concentrations, and morphometric measurements (*Ecological Archives* XXXXXX).

Appendix 1. Geographic location of Cory's and Cape Verde shearwaters and sample sizes for each analysis

Species	Geographic location	Archipelago	Colony	Latitude	Longitude	Genetics (n)	Biometrics (n)	Traces elements (n)	Isotopes (n)
Calonectris d. diomedea	Mediterranean basin	Balearic Is.	Mallorca	39.58	2.37	11	45	11	11
"	Mediterranean basin	Balearic Is.	Menorca	39.80	4.29	10	42	15	15
"	Mediterranean basin	Balearic Is.	Ibiza	38.96	1.20	12	45	16	16
"	Mediterranean basin	–	Murcia	37.58	-0.98	10	13	15	15
"	Mediterranean basin	–	Chafarinas	35.18	-2.42	10	59		23
"	Mediterranean basin	–	Creta	36.44	25.23	9	17	15	15
"	Mediterranean basin	–	Tremiti	42.13	15.49	10		5	5
Calonectris d. borealis	Mediterranean basin	–	Almeria	37.35	-1.65	10	28	15	15
"	Atlantic basin	Azores Is.	St. Maria	36.94	-25.17	9	94	13	13
"	Atlantic basin	Azores Is.	Graciosa	39.06	-27.95	10	26	17	17
"	Atlantic basin	Azores Is.	Corvo	39.67	-31.11	10	22	13	13
"	Atlantic basin	Azores Is.	Flores	39.37	-31.20	9	8		
"	Atlantic basin	Azores Is.	Faial	38.52	-28.75	7	13		
"	Atlantic basin	Azores Is.	S.Miguel	37.71	-25.44	10	16		
"	Atlantic basin	Canary Is.	Lanzarote	29.29	-13.54	9	25	18	18
"	Atlantic basin	Canary Is.	Tenerife	28.11	-16.76	10	9	9	9
"	Atlantic basin	Canary Is.	G.Canaria	27.85	-15.79	5	168	4	4
"	Atlantic basin	–	Selvagens	30.13	-15.87	7		8	8
"	Atlantic basin	–	Madeira	32.34	-16.49	10	26	18	18
"	Atlantic basin	–	Berlengas	39.41	-9.49	10	21	18	18
Calonectris edwardsii	Atlantic basin	Cape Verde	Boavista	15.98	-22.78	10	26	15	15

Appendix 2. Morphological measurements (mm) for all *Calonectris* breeding colonies included in the biometric analysis. Values are means ± standard deviations. Sample size for each sex is shown in brackets (males; females).

Island breeding colony	Tarsus		Wing		Head-bill lenght		Bill lenght		Bill depth		Bill depth at nostril	
	Males	*Females*	*Males*	*Females*	*Males*	*Females*	*Males*	*Females*	*Males*	*Females*	*Males*	*Females*
St. Maria- Azores Is. (47; 47)	59.13 ± 1.60	56.67 ± 1.30	370.74 ± 6.46	362.77 ± 8.78	117.45 ± 2.60	110.79 ± 1.80	56.57 ± 1.79	53.06 ± 1.41	21.86 ± 0.86	19.99 ± 0.80	16.27 ± 0.89	14.58 ± 0.61
S. Miguel- Azores Is. (6; 4)	59.70 ± 0.97	57.72 ± 1.75	373.50 ± 10.27	366.50 ± 3.87	117.44 ± 1.52	111.57 ± 0.86	56.39 ± 1.37	53.50 ± 1.21	21.88 ± 0.58	19.86 ± 0.86	16.40 ± 0.50	14.82 ± 0.62
Graciosa- Azores Is. (13; 13)	59.66 ± 1.42	56.69 ± 1.23	375.85 ± 6.56	361.15 ± 5.81	116.88 ± 1.19	110.00 ± 2.45	55.59 ± 1.17	51.94 ± 1.77	21.14 ± 0.58	19.23 ± 0.39	15.56 ± 0.45	14.14 ± 0.49
Flores- Azores Is. (5; 3)	60.42 ± 1.40	57.88 ± 2.24	376.80 ± 7.19	365.67 ± 4.04	118.22 ± 3.30	112.85 ± 1.51	57.50 ± 1.80	54.02 ± 0.73	21.07 ± 0.92	19.53 ± 0.78	15.52 ± 0.87	14.53 ± 0.70
Corvo- Azores Is. (12; 10)	58.75 ± 1.28	56.64 ± 2.02	373.08 ± 6.58	364.30 ± 8.42	116.72 ± 2.82	111.48 ± 3.23	55.77 ± 2.09	53.34 ± 1.59	21.35 ± 0.90	18.93 ± 1.03	15.43 ± 0.84	13.98 ± 0.95
Faial- Azores Is. (8; 5)	59.59 ± 1.24	56.65 ± 0.95	373.38 ± 6.70	361.60 ± 6.50	116.23 ± 3.02	111.52 ± 1.18	55.57 ± 2.47	52.98 ± 1.86	21.65 ± 0.37	21.21 ± 1.47	15.71 ± 0.49	14.33 ± 1.09
Madeira- Portugal (9; 17)	59.26 ± 1.74	56.92 ± 2.34	373.11 ± 7.23	362.71 ± 7.06	119.24 ± 1.98	111.48 ± 3.80	57.52 ± 1.42	52.86 ± 2.26	21.68 ± 0.58	19.45 ± 0.99	15.49 ± 0.85	14.08 ± 0.88
Berlengas- Portugal (12; 9)	59.33 ± 1.37	57.75 ± 1.97	375.58 ± 7.01	364.11 ± 6.17	116.88 ± 2.31	111.21 ± 2.12	56.83 ± 1.37	52.98 ± 1.85	21.94 ± 0.86	19.60 ± 0.86	16.01 ± 0.55	14.43 ± 0.71
G.Canaria- Canary Is. (81; 87)	57.82 ± 1.54	56.07 ± 1.20	367.78 ± 7.66	360.98 ± 6.86	114.43 ± 2.82	108.94 ± 2.37	55.43 ± 2.08	52.38 ± 1.69	21.34 ± 0.74	19.20 ± 1.17	15.56 ± 0.73	14.17 ± 0.74
Lanzarote- Canary Is. (14; 11)	59.21 ± 1.37	56.77 ± 1.42	374.28 ± 9.77	366.91 ± 0.62	115.95 ± 2.69	111.72 ± 2.84	55.14 ± 1.91	53.35 ± 2.42	21.39 ± 0.99	19.79 ± 0.82	15.58 ± 1.04	14.23 ± 0.70
Tenerife- Canary Is. (4; 5)	57.84 ± 1.21	55.97 ± 0.97	368.25 ± 12.76	360.40 ± 2.88	115.03 ± 1.44	109.35 ± 1.46	54.78 ± 0.63	51.65 ± 0.72	20.79 ± 0.97	18.98 ± 0.61	15.11 ± 0.81	13.50 ± 0.47
Almeria- Spain (12; 16)	58.66 ± 1.85	56.19 ± 1.51	372.83 ± 6.51	366.00 ± 5.25	112.43 ± 4.37	109.28 ± 3.17	53.27 ± 2.50	51.40 ± 2.15	21.21 ± 1.47	19.05 ± 1.03	15.47 ± 1.19	14.10 ± 0.93
Mean for *C.d. borealis* (223)	**58.70 ± 1.66**	**56.49 ± 1.50**	**371.04 ± 7.88**	**362.56 ± 7.28**	**115.89 ± 3.14**	**110.10 ± 2.67**	**55.79 ± 2.08**	**52.61 ± 1.79**	**21.49 ± 0.86**	**19.42 ± 1.02**	**15.74 ± 0.85**	**14.25 ± 0.76**
Chafarinas Is.- Spain (29; 30)	56.52 ± 2.02	54.67 ± 2.43	353.86 ± 8.08	347.52 ± 12.57	110.50 ± 3.59	106.65 ± 4.14	52.93 ± 2.04	50.76 ± 3.37	20.38 ± 1.22	18.72 ± 1.29	15.08 ± 0.86	13.52 ± 0.99
Murcia- Spain (9; 4)	57.18 ± 1.04	53.89 ± 1.03	353.67 ± 7.53	346.25 ± 4.19	111.36 ± 2.24	101.96 ± 1.27	52.75 ± 0.73	47.79 ± 1.13	19.96 ± 1.16	17.56 ± 0.64	14.13 ± 0.92	12.14 ± 0.23
Mallorca- Balearic Is. (24; 21)	54.60 ± 1.69	53.66 ± 2.09	352.79 ± 7.79	349.14 ± 6.05	107.70 ± 3.30	104.85 ± 3.57	50.24 ± 2.14	48.52 ± 1.45	19.18 ± 2.20	17.59 ± 1.29	14.01 ± 0.74	12.86 ± 0.99
Ibiza- Balearic Is. (23; 22)	55.61 ± 1.26	53.46 ± 1.11	360.04 ± 5.70	347.55 ± 5.84	109.08 ± 2.17	104.38 ± 1.53	51.22 ± 1.70	48.40 ± 1.12	19.13 ± 0.69	17.57 ± 0.68	13.93 ± 0.71	12.80 ± 0.52
Menorca- Balearic Is. (19; 23)	55.96 ± 1.43	53.29 ± 1.21	359.47 ± 7.22	350.26 ± 5.68	109.17 ± 1.01	103.88 ± 2.71	51.58 ± 2.57	48.13 ± 1.77	19.00 ± 1.01	17.32 ± 0.77	13.91 ± 0.88	12.42 ± 0.56
Crete- Greece (13; 4)	54.14 ± 1.35	52.03 ± 1.64	352.69 ± 6.03	336.25 ± 9.98	108.25 ± 1.40	102.98 ± 1.60	51.63 ± 1.07	47.79 ± 1.08	18.59 ± 0.51	16.90 ± 0.27	13.18 ± 0.34	12.14 ± 0.48
Mean for *C.d. diomedea* (117)	**55.64 ± 1.81**	**53.77 ± 1.90**	**355.62 ± 7.73**	**347.97 ± 8.70**	**109.25 ± 3.15**	**104.88 ± 3.34**	**51.67 ± 2.14**	**49.00 ± 2.43**	**19.44 ± 1.42**	**17.83 ± 1.17**	**14.16 ± 0.96**	**12.89 ± 0.89**
Boavista- Cape Verde (15; 11)												
Mean for *C.edwarsii* (26)	**48.93 ± 1.36**	**46.68 ± 1.35**	**317.60 ± 5.60**	**307.64 ± 4.52**	**94.83 ± 1.35**	**90.71 ± 1.02**	**45.20 ± 0.78**	**43.12 ± 1.02**	**16.29 ± 0.84**	**14.49 ± 0.62**	**11.81 ± 0.88**	**10.35 ± 0.31**

Appendix 3. Mean and standard deviation of stable isotopes signatures (‰) and trace elements concentrations (ppm) of Cory's shearwater and the Cape Verde shearwater breeding colonies. Sample size for each breeding colony is shown in brackets. Trace elements concentrations were log_{10} transformed. Latitude and longitude correspond to geographic coordinates.

Breeding colony	Geographic location	Long	Lat	$\delta^{15}N$	$\delta^{13}C$	B	Na	Mg	Al	P	Ca	Cr	Mn
St.Maria (13)	Azores Is.	36.94	-25.17	13.12 ± 0.79	-16.08 ± 0.33	3.39 ± 0.09	6.31 ± 0.07	6.07 ± 0.09	4.44 ± 0.38	4.53 ± 0.11	5.86 ± 0.07	3.54 ± 0.36	2.98 ± 0.33
Graciosa (17)	Azores Is.	39.06	-27.95	12.73 ± 0.77	-16.05 ± 0.49	3.35 ± 0.08	6.54 ± 0.06	6.07 ± 0.06	4.62 ± 0.21	4.50 ± 0.10	5.88 ± 0.10	3.42 ± 0.11	3.08 ± 0.14
Corvo (13)	Azores Is.	39.67	-31.11	12.52 ± 0.96	-16.15 ± 0.35	3.21 ± 0.10	6.50 ± 0.05	6.03 ± 0.08	4.12 ± 0.32	4.47 ± 0.05	5.88 ± 0.11	3.53 ± 0.15	2.62 ± 0.12
Lanzarote (18)	Canary Is.	29.29	-13.54	13.16 ± 0.32	-14.05 ± 1.09	3.40 ± 0.06	6.52 ± 0.06	6.11 ± 0.09	4.49 ± 0.36	4.50 ± 0.06	6.06 ± 0.17	3.38 ± 0.09	3.00 ± 0.19
Tenerife (9)	Canary Is.	28.11	-16.76	12.87 ± 0.27	-13.87 ± 0.97	3.37 ± 0.04	6.61 ± 0.02	6.11 ± 0.09	4.61 ± 0.44	4.41 ± 0.09	5.92 ± 0.15	3.47 ± 0.11	2.98 ± 0.16
G.Canaria (4)	Canary Is.	27.85	-15.79	13.45 ± 0.84	-13.34 ± 0.69	3.49 ± 0.05	6.51 ± 0.17	6.06 ± 0.09	4.55 ± 0.27	4.54 ± 0.08	5.90 ± 0.10	3.80 ± 0.16	3.36 ± 0.31
Selvagens (8)	Portugal	30.13	-15.87	12.58 ± 0.36	-15.25 ± 0.61	3.42 ± 0.09	6.57 ± 0.05	6.12 ± 0.05	4.66 ± 0.45	4.46 ± 0.07	6.03 ± 0.14	3.48 ± 0.11	3.02 ± 0.35
Madeira (18)	Portugal	32.34	-16.49	13.00 ± 0.95	-16.05 ± 0.57	3.39 ± 0.07	6.51 ± 0.05	6.09 ± 0.08	4.45 ± 0.20	4.54 ± 0.08	6.04 ± 0.19	3.43 ± 0.12	2.90 ± 0.21
Berlengas (18)	Portugal	39.41	-9.49	13.28 ± 0.63	-16.36 ± 0.55	3.36 ± 0.08	6.54 ± 0.04	6.08 ± 0.06	4.21 ± 0.20	4.58 ± 0.14	6.02 ± 0.14	3.43 ± 0.09	2.65 ± 0.23
Almeria (15)	Spain	37.35	-1.65	12.58 ± 0.58	-16.15 ± 0.92	3.36 ± 0.05	6.49 ± 0.04	6.00 ± 0.07	4.57 ± 0.37	4.52 ± 0.08	6.13 ± 0.13	3.44 ± 0.05	3.26 ± 0.27
C. d. borealis (133)				***12.97 ± 0.74***	***-15.49 ± 1.18***	***3.38 ± 0.10***	***6.51 ± 0.11***	***6.08 ± 0.08***	***4.45 ± 0.34***	***4.51 ± 0.09***	***5.96 ± 0.15***	***3.49 ± 0.19***	***2.94 ± 0.31***
Chafarinas* (23)	Morocco coast	35.18	-2.41	11.80 ± 0.63	-17.27 ± 0.61								
Murcia (15)	Spain	37.58	-0.98	10.72 ± 0.46	-16.34 ± 0.23	3.26 ± 0.29	6.39 ± 0.05	5.72 ± 0.10	4.15 ± 0.28	4.65 ± 0.21	5.72 ± 0.08	3.50 ± 0.12	2.64 ± 0.17
Mallorca (11)	Balearic Is.	39.58	2.37	10.27 ± 0.80	-16.22 ± 0.27	3.40 ± 0.06	6.44 ± 0.04	6.00 ± 0.07	4.10 ± 0.14	4.48 ± 0.06	5.83 ± 0.07	3.48 ± 0.06	2.58 ± 0.11
Ibiza (16)	Balearic Is.	38.96	1.20	10.41 ± 0.54	-16.40 ± 0.29	3.34 ± 0.07	6.45 ± 0.06	6.02 ± 0.08	4.12 ± 0.16	4.50 ± 0.07	5.92 ± 0.09	3.50 ± 0.14	2.64 ± 0.11
Menorca (15)	Balearic Is.	39.80	4.29	9.73 ± 0.69	-16.23 ± 0.32	3.37 ± 0.08	6.45 ± 0.04	6.01 ± 0.09	4.36 ± 0.23	4.50 ± 0.11	5.86 ± 0.11	3.52 ± 0.15	2.84 ± 0.17
Tremiti (5)	Italy	42.13	15.50	12.28 ± 1.03	-15.90 ± 0.25	3.43 ± 0.12	6.50 ± 0.02	6.02 ± 0.05	4.26 ± 0.18	4.56 ± 0.08	6.02 ± 0.21	3.48 ± 0.10	2.94 ± 0.22
Creta (15)	Greece	36.44	25.23	9.10 ± 1.04	-16.30 ± 0.53	3.42 ± 0.08	6.53 ± 0.06	6.11 ± 0.12	4.25 ± 0.23	4.57 ± 0.16	5.96 ± 0.12	3.55 ± 0.14	2.71 ± 0.11
C. d .diomedea (77)				***10.54 ± 1.21***	***-16.51 ± 0.59***	***3.36 ± 0.14***	***6.46 ± 0.06***	***5.98 ±0.15***	***4.26 ± 0.29***	***4.54 ± 0.13***	***5.91 ± 0.16***	***3.50 ±0.12***	***2.78 ± 0.28***
Boavista (15)													
C. edwardsii (15)	Cape Verde	**15.98**	**-22.78**	***12.33 ± 1.17***	***-15.66 ± 0.76***	***3.49 ± 0.10***	***6.33 ± 0.08***	***6.12 ± 0.11***	***4.35 ± 0.23***	***4.72 ± 0.18***	***5.89 ± 0.11***	***3.60 ± 0.11***	***3.08 ± 0.24***

*Forero, M.G., J.González-Solís, J.M. Igual, K.A.Hobson, X. Ruíz & G.Viscor. Ecological variance in T-cell mediated immune response in Cory's shearwaters *Calonectris diomedea*. Condor 108, in press.

Appendix 3 (continued).

Breeding colony	**Zn**	**Rb**	**Sr**	**Sb**	**Ba**	**La**	**Ce**	**Pr**	**Nd**	**Hg**	**U**
St.Maria (13)	4.81 ± 0.23	1.94 ± 0.13	4.13 ± 0.08	1.72 ± 0.39	3.00 ± 0.51	1.34 ± 0.36	1.58 ± 0.38	0.74 ± 0.38	0.88 ± 0.68	3.69 ± 0.17	1.66 ± 0.11
Graciosa (17)	4.77 ± 0.12	1.58 ± 0.24	4.05 ± 0.08	1.16 ± 0.17	2.19 ± 0.75	1.53 ± 0.24	1.75 ± 0.19	0.98 ± 0.21	1.43 ± 0.25	3.70 ± 0.11	1.48 ± 0.11
Corvo (13)	4.78 ± 0.10	1.52 ± 0.11	4.04 ± 0.11	1.18 ± 0.21	0.93 ± 1.18	1.00 ± 0.15	1.23 ± 0.13	0.40 ± 0.18	0.30 ± 0.55	3.86 ± 0.20	1.51 ± 0.11
Lanzarote (18)	4.78 ± 0.09	1.63 ± 0.20	4.15 ± 0.16	1.33 ± 0.33	1.96 ± 1.10	1.46 ± 0.16	1.70 ± 0.18	0.88 ± 0.16	1.31 ± 0.18	3.61 ± 0.10	1.56 ± 0.15
Tenerife (9)	4.79 ± 0.14	1.65 ± 0.12	4.08 ± 0.12	0.90 ± 0.21	1.27 ± 1.26	1.34 ± 0.15	1.63 ± 0.17	0.75 ± 0.09	1.22 ± 0.18	3.54 ± 0.12	1.37 ± 0.12
G.Canaria (4)	4.87 ± 0.06	1.90 ± 0.19	4.13 ± 0.11	1.01 ± 0.66	2.63 ± 0.44	1.68 ± 0.34	2.08 ± 0.36	1.16 ± 0.34	1.48 ± 0.49	3.56 ± 0.17	1.56 ± 0.14
Selvagens (8)	4.82 ± 0.06	1.64 ± 0.19	4.15 ± 0.07	1.42 ± 0.39	2.43 ± 1.08	1.61 ± 0.20	1.83 ± 0.20	1.03 ± 0.19	1.50 ± 0.20	3.66 ± 0.18	1.71 ± 0.12
Madeira (18)	4.84 ± 0.08	1.68 ± 0.15	4.17 ± 0.17	1.40 ± 0.44	1.18 ± 1.30	1.32 ± 0.12	1.52 ± 0.14	0.77 ± 0.17	1.19 ± 0.19	3.82 ± 0.19	1.62 ± 0.13
Berlengas (18)	4.78 ± 0.05	1.35 ± 0.65	4.09 ± 0.07	0.98 ± 0.13	2.74 ± 0.47	1.06 ± 0.26	1.29 ± 0.19	0.50 ± 0.20	0.67 ± 0.42	3.71 ± 0.11	1.52 ± 0.10
Almeria (15)	4.85 ± 0.12	1.35 ± 0.68	4.03 ± 0.08	1.21 ± 0.26	2.32 ± 0.63	1.32 ± 0.15	1.49 ± 0.14	0.74 ± 0.11	1.10 ± 0.22	3.96 ± 0.10	1.37 ± 0.13
C. d. borealis (133)	***4.81 ± 0.08***	***1.65 ± 0.36***	***4.11 ± 0.12***	***1.23 ± 0.43***	***2.07 ± 1.13***	***1.37 ± 0.32***	***1.61 ± 0.34***	***0.80 ± 0.32***	***1.10 ± 0.54***	***3.69 ± 0.18***	***1.55 ± 0.15***
Murcia (15)	4.97 ± 0.08	1.66 ± 0.28	3.47 ± 0.12	1.30 ± 0.21	1.60 ± 0.83	0.91 ± 0.32	1.15 ± 0.27	0.37 ± 0.32	0.32 ± 0.59	4.01 ± 0.33	0.75 ± 0.24
Mallorca (11)	4.80 ± 0.14	1.52 ± 0.08	3.94 ± 0.07	1.08 ± 0.19	1.26 ± 1.08	0.94 ± 0.14	1.17 ± 0.18	0.45 ± 0.15	0.62 ± 0.33	3.98 ± 0.15	1.26 ± 0.06
Ibiza (16)	4.87 ± 0.05	1.52 ± 0.15	4.00 ± 0.10	1.00 ± 0.30	1.25 ± 1.25	0.92 ± 0.11	1.11 ± 0.13	0.35 ± 0.11	0.55 ± 0.44	4.02 ± 0.14	1.34 ± 0.15
Menorca (15)	4.77 ± 0.15	1.53 ± 0.54	3.98 ± 0.11	0.94 ± 0.27	2.17 ± 0.51	1.06 ± 0.29	1.27 ± 0.21	0.50 ± 0.20	0.66 ± 0.56	3.98 ± 0.15	1.32 ± 0.14
Tremiti (5)	4.80 ± 0.04	1.56 ± 0.05	3.91 ± 0.07	1.81 ± 0.47	2.44 ± 0.11	1.31 ± 0.07	1.40 ± 0.14	0.65 ± 0.08	0.96 ± 0.30	3.80 ± 0.06	1.32 ± 0.09
Creta (15)	4.78 ± 0.06	1.64 ± 0.19	4.08 ± 0.14	1.06 ± 0.25	1.57 ± 1.13	1.01 ± 0.23	1.29 ± 0.19	0.47 ± 0.23	0.81 ± 0.48	4.05 ± 0.23	1.52 ± 0.20
C. d .diomedea (77)	***4.84 ± 0.08***	***1.54 ± 0.38***	***3.92 ± 0.22***	***1.14 ± 0.31***	***1.74 ± 1.00***	***1.04 ± 0.26***	***1.26 ± 0.22***	***0.49 ± 0.23***	***0.69 ± 0.50***	***3.99 ± 0.20***	***1.27 ± 0.29***
Boavista (15)	**4.85 ± 0.13**	**1.78 ± 0.61**	**4.16 ± 0.09**	**1.95 ± 0.23**	**3.20 ± 0.58**	**1.21 ± 0.25**	**1.49 ± 0.16**	***0.77 ± 0.18***	***0.27 ± 0.74***	***3.45 ± 0.29***	***1.59 ± 0.12***
C. edwardsii (15)											

Appendix 4. Regression models on the latitudinal and longitudinal gradients in stable isotopes signatures (‰), trace elements concentrations (ppm) and morphometric measurements (1 males; 2 females). Only those elements showing R^2 greater than 0.2 in either cubic or linear regression models are shown.

Geographic range	**Least squares regression**					**Polynomial regression**						
All Breeding colonies	**a**	**b**	**R^2**	**F**	**P**	**a_1**	**a_2**	**a_3**	**b**	**R^2**	**F**	**P**
$\delta^{13}C$ · Latitude	-0.0708	-13.4777	0.1667	49.41	<0.001	0.2798	-0.0060		-16.5033	0.2438	36.39	<0.001
$\delta^{15}N$ · Longitude	-0.0713	11.3113	0.4532	204.72	<0.001	-.143537	0.0001	0.0001	11.2123	0.5573	102.79	<0.001
$\delta^{13}C$ · Longitude	-0.0187	-16.1143	0.0636	16.77	0.001	-0.0815	0.0008	0.0001	-16.3749	0.2158	22.48	<0.001
$\delta^{15}N$ · Latitude	-0.0666	14.2480	0.0721	19.19	<0.001	0.1090		-0.0001	11.0711	0.1053	13.95	<0.001
Hg · Longitude	154.9981	8737.2275	0.2798	94.39	<0.001	345.5694	-1.7923	-0.3295	9491.4790	0.3708	47.33	<0.001
Mg · Longitude	-2885.9637	1123632.6717	0.0178	4.41	0.037	-17198.0102	529.3873	30.3593	974585.8251	0.2033	20.50	<0.001
Sr · Longitude	-80.7779	11197.9833	0.0534	13.70	<0.001	-357.6300	6.6719	0.5365	9150.0608	0.2261	23.47	<0.001
U · Longitude	-0.3247	28.8900	0.0956	25.68	<0.001	-1.1741	0.0288	0.0018	20.6602	0.3440	42.12	<0.001
Mediterranean subspecies range												
$\delta^{15}N$ · Longitude	-0.0622	10.8452	0.2328	30.04	<0.001	-0.3581	0.0590	-0.0019	10.5337	0.6362	56.53	<0.001
$\delta^{13}C$ · Latitude	0.1750	-23.1350	0.3470	52.60	<0.001	2.5742	-0.0316		-68.5615	0.3939	31.84	<0.001
Mg · Longitude	19717.9382	864193.8793	0.2727	29.62	<0.001	138286.6004	-13301.1461	346.4521	761272.6409	0.4206	18.63	<0.001
Sr · Longitude	218.9389	7325.8117	0.2513	26.52	<0.001	1814.7951	-186.7916	4.9746	6003.7343	0.4570	21.60	<0.001
U · Longitude	0.8349	15.4245	0.3538	43.26	<0.001	3.9678	-0.3932	0.0108	13.0445	0.4371	19.93	<0.001
Tarsus[1] · Longitude	-0.0837	56.1214	0.1632	20.67	<0.001	-0.1649	0.0034		56.1346	0.1750	11.14	<0.001
Bill depth[1] · Longitude	-0.0534	19.5870	0.1291	15.71	<0.001	-0.2600	0.0087		19.6207	0.2784	20.26	<0.001
Bill depth[1] at nostril · Longitude	-0.0571	14.3983	0.2364	32.82	<0.001	-0.1874	0.0055		14.4196	0.3317	26.06	<0.001
Tarsus[2] · Longitude	-0.1043	53.9504	0.0962	9.79	0.002	-0.2290	0.0061		53.9736	0.1290	6.74	0.002
Bill length[2] · Longitude	-0.0765	18.0216	0.1419	15.22	<0.001	-0.2149	0.0068		18.0473	0.2530	15.41	<0.001
Bill length at nostril[2] · Longitude	-0.0575	13.0178	0.3636	14.01	<0.001	-0.1536	0.0047		13.0357	0.2206	12.88	<0.001
Atlantic subspecies range												
$\delta^{15}N$ · Longitude	0.0070	13.0416	0.0071	0.94	0.335	-0.1103	-0.0056	-0.0001	12.4682	0.0750	3.49	0.018
$\delta^{13}C$ · Latitude	-0.1866	-9.0469	0.4879	124.82	<0.001	-2.8887	0.0395	0.0000	36.3540	0.6128	102.86	<0.001
B · Latitude	-53.6746	4282.4303	0.2271	43.18	<0.001	-17.6494		-0.0103	3486.7761	0.2276	21.51	<0.001

Discussió

Filogeografia i estructuració genètica de les poblacions d'hoste

Els principals objectius de la filogeografia són reconstruir l'historia biogeogràfica de les poblacions i identificar les subdivisions genètiques dins d'una espècie (Avise 2000). L'història biogeogràfica de les espècies del complex *Calonectris* suggereix un fort component geogràfic en la cladogènesis del grup. L'espècie del Pacífic, la baldriga canosa, apareix com el clade més basal i distant. Mentre que la resta d'espècies, la baldriga de Cap Verd i les dues subespècies de baldriga cendrosa, s'agrupen en un únic clade corresponent al Paleàrtic. Els temps de separació estimats indiquen que la separació entre els clades Pacífic i Paleàrtic es va produir per un fenomen de vicariància coincidint amb el tancament de l'Istme de Panamà aproximadament tres milions d'anys enrere (Coates *et al.* 1992). Dins del Paleàrtic, la separació entre els tres subclades principals, l'Atlàntic, el Mediterrani i el de Cap Verd; data de fa entre 600.000 i 800.000 anys aproximadament. Aquesta separació coincideix amb els fenòmens paleogeogràfics durant les glaciacions del Pleistocè i ha estat previament documentada en altre espècies de petrells i baldrigues (Cagnon *et al.* 2004, Austin 2004). En aquest context, els clades Atlàntic i Mediterrani haurien evolucionat en al·lopatria per una contracció del rang de distribució de l'espècie, i tot seguit, una adaptació local a condicions oceanogràfiques particulars en cadascun dels oceans. En canvi, la baldriga de Cap Verd probablement va evolucionar com a forma endèmica en l'arxipèlag de Cap Verd per un procés de divergència ecològica, el què explicaria un major grau de diferenciació morfològica en relació a les dues subespècies de baldriga cendrosa.

Globalment, l'estructura filogenètica de la baldriga cendrosa està d'acord amb la distribució espacial segregada dels quatre clades de *Calonectris* i es correspon amb els quatre taxons acceptats per la taxonomia convencional. No obstant, la filogènia obtinguda no encaixa del tot amb els límits tradicionals d'espècies i subespècies dins del complex (Bourne 1955, Porter *et al.* 1997, Cramp and Simmons 1977, Oustalet 1983, Hazevoet 1995). Mentre que els anàlisis biomètrics separen clarament les dues subespècies de baldriga cendrosa de les baldrigues de Cap Verd i canosa com a morfoespècies separades; els anàlisis filogenètics indiquen una divergència genètica equivalent entre els tres subclades dins del Paleàrtic (menor

al 1%) i que contrasta amb la divergència entre les dos espècies reconegudes, la baldriga cendrosa i la baldriga canosa (del 3%). De fet, i contràriament a la classificació actual, el clade que compren la baldriga cendrosa Atlàntica (*C. d. borealis*) i la Mediterrània (*C. d. diomedea*) es parafilètic. Això, per tant, posaria en dubte el status actual d'espècie de la baldriga de Cap Verd o bé el de subespècies de les baldrigues cendroses Atlàntica i Mediterrània.

Son molts els estudis que mostren una incongruència entre les distàncies genètiques i les separacions a nivell de morfoespècies (Ball *et al.* 1988, Ball & Avise 1992, Avise 2000). Actualment, hi ha un solapament considerable en el rang de distàncies que separen subespècies respecte al que separa espècies (Sangster 2000), un fenomen que es particularment manifest en el cas del ocells marins (Liebers *et al.* 2001, Austin 1996, Austin 2004, Heidrich *et al.* 1998). Austin (2004) va classificar la divergència genètica entre parells d'espècies germanes de cero a 14.9%. Dins d'aquest rang, mentre que algunes subespècies estaven separades per només un 1.0%, d'altres divergien fins a un 5.8%. Al contrari, espècies reconegudes, mostraven distàncies genètiques des de 0 a gairebé un 11.1%. Les evidències actuals indiquen que encara que la divergència genètica avança al llarg de milions d'anys, els canvis morfològics, fisiològics i de comportament, poden donar lloc a espècies i subespècies molt més ràpidament (Hewitt 2000). Així doncs, mentre que la divergència genètica entre dos taxons reflecteix el temps des de que es van separar, la divergència morfològica, comportamental i ecològica pot donar-se per una selecció natural diferencial sota un ambient determinat (Schluter 2000). Això explicaria la diferència de magnitud entre la divergència genètica i morfològica de la baldriga de Cap Verd. En aquest context, i a la llum dels nostres resultats, es necessària una reavaluació de la sistemàtica i taxonomia del grup. I la combinació de caràcters genètics concordants amb caràcters morfològics i de comportament, seria el més desitjable per una definició taxonòmica acurada dins del complex *Calonectris*.

Quant a l'estructuració genètica de les poblacions de baldriga cendrosa, hem trobat una forta estructuració genètica al llarg del rang de distribució de l'espècie com dos grups de poblacions corresponents a cadascuna de les dues subespècies. Tanmateix, no només la distribució geogràfica de les poblacions, sinó també les característiques oceanogràfiques de

l'hàbitat sembla que influencien els patrons d'estructuració genètica en la baldriga cendrosa. De fet, la separació genètica entre la subespècie Atlàntica i la Mediterrània es correspon amb el límit oceanogràfic que separa ambdós mars (Longhurst 1998). No obstant, tot i que l'estret de Gibraltar constitueix una frontera física, els nostres resultats suggereixen que la barrera efectiva entre les dues subespècies es situaria en el front oceanogràfic Almeria- Orà (AOOF).

Dins de cada regió, la diferenciació genètica és menor però tot i així existeix un cert grau d'estructura tant al Mediterrani com a l'Atlàntic. Curiosament, mentre que les colònies dins de l'Atlàntic sembla que s'estructuren en arxipèlags, dins del Mediterrani existeix una certa diferenciació entre la regió occidental i oriental. D'acord amb això, el model d'aïllament per distància explicaria el patró observat a l'Atlàntic. En canvi, al Mediterrani la distància no juga un paper important, sinó que sembla que existeix un efecte combinat de l'oceanografia i la geografia.

Ecologia i estructuració de la comunitat de ectoparàsits

Les comunitats d'ectoparàsits s'estructuren en diferents nivells jeràrquics en base a l'organització espacial de l'hoste (infracomunitat, comunitats component i comunitat regional). Els factors ecològics que afectin a la distribució i l'abundància d'hostes i paràsits en cadascun d'aquets nivells, poden influenciar els patrons de congruència entre ambdós organismes (Clayton *et al.* 2004, Rannala & Michalakis 2003). En el nostre estudi, hem pogut detectar la influencia de diversos factors en l'abundància i distribució de les espècies d'ectoparàsits a cadascun dels nivells d'organització de la comunitat.

En primer lloc, les infracomunitats (la comunitat d'ectoparàsits dins d'un individu hoste) estan temporalment i espacialment estructurades. Temporalment, els patrons de distribució i abundància varien al llarg del cicle de cria de l'hoste. Tot i així, existeix força solapament en els patrons temporals entre les diferents espècies d'ectoparàsits. Aquesta variació pot estar relacionada amb els canvis de comportament de l'hoste i les oportunitats per la dispersió en les diferents etapes del cicle reproductor (Proctor 2003). A més a més, moltes espècies d'ectoparàsits mostren cicles vitals sincronitzats amb l'hoste (Marshall 1981).

Nosaltres hem detectat una major abundància d'ectoparàsits durant el període de posta i incubació de l'hoste que podria reflectir el moment de reproducció dels ectoparàsits, mentre que l'abundància cau de nou durant l'època de cria del poll probablement degut a una transmissió vertical d'ectoparàsits de pares a fills. Espacialment, la puça i les tres espècies de polls de la ploma mostren una forta segregació dintre del cos de l'hoste. Així per exemple, les puces es concentren principalment a l'àrea del ventre. Quant a les espècies de polls; *S.peusi* es distribueix casi exclusivament al cap, mentre que *H.abnormis* ocupa de forma més o menys homogènia la resta del cos de l'hoste. Aquesta segregació probablement resulta de l'heterogeneïtat d'hàbitat que proporcionen els diferents tipus de ploma i que varien de forma predictible amb la regió del cos (Reed *et al.* 2000).

A nivell de la comunitat component (les infracomunitats dins d'una població d'hoste), el nostre estudi mostra que els ectoparàsits es distribueixen d'acord amb un patró agregat, però el grau d'agregació varia significativament entre els polls i la puça. En la puça aquest patró és més marcat, mentre que l'agregació és menor en els polls de la ploma, especialment en les espècies menys abundants (*S. peusi* and *A. echinatum*). La distribució agregada dels paràsits pot resultar de l'agregació espacial de l'hoste, és a dir, de la densitat (Whiteman & Parker 2004). De fet, la densitat d'hostes sembla que influencia els patrons d'abundància de les espècies més mòbils, com la puça. No obstant, aquesta relació no es dona per la resta d'espècies d'ectoparàsits.

Finalment, a nivell de la comunitat regional (les comunitats components al llarg del rang de distribució de l'hoste), les comunitats d'ectoparàsits difereixen significativament entre les dues subespècies de baldriga cendrosa i la baldriga de Cap Verd, així com entre colònies dins de cada espècie d'hoste. Aquestes diferències sovint responen a un patró geografic (Goüy de Bellocq *et al.* 2002, Krasnov *et al.* 2005c, Poulin & Morand 1999, Poulin 2003). D'acord, els nostres resultats mostren que l'estructura de la comunitat regional està influenciada per la distribució geogràfica de les colònies d'hoste de manera que la similitud en els patrons d'abundància i la composició d'espècies disminueix amb la distància geogràfica. Aquesta similitud pot estar mediatitzada per una dispersió del paràsit hoste- depenent si la dispersió de

l'hoste i la connectivitat entre colònies de l'hoste actua com a factor limitant per a la dispersió del paràsit (Poulin & Morand 1999, Poulin 2003). No obstant, no hem trobat cap relació entre els patrons de connectivitat genètica entre colònies d'hoste i els patrons de similitud en les comunitats d'ectoparàsits. En canvi, sembla que existeix certa influencia del clima. Per tant, la similitud en els patrons d'abundància i distribució estaria mediatitzada més per una semblança climàtica, que no pas per una dispersió hoste- depenent del paràsit.

Tot plegat, els nostres resultats ressalten el paper dels factors espacials com a principals determinats en l'estructuració ecològica de les comunitats d'ectoparàsits als diferents nivells jeràrquics d'organització.

Coevolució del complex Calonectris i els seus ectoparàsits

Els polls de la ploma sempre han estat considerats com organismes model en estudis coevolutius, i la coespeciació entre els ocells marins i els seus polls ha estat prèviament provada (Paterson *et al.* 1993, Page 1994, Paterson & Gray 1997, Paterson *et al.* 2000, Page *et al.* 2004, Banks *et al.* 2006). A més, nombroses característiques ecològiques dels ocells marins, com una forta filopatria, fidelitat als llocs de cria així com el fet de viure en illes, suggereix que els ocells marins i els seus polls representen un model hoste- paràsit en el qual la codivergència estaria afavorida.

En el cas de l'hoste, els nostres resultats mostren una estructuració filogeogràfica pronunciada entre les dues subespècies de baldriga cendrosa i l'espècie de Cap Verd. A més a més, dins de cada subespècie existeix una certa estructuració genètica de poblacions tant a l'Atlàntic com al Mediterrani. D'acord amb això, esperaríem trobar patrons d'estructuració genètica congruent entre les baldrigues i els seus ectoparàsits al menys a dos nivells; ja sigui a nivell de subespècies, entre les dues subespècies de baldriga cendrosa i la baldriga de Cap Verd, així com a nivell de poblacions dintre de cada subespècie. No obstant no hem trobat estructuració genètica en cap de les tres espècies de polls de la ploma que, al contrari, apareixen genèticament indiferenciats en comparació amb la seva espècie d'hoste.

Sota un escenari de coespeciació, esperaríem canvi genètic sincrònic i taxes equivalents d'evolució molecular entre hoste i ectoparàsits (Page 1996). En aquest cas, potser els polls experimenten un fenòmen de "*fracàs per especiar-se*" en resposta a l'especiació del seu hoste (Paterson and Banks 2001, Johnson *et al.* 2003), donat que tots tres taxons de *Calonectris* ja han començat a especiar-se. Que el temps d'aïllament hagi estat insuficient pels polls, explicaria l'escasa divergència genètica observada (Rannala and Michalakis 2003). D'altrament, un endarreriment evolutiu dels polls respecte a l'hoste es pot donar si el flux gènic entre les poblacions del paràsit és molt superior al del seu hoste (Johnson *et al.* 2003). Però, donat que l'especiació de *Calonectris* és més aviat al·lopàtrica i que els polls de la ploma són relativament inmòvils, el flux gènic entre polls de tots tres taxons de *Calonectris* és virtualment impossible. A més a més, per una espècie d'ocell marí que viu en illes oceàniques remotes i que passa la major part de la seva vida en alta mar, la dispersió pels polls està temporalment i espacialment limitada. No obstant, en algunes localitats fins a quatre espècies d'ocells marins poden criar de forma simpàtrica amb *Calonectris*, el que podria promoure la dispersió d'ectoparàsits entre diferents localitats. Però tot i així, no existeixen registres de cap poll de *Calonectris* parasitant a altres espècies d'ocells marins (see Price *et al.* 2003). Com ja hem mencionat previament, una condició necessària per la congruència, és que la dispersió del paràsit estigui mediatitzada per la dispersió de l'hoste (McCoy *et al.* 2003). Però, en el casos en què el flux gènic del paràsit sigui independent, les distàncies geogràfiques entre localitats poden ser una millor aproximació a l'hora d'examinar l'estructuració genètica de les poblacions dels paràsits (Blouin 1995, Weckstein 2005, McCoy *et al.* 2005). De fet les nostres dades ecològiques indiquen una relació entre la distribució geogràfica de les poblacions i l'estructuració de les comunitats d'ectoparàsits (veure l'apartat anterior). Tot i així, la variabilitat genètica en els polls de la ploma no s'estructura d'acord ni amb l'hoste ni tampoc amb les distàncies geogràfiques entre colònies. A part dels modes de dispersió d'hoste i paràsits, altres factors que afectin a la mida efectiva de les poblacions, com per exemple fenòmens d'extinció i colonització freqüents, també poden actuar reduint l'estructuració genètica de les poblacions d'ectoparàsits i, per tant, en el grau de congruència observat (Nadler 1995). De fet, les dades d'abundàcia de la comunitat

d'ectoparàsits indiquen que totes tres espècies de polls experimenten forts cicles d'extinció i colonització coincidint amb el cicle reproductor de l'hoste. Tot i així les nostres dades genètiques no ens poden ajudar a confirmar aquesta hipòtesi.

D'acord amb la nostra segona hipòtesi, donat que existeix un grau d'especificitat d'hoste diferencial entre les espècies d'ectoparàsits de les baldrigues (polls i puces), esperaríem trobar diferencies en el grau de congruència observat (Clayton *et al.* 2004). A més a més, evidencies prèvies suggereixen que els polls corporals (Amblycera) serien més hoste- específics que els polls de l'ala (Ischnocera), i de fet s'han documentat diferències en el grau de congruència entre els dos grups (Johnson *et al.* 2002). Així doncs, la filopatria de l'hoste i la fidelitat als llocs de cria limitaria la dispersió d'espècies més especifiques que passen tot els seu cicle vital associats a l'hoste i mostren transmissió vertical (per exemple les tres espècies de polls de la ploma). Al contrari, les puces, més generalistes i amb una major capacitat de dispersió, es transmetrien horitzontalment entre hostes en el mateix niu i nius veïns així com entre espècies d'ocells marins que comparteixen l'àrea de cria amb les baldrigues *Calonectris*. De fet, les nostres dades ecològiques en quant als patrons d'abundància i distribució de la comunitat d'ectoparàsits de *Calonectris* corroboren aquestes premisses. Mentre que en el cas dels polls de la ploma l'estructuració genètica hauria de ser congruent, en les puces l'estructuració hauria de ser menor que l'observa't per *Calonectris*. Tot i així, en ambdós casos, els resultats genètics estan en desacord amb les prediccions de partida. En primer lloc, no hem detectat diferencies significatives en els patrons de diferenciació genètica entre els polls de l'ala (*Halipeurus* i *Saedmundssonia*) i els polls del cos (*Austromenopon*). En segon lloc, quant a les diferències de congruència entre polls i puces, curiosament mentre que totes tres espècies de polls mostren nivells baixos de diferenciació genètica entre poblacions i l'estructuració filogeogràfica és inexistent, en la puça existeix una estructuració genètica força marcada. Estudis genètics previs mostren que la diferenciació del DNA mitocondrial en polls que parasiten diferents espècies d'hoste pot ser forta comparada amb polls que són específics d'un sol hoste (Johnson *et al.* 2002). En el nostre cas, per totes tres espècies de polls, les baldrigues *Calonectris* en són l'hoste primari, mentre que l'espècie de puça *X. gratiosa* és específica de

tres gèneres d'ocells marins diferents (*Calonectris*, *Puffinus,* i *Hydrobates*) (Beaucournu *et al.* 2005). En aquest context, els nostres resultats coincideixen amb descobriments previs, ja que la diferenciació genètica en la puça és aproximadament 10 vegades major que en els polls. Per tat, l'estructuració genètica de les poblacions de puça poder és el resultat d'una adaptació local a diferents espècies d'hoste. És a dir, per un paràsit generalista, nivells grans de variabilitat genètica poden proporcionar el potencial evolutiu per la formació local de races d'hoste. Exemples similars han estat documentats en paparres i polls que parasiten hostes simpàtrics (McCoy *et al.* 2001, Johnson *et al.* 2002, McCoy *et al.* 2002, McCoy *et al.* 2003). A més, evidències empíriques confirmen que *X. gratiosa* parasita diverses espècies d'ocells marins simpàtrics (compartint habitat) i sintòpics (compartint niu) de les baldrigues *Calonectris* (Beaucournu *et al.* 2005; observació personal). En aquest cas i d'acord amb la hipòtesi de l'adaptació local, esperaríem una major similitud genètica entre individus parasitant diferents espècies d'hoste en la mateixa àrea de cria, espècies hàbitat específiques, que entre individus de diferents poblacions d'hoste d'una mateixa espècie, espècies hoste- específiques. Però que les puces siguin habitat- específiques més que hoste- especifiques encara no esta del tot clar. En aquest sentit, són necessaris estudis genètics més detallats sobre puces que parasitin diferents espècies d'hostes simpàtrics al llarg del rang de distribució de les baldrigues *Calonectris* (*Puffinus*, *Hydrobates*, *Oceanodroma* i *Bulweria* sp.). Tanmateix, les dades preliminars en aquesta línea suggereixen que els patrons d'estructuració genètica en la puça responen més a un factor d'hàbitat i que la variabilitat observada seria resultat d'una adaptació local a les diferents espècies d'hoste.

Implicacions per la conservació

En el context de les implicacions per la conservació dels ocells marins, en aquest tesi hem avaluat la utilitat de diversos tipus de marcadors intrínsecs: DNA mitocondrial, morfologia, isòtops estables i elements traça, per l'assignació de baldrigues cendroses a les seves poblacions d'origen. L'aplicació amb èxit d'aquets marcadors intrínsecs depèn de que existeixin diferències

suficients entre les colònies de cria potencials de forma que els individus puguin ser assignats de manera inequívoca al seu origen.

Globalment, l'efectivitat dels quatre tipus de biomarcadors avaluats: genètics, biomètrics, elements traça i isòtops estables, per l'assignació geogràfica de baldrigues varia amb l'escala espacial de l'anàlisi. Mentre que totes quatre aproximacions són efectives a l'hora de diagnosticar el taxó d'origen, existeixen diferències importants quant a l'assignació correcte de la colònia de cria. L'aplicació tant de marcadors biomètrics com moleculars resulta inefectiva per discriminar entre colònies de cria geogràficament properes. Quant als marcadors biogeoquímics, els isòtops estables mostren patrons de variabilitat latitudinal i longitudinal però el poder predictiu d'aquetes clines per l'assignació d'individus és molt dèbil. Al contrari, els elements traça són més sensibles a les diferències micro- geogràfiques que els isòtops estables, i confereixen la millor taxa d'assignació correcte a escala local. No obstant, els elements traça no mostren gradients geogràfics i per tant l'existència de poblacions fantasma per un mostreig incomplert, podria forçar al model a assignar erròniament individus a colònies molt distants respecte a l'origen verdader. Així doncs, la incorporació d'isòtops al model basat en elements traça, tot i no incrementar la taxa d'assignació correcta, millora la robustesa i l'exactitud del model d'assignació en totes les escales espacials de l'anàlisi.

En un context de coevolució, els ectoparàsits de les baldrigues inclús es podrien utilitzar com a traces biològiques per assignar l'origen dels seus hostes (Criscione *et al*. 2005b, Criscione *et al.* 2006). No obstant, l'aplicació dels ectoparàsits com etiquetes biològiques depèn de l'existència d'una estructuració genètica poblacional més pronunciada en el paràsit en relació al seu hoste. En el nostre cas, la manca de diferenciació genètica dels polls de la baldriga a tots els nivells, no només entre poblacions sinó també a nivell d'espècie d'hoste, fa que aquesta aproximació sigui impracticable.

Conclusions

1. Les baldrigues del complex *Calonectris* mostren una estructura filogeogràfica molt marcada. Tant les dades morfològiques com els anàlisi filogenètics agrupen les poblacions en quatre clades principals d'acord amb les seves distribucions espacials segregades, els quals corresponen als quatre taxons acceptats per la taxonomia convencional.

2. Mentre que l'espècie pacífica de baldriga apareix com el clade més basal i distant, els subclades de les dues subespècies de baldriga cendrosa i la baldriga de Cap Verd s'agrupen en un únic clade Paleàrtic. En contradicció amb la classificació taxonòmica actual, les relacions genètiques de la baldriga de Cap Verd amb les dues subespècies de baldriga cendrosa, posen en dubte el seu status actual de subespècie.

3. Les poblacions de baldriga cendrosa s'estructuren genèticament en dos grups corresponents a cadascuna de les dues subespècies; la baldriga cendrosa Atlàntica i la Mediterrània. Però contrariament al que esperaríem, el front oceanogràfic Almeria- Orà apareix com la barrera efectiva entre les dues subespècies.

4. Dins de cada subespècie, tot i ser més dèbil, també existeix certa estructuració genètica. Mentre que el model d'aïllament per distància explicaria el patró de variabilitat observat a l'Atlàntic, dins del Mediterrani l'oceanografia sembla que també hi juga un paper important.

5. Les comunitats d'ectoparàsits de la baldriga cendrosa i la baldriga de Cap Verd s'estructuren a diferents nivells jeràrquics d'organització i diversos factors espacials hi juguen un paper molt important. Primer, els patrons de distribució i abundància de la comunitat d'ectoparàsits estàn correlacionats amb la distribució geogràfica de les colònies de l'hoste; i segon, les espècies d'ectoparàsits mostren una segregació espacial i una dinàmica temporal molt marcada dins del cos de l'hoste.

6. Tot i l'estructuració filogeogràfica i poblacional de l'hoste, no hem trobat estructuració genètica en cap de les tres espècies de polls de la ploma que apareixen genèticament indiferenciats. Contràriament, la puça mostra una nivells de variabilitat interespecífica alts, però ni l'estructuració genètica de l'hoste ni la distribució geogràfica de les poblacions explica els patrons de variabilitat genètica observats.

7. Mentre que un fenomen de "*fracàs per especiar-se*" pot explicar la incongruència entre les baldrigues i els seus polls, el patró observat per la puça pot ser el resultat de fenòmens d'adaptació local a l'hàbitat i d'especialització simpàtrica a diferents espècies d'hostes.

8. L'avaluació de diversos marcadors intrínsecs per l'assignació de baldrigues cendroses a les seves colònies de cria, demostra que és possible utilitzar marcadors biogeoquímics en plomes, elements traça i isòtops estables, com una eina efectiva en l'anàlisi de la mortalitat d'ocells marins al mar a una escala local.

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