Coevolutionary analysis of the transposon Galileo in the genus Drosophila

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Análisis coevolutivo del transposon Galileo en el género *Drosophila*

–TESIS DOCTORAL–

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El Doctor Alfredo Ruiz, Catedràtic del Departament de Genètica i de Microbiologia de la Facultat de Biociències de la Universitat Autònoma de Barcelona**,**

CERTIFICA: que Andrea E. Acurio Armas ha dut a terme sota la seva dirección el treball de recerca realizat al Departament de Genètica i de Microbiologia de la Facultat de Biociències de la Universitat Autònoma de Barcelona que ha portat a l'elaboració d'aquesta Tesi Doctoral, titulada "Coevolutionary analysis of the transposon Galileo in the genus *Drosophila*".

I per què consti als efectes oportuns, signa el present certificat a Bellaterra, 1 de desembre Noviembre de 2014.

Dr. Alfredo Ruiz.

DEDICATORIA

Esta disertación está dedicada a:

Mi familia por que su apoyo y cariño me acompañan siempre.

Los amigos que se han convertido en mi segunda familia.

Gloria Luo R.I.P

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ABBREVIATIONS

aa	amino acid
AIC	Akaike Information Criterion
BI	Bayesian Inference
bp	base pairs
COI	Cytochrome oxidase subunit I gene
COII	Cytochrome oxidase subunit II gene
CPT	Cherry-picking test
DNA	Deoxyribonucleic acid
d_S	Average number of nucleotide differences between sequences per synonymous site
GTR	General Time Reversible model
GTR+I	GTR + invariable sites
HRR	Historical Range Reconstruction
HT	Horizontal Transfer
ICZN	International Committee of Zoological Nomenclature
LTR	Long Terminal Repeat
Marf	Mitochondrial assembly regulatory factor gene
ML	Maximum Likelihood
MLAR	ML Ancestral Reconstruction
MLE	Mariner Like Element
MRCA	Most Recent Common Ancestor
Mya	Million years ago
ND2	Dehydrogenase subunit 2 gene
p	Probability (p-value)
P	Parsimony
PAR	P Ancestral Reconstruction
PCR	Polymerase chain reaction
SinA	Seven in Absentia gene
TE	Transposable Element
TIR	Terminal Inverted Repeat
TPase	Transposase
TSD	Target Site Duplication

I. INTRODUCTION

I.1 The genus *Drosophila*

I.1.1 Phylogenetic taxonomy

The family Drosophilidae encompasses over 3600 valid binomial Latin names and includes about 2000 species[1] belonging to the genus *Drosophila* (Powell 1997; O'Grady & Markow 2009). Because of both, its great diversity, increasing every year with the description of new species, and controversy on their evolutionary relationships, the systematics of *Drosophila* is complicated. Initially, the tradition to systematize this large amount of taxa started with Sturtevant (1942) and Patterson & Stone (1952), who set forth several taxonomical ranks (Figure I-1), in addition to those formally recognized (family, genus and species) by the International Committee of Zoological Nomenclature (ICZN).

For decades, the species group and subgenus ranks were conveniently accepted by a wide community of *Drosophila* workers in several study fields. One of the first assessments to evolutionary relationships across species groups was performed by Lynn Throckmorton (1962; 1975), who, using morphological, behavioral and biogeographical data, produced genealogical trees consisting in nested groups of species or genera named "radiations" (Figure I-1). Currently, most of Throckmorton's findings have been corroborated by molecular phylogenetic approaches; althought the term radiation started a long controversy in the systematics of the Drosophilidae.

Main reason of this controversy was that Throckmorton's radiations were taxonomical ranks instead of monophyletic[2] groups, for example *Chymomyza* inside

[1] The biological species concept defined as groups of interbreeding natural populations that are reproductively isolated from other such groups has been used in this study (Mayr 1996).
[2] Monophyletic: A group composed of a collection of organisms, including the most recent common ancestor of all those organisms and all the descendants of that most recent common ancestor.

the genus *Drosophila*. Thus, the *Drosophila* radiation was clearly a paraphyletic[3] group (Brake & Bächli 2008; Powell 1997).

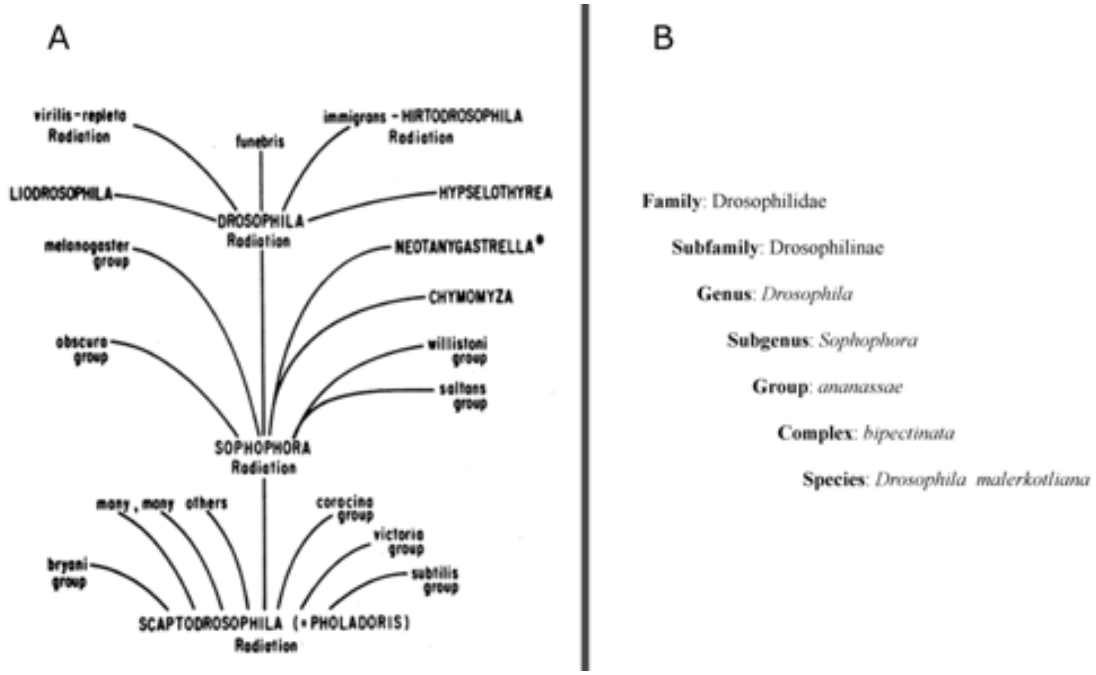

Figure I-1**.** Taxonomic ranks used in the nomenclature of Drosophilidae. (A) Throckmorthon's radiations [modified from Throckmorthon, 1975]. (B) Classification of *Drosophila malerkotliana* is shown as an example [modified from Powell, 1997].

I.1.2 Evolutionary relationships

Subsequent advances in the field of Phylogenetics, first using morphological traits (Grimaldi 1990; Okada 1989), and then analyzing molecular data (Russo et al. 1995; Pélandakis et al. 1991; DeSalle 1992; Da Lage et al. 2007; Remsen & O'Grady 2002; Robe et al. 2002; O'Grady & Kidwell 2002) helped to define better the relationships between species groups, rather than resolve the paraphyletic status of the genus *Drosophila*.

[3] Paraphyletic: A group composed of a collection of organisms, including the most recent common ancestor of all those organisms. Unlike a monophyletic group, a paraphyletic taxon does not include all the descendants of the most recent common ancestor.

Thereby, the taxonomic structure of *Drosophila*, one of the best-studied model systems in modern biology, does not reflect its evolutionary relationships. The release of 12 whole *Drosophila* genome sequences on 2007, and the promise of several more in the future─currently 23 sequenced genomes are available (St Pierre et al. 2014)─ stimulated even more comparative studies in this genus. Such studies can only be sustained by clear, stable taxonomy and well resolved evolutionary relationships of this group. On this scenario, emerged the proposal of Van Der Linde et al. (2007) to the ICZN to splits this genus on three or more separate genera, the proposal included an exemption to the nomenclature rules asking for the change of the genus type (*D. funebris*) to *D. melanogaster* to preserve its name.

This proposal was highly debated by the whole community of *Drosophila* researchers. Some of them supported the proposition (van der Linde & Houle 2008; Roisin 2008; Polaszek 2008; van der Linde et al. 2010), whereas many other opposed it (O'Grady & Markow 2009; McEvey et al. 2008; O'Grady 2010; O'Grady et al. 2008; Yassin 2008). After three years of deliberations, the ICZN rejected the proposal based on three main arguments (1) Exceptions can destabilize names across animal taxa. (2) The proposal was a debate dealing with Systematics and Taxonomy instead to be a nomenclatural issue. (3) The relationships within and between many lineages from the genus *Drosophila*, as currently defined, are poorly understood (ICZN 2010). In addition, many of the putative genera within *Drosophila* lacked of phylogenetic support, taxonomic revisions, morphological synapomorphies, or all three (O'Grady 2010).

Recently, two studies (Yassin 2013; Russo et al. 2013) tried to address this problem and gave important steps in the understanding of the evolutionary relationships of Drosophilidae (Figure I-2). The study from Yassin (2013) analyzed

seven partial coding-regions from 126 taxa and defined morphological synapomorphies for each molecular clade. The resulting monophyly grouping was similar to the one suggested by Throckmorton (1975), based on this and to preserve the binomina of model species (*e.g*., *Drosophila melanogaster*), Yassin advocates that nomenclatural changes be restricted to the subgeneric level by means of the division of the genus *Drosophila* into five subgenera: *Dorsilopha*, *Drosophila*, *Dudaica*, *Siphlodora* and *Sophophora*.

Almost simultaneously, Russo et al. (2013) analyzed nine partial coding-regions from 358 taxa including biogeographic data in their approach. They obtained a relatively well supported phylogeny and were able to give estimates of the time of divergence for major clades in the family. Russo et al. (2013) determined that the Drosophilidae diversification began during the Palaeocene in Eurasia and that the most recent common ancestor (MRCA) from subgenera *Sophophora* and *Drosophila* lived approximately 56 million years ago (Mya). Despite using different taxa, there is some consistency with Yassin's phylogenetic hypothesis. Russo et al. (Figure 1) recovered the family Drosophilidae as a monophyletic clade althought comparatively less support was found in internal nodes, the *Drosophila* radiation is recovered encompassing other genera such as Hawaiian *Drosophila* (*Idiomyia*, *Scaptopmyza*) or *Zaprionus*.

The *Siphlodora* subgenus (*sensu* Yassin 2013) was recovered by Russo et al. 2013 study that named clade A. Interestingly, most of the radiations proposed by Throckmorton (1975) were recovered by both studies, one of them was the *virilis-repleta* radiation.

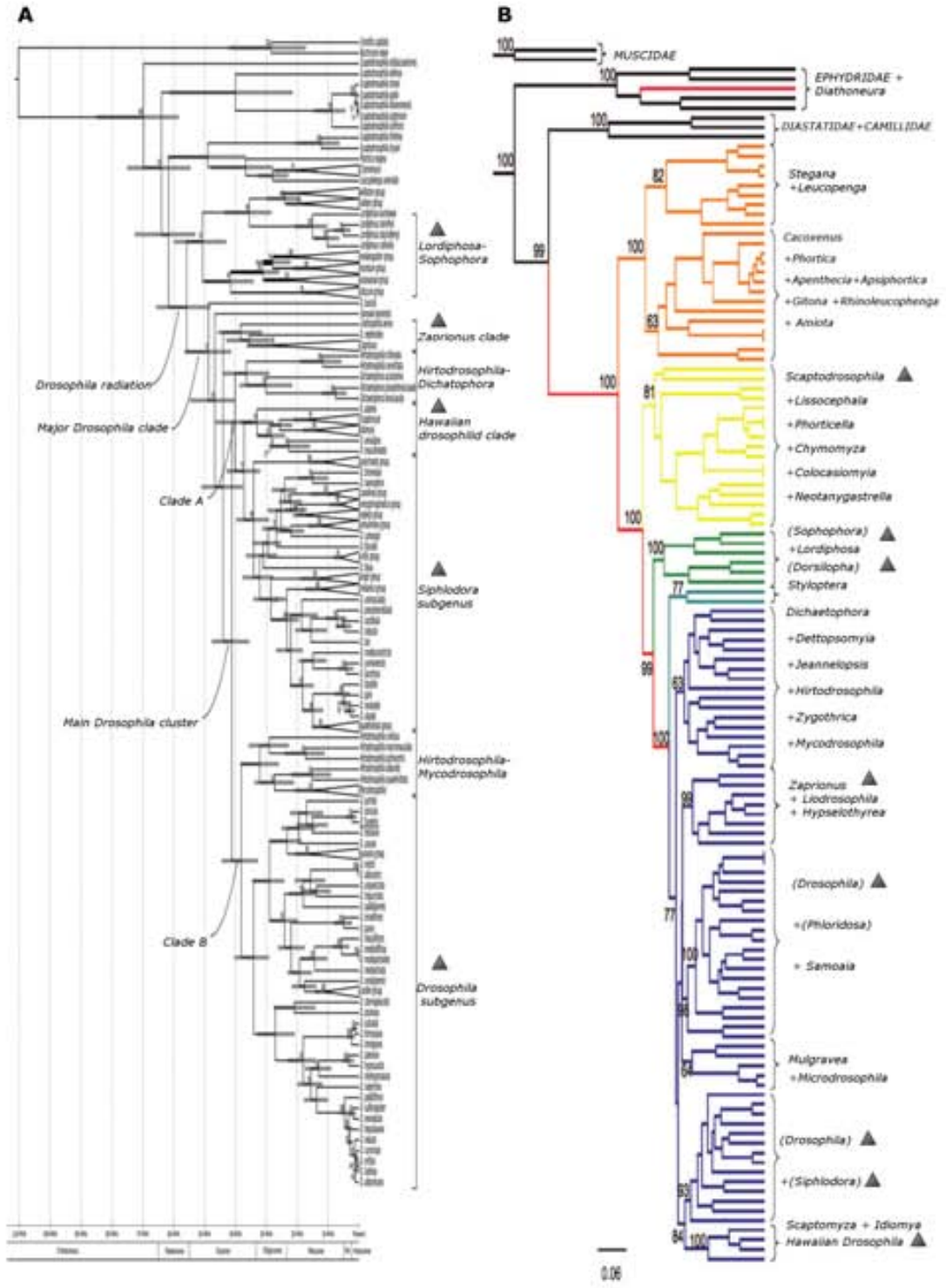

Figure I-2. Latest phylogenetic hypotheses inferred for the Drosophilidae. A) Timescale and ML tree from Russo et al. 2013. B) Bayesian tree from Yassin 2013, new subgenera proposed in parenthesis. Gray triangles denote main clades analyzed in this study.

I.1.3 The *Drosophila repleta* species group

For almost a century the *repleta* species group has been used as a model system for studies of ecological adaptation, evolution and speciation (Sturtevant 1915; Wharton 1942; Wasserman 1982; Ruiz et al. 1997; Vilela 1983; Oliveira et al. 2005). This lineage includes *ca*. 100 species and it is considered one of the most successful radiations among *Drosophila* (Powell 1997). Mainly based on cytological evidence, five subgroups have been traditionally recognized within the *repleta* lineage: *fasciola*, *hydei*, *mercatorum*, *repleta* and *mulleri* (Wasserman 1982, 1992). A sixth subgroup, *inca*, encompassing three species endemic to Ecuador and Peru, has been the latest to be defined using morphological characters (Rafael and Arcos 1989).

A revised molecular phylogeny including representative taxa from the five traditionally recognized subgroups, which also included divergence time estimates for such species, has suggested a South American origin of this group (Oliveira et al. 2012). Several *repleta* species have adaptations to live on cactus, thus it was postulated that this radiation occurred when cacti from the genus *Opuntia* moved to other localities and *Drosophila* species associated with the cacti, spread with them. The fact that South America is the region where the *Opuntia* genus originated leads to propose the same origin for the *repleta* lineage.

This hypothesis brought some debate because the trans-volcanic region from Mexico had been considered for decades the center of diversification of the group (Patterson & Stone 1952, Throckmorton 1975). A subsequent biogeographical assessment of several *Drosophila* species groups performed by Morales-Hojas and Vieira (2012) neither was able to accept or reject the South American origin suggested by Oliverira et al- (2012). Despite the significant contribution of these two

recent studies, neither of them has been able to resolve the origin of the *repleta* radiation. Perhaps the most critical point is that none of the previous studies has included representatives of the *inca* lineage. The *inca* subgroup (Rafael and Arcos 1989; Mafla and Romero 2009) comprises three cactophilic species (*D. inca*, *D. huancavilcae* and *D. yangana*) with an endemic narrow distribution and that live in sympatry with other members of the *repleta* radiation such as the *hydei* subgroup, a clade considered basal in the *repleta* lineage (Oliveira, Almeida, O'Grady, Armella, Desalle, et al. 2012). Inclusion of *inca* species could potentially help to resolve issues such as, low statistical support in phylogenetic trees from previous studies, or the geographical origin of the *repleta* lineage.

I.2 Transposable elements

I.2.1 Abundance and impact on host genomes

Transposable elements (TEs) are short DNA fragments competent to integrate into new positions in the genome, increase their copy number over time and that rely on the enzymatic function provided by an autonomous element[4] (Lisch 2013). TEs were discovered by Barbara McClintock, who was awarded the Nobel Prize in 1983 for her work with instability factors at maize chromosomes, what is currently known as the Ac/Ds system (McClintock 1950; McClintock 1984). Regardless of TEs were initially discovered in plants, it is currently known that TEs are broadly distributed across the eukaryotic tree of life (Feschotte & Pritham 2007) and majority of eukariots and represent a dynamic component on their genomes (Hua-Van et al. 2011).

[4] Definition used in this study.

The possible role that TEs might play in their host genomes has been a matter of discussion since they were discovered. A theory emphasizing the parasitic nature of TEs ─the selfish DNA theory of TEs─ was proposed and theoretically demonstrated during the 80's (Doolittle & Sapienza 1980; Orgel & Crick 1980; Hickey 1982). This idea implies that the emergence and spread of TEs could be explained solely by their ability to replicate themselves in the genome. The underlying logic and coherence of this theory led to a drastic stance on the evolutionary significance of TEs. Subsequent accumulation of molecular evidence demonstrated that, while TEs are by and large genomic parasites, they have been co-opted many times and in a number of different ways to serve the interests of their hosts (Bowen 2002; Kazazian 2004; Feschotte & Pritham 2007; Capy et al. 1998). TEs can be involved in changes that include knockout of gene function, introduction of new functions, changes in the structure of genes, epigenetic silencing of genes and mobilization/rearrangement of gene fragments (Lisch 2013).

I.2.2 Classification

Based on their mechanism of transposition, TEs can be categorized on two major groups (Kapitonov & Jurka 2008; Wicker et al. 2007): (1) Retrotransposons, mobilized by a replicative mechanism that requires the reverse transcription of RNA intermediate also named "copy-and-paste" mechanism[5]. (2) DNA transposons, which usually consist of a transposase (TPase) gene flanked by a terminal inverted repeat (TIR) of variable length. Inside this group, TEs can be divided into: rolling-circle (Helitrons), self-synthesizing (Polintons) and cut-and-paste transposon (Bao et al. 2009).

[5] in which mRNA transcribed from the element by RNA polymerase II (RNA Pol II) is converted into a cDNA by reverse transcription and then integrated by an integrase enzyme at a new position in the genome (Lisch 2013)

In the cut-and-paste transposon reaction, the element is excised from the donor site, causing a double strand break, and inserted elsewhere in the genome. The TE sequence can be restored to the empty donor site by the host repair machinery, leading to an increase in copy number. The integration of the elements into a new genomic location usually generates a short (2-10 bp) target site duplication (TSD) from host sequences (Yuan & Wessler 2011).

The system of classification applied in this study is that proposed by Wicker et al. (2007). This classification includes hierarchical levels (Table I.1). The superfamily level is characterized by a superfamily-specific TPase. Families are defined as a set of phylogenetically close TE copies that share >80% sequence identity (Wicker et al. 2007). Subsequently families can be divided in subfamilies, which are groups of sequences that share specific insertions, deletions or substitutions (Venner et al. 2009). Autonomus elements encode all the necessary proteins for transposition. Non-autonomus elements carry the minimum sequences necessary for transposition but do not encode functional proteins; therefore they require the presence of proteins encoded by autonomous elements.

Table I-1.Examples of the hierarchical classification for Barbara, Talos and Galileo TEs (modified from Wicker et al. 2007).

	Barbara	**Thalos**	**Galileo**
Class:	Retrotransposon	DNA transposon	DNA transposon
Subclass:	N/A	1	1
Order:	LTR	TIR	TIR
Superfamily:	retrotransposon	Mariner	
Family:	Copta	Stowaway	Galileo
Subfamily:	Barbara	Thalos	Newton

I.2.3 DNA transposons

DNA transposons are characterized by a TPase encoded by autonomus copies and with a few exceptions, by the presence of TIRs. The TPases encoded by cut-and-paste DNA transposons are also called DDE/DDD TPases (Bao et al. 2009), due to the universal occurrence of three conserved acidic catalytic residues: two aspartates (D) and one glutamate (E), or three aspartates (DDD). To-date, 17 superfamilies of cut-and-paste DNA transposons are recognized (Yuan & Wessler 2011). Traditionally, monophyletic ancestry of TPase superfamilies is determined by the phylogenetic analysis of their core catalytic region. In some cases (*e.g.*, Tc1/mariner) the superfamily can be further divided into monophyletic groups that have diverged across eukaryotic phyla (Feschotte & Pritham 2007).

I.2.4 Dynamics of DNA transposons

Presence of TEs in a new host genome may have two origins (1) Horizontal Transfer (HT), the transmission of DNA between different genomes in a manner other than traditional reproduction, in which an active copy of the element enter into the germ line, and (2) *de novo* emergence or re-emergence of autonomous sequences as a results of recombination between inactive copies (Hua-Van et al. 2011; Kidwell 2002).

Once arrived in the host genome, the new element has to face the challenge of spreading at levels of the individual and the population. Theoretical approaches of long-term dynamics have suggested at least two possible scenarios: a transposition-selection equilibrium or succession of burst and decay stages. Modelizations have suggested that TEs experience bursts of amplification by which its number of copies

increase (Le Rouzic & Capy 2005). This high rate of transposition is opposed by several other restraining factors such as deletion, selection and regulation, the latest restraint attributed to both, element self-regulation or host genome regulation (Charlesworth et al. 1994; Rouzic & Deceliere 2005; Capy et al. 1998). Although it is widely accepted that transposition is balanced by selection or self-regulation, the persistence of TEs on host genomes over very long periods of time does not necessarily imply a stable copy number equilibrium (Le Rouzic et al. 2007).

As is established by Daniels et al. (1990), to fully understand the evolutionary history of a particular TE within a phylogenetic lineage, it is necessary to determine: (a) its initial point of entry, (b) its subsequent distribution and (c) its mode of transmission between species. When TE transmission has been strictly vertical, the descendants of an ancestral species bearing the element should also possess homologues of the element, if during evolution the element has been lost from one species, then all of its descendants should be element-free. This mode of transmission results in a distribution pattern that is virtually discontinuous.

Alternatively, if transmission has occurred horizontally between reproductively isolated species, the distribution patterns may not follow phylogenetic groupings, for instance, they may be discontinuous. Inconsistences between phylogenies of TEs and host species generally are interpreted as resulting from HT of TEs across species boundaries (Capy et al. 1998). However, other processes that can lead to incongruences between phylogenetic trees include stochastic losses, variation in evolutionary rates and ancestral polymorphism (Capy et al. 1994; Clark et al. 1994).

It has been proposed that HT is an essential step in the TE “life cycle” because it is thought that in this way transposons can escape from the host-defense mechanisms

that lead to its eminent deletion of the genome (Schaack et al. 2010). According to Loreto et al. (2008) from 98 putative cases of HT reported on *Drosophila*, 51% belong to DNA transposons and 49% are from retrotransposons.

Several studies evaluating the impact of TEs on host genomes (Lee & Langley 2012; Lisch 2013) conclude that genomes are quite flexible entities and that TEs can affect gene regulation, composition and structure. Nevertheless only a few studies have looked at the impact of the genomic environment have on TE evolution. TE dynamics is usually inferred from population genetics and the use of simulation models (Rouzic & Deceliere 2005), but there are few experimental studies or biological data (Hua-Van et al. 2011). An emerging approach is exploring this issue from an ecological point of view, using the analogy of TEs as individuals living in the genome.

The term "ecology of the genome" was for first time used by Kidwell & Lisch (1997) to illustrate the complexity of interactions between TEs and their host from an evolutionary perspective. This concept implies an analogy between community ecology and population genetic of TEs. A list of the terms to which the genome is compared with an ecosystem is detailed in the review of Venner et al. (2009). In such analogy, a copy of TE is considered as an individual, one TE species comprises closely genetically related TE copies sharing same interactions with their environment. Genomes could be seen as ecosystems in which TEs families are co-evolving species (Brookfield 2005; Le Rouzic et al. 2007; Venner et al. 2009).

I.2.5 The transposon Galileo

The transposon Galileo was discovered in the breakpoints of the chromosomal inversion 2j on *Drosophila buzzatii* (Cáceres et al 1999). Subsequent analyses of the

same inversion breakpoints in a large set of chromosomal lines discovered another two elements, Kepler and Newton. Because of their structural similarities, these three elements were tentatively classified as Foldback-like elements (Cáceres et al. 2001). Further investigation determined that Galileo was also involved in the generation of another two *D. buzzatii* chromosomal inversions: $2q^7$ and $2z^3$ (Casals et al. 2003; Delprat et al. 2009).

A Galileo screening by both Sothern blot and *in situ* hybridization methods on 23 lines of *D. buzzatii* and 12 lines of closely related species, detected this element in another five species of the *buzzatii* cluster (*D. antonietae*, *D. gouveai*, *D. koepferae, D. serido* and *D. seriema*), three species of the *martensis* cluster (*D. martensis*, *D. venezolama* and *D. uniseta*) and *D. stalkeri*, from the *stalkeri* cluster. Galileo was not detected in species of more distantly related species such as those of the *mulleri* and *repleta* subgroups (Casals et al. 2005).

A subsequent experimental approach of this element in the genome of *D. buzzatii,* (Marzo et al. 2008), characterized an almost complete copy of Galileo with a length of 5406 bp that had TIRs of 1229 bp and an intronless 2738 bp ORF encoding a 912 aminoacids protein (after fixing two stop codons and 1 bp deletion that causes a frameshift mutation).

The fact that Galileo encode a TPase similar to those encoded by other elements of the P superfamily (P and 1360) led to the reclassification of this element inside the P superfamily of cut and paste transposons. In addition, Marzo et al. (2008) performed a *in sili*co search of Galileo and the element 1360 (previously named Hoppel element) on the genomes of the 12 *Drosophila* species sequenced. The results showed that Galileo is present in six species (*D. ananassae, D. willistoni, D.*

psedoobscura, D. persimilis, D. virilis and D. mojavensis) from the two main subgenera, *Sophophora* and *Drosophila.* The most complete copies characterized had a length ranging from *ca*. 4.3 to 5.9 kb and TIRs from *ca*. 0.6 to 0.8 kb. All of them are flanked by 7 bp TSD. However, none of them contains a full ORF encoding a potentially functional TPase because all bear stop codons, deletions or frame shift mutations.

The analysis of the Galileo TPases determined the presence of a THAP domain, a 22 aa long coiled motif and the closely relationship with the 1360 element. In addition, the analysis of TIRs from non-autonomous copies revealed that, in some cases, inside each host genome, Galileo copies clustered in different groups. For instance, *D. mojavensis* harbor four groups C, D, E and F, two of them (C and D) including copies with nearly-complete TPase coding-regions. A fifth Galileo subfamily has subsequently characterized in *D. mojavensis* (Marzo et al. 2013a). A similar subfamily pattern of diversification also has been found in the genome of *D. willistoni*, which harbor V and W subfamilies (Gonçalves et al. 2014).

Based in the comparison of homologous regions of the TIRs (that include the almost identical terminal 40 bp), and that Galileo, Kepler and Newton generate a 7 bp TSD with the same consensus sequence, Marzo et al. (2008) proposed that Galileo is a family of transposons comprising three subfamilies denoted with the letters G, N and K. In fact, this classification was already taken into account by Delprat et al. (2009), who demonstrated that a copy of GalileoN (Newton) has a primary role in the generation of a chromosomal rearrangement through the mechanism of ectopic recombination.

The transposition activity of Galileo was later tested using the THAP DNA-binding domain, which was expressed and purified to test its binding activity towards the respective TIR. In spite that no transposition events were detected, their results revealed an existing ability of the THAP domain to bind different Galileo TIR subfamilies (cross-reactivity), despite to be significantly weaker than binding to their cognate TIR (Marzo et al. 2013b).

I.3 Reconstructing the history of Galileo-*Drosophila* association

I.3.1 Coevolution, Codivergence and Cospeciation

Since Darwin's attempts to show how animal and plants are bound together by a complex web of relations (Darwin 1859; Darwin 1877), coevolution is a fundamental part of the evolutionary theory. The conceptual framework of coevolution appeared in several previous studies (Fahrenholz 1913; Hennig 1966; Ehrlich & Raven 1964), but it is formally defined in the 80's. According Thompson (1982), coevolution is the reciprocal evolutionary change between interactive species driven by natural.

Coevolution is used to explain a great variety of coevolutionary process that can occur between two interacting entities, for instance: prey-predator, plant-herbivore and host-pathogen systems (Woolhouse et al. 2002).

Codivergence is the parallel divergence of two associated lineages within two distinct phylogenies and it is considered as one of the strongest available evidences for coevolution (Page 2003).

Cospeciation is inferred when exist topological congruence between host and associates phylogenetic histories (Page 2003). Cospeciation confirm a long and intimate association between organisms that may be biologically very distinct (Page

& Hafner 1996). The terms coevolution, codivergence and cospeciation are adopted from here on.

Associations between two organisms, for instance viruses in their host, can have a long evolutionary history, which can be reflected in similarities between their evolutionary trees, in this kind of interactions one entity (associate) tracks the other (host) with a degree of fidelity that depends of the evolutionary dynamic of the two organisms associated (Page & Charleston 1998), thus cospeciation can be determined whether matching of phylogenies is greater than that expected by random associations on two clades of interacting species.

The primary goal of comparing associate and host phylogenies (cophylogenetic analysis) is to document the history of their association (Page 2003). Four prerequistes, according Page & Hafner (1996), are necessary to perform a cophylogenetic analysis of two associated entities: (i) well stablished taxonomy (ii) robust phylogenies (iii) wide taxon sampling and (iv) phylogenetic comparison by means of explicit statistical test. In addition, concordance of the two phylogenies could only be expected if sufficient time elapsed between successive host speciation events for lineage sorting to have occurred. (Figure 1-3).

Natural processes such as: gene duplication, lineage sorting, ancestral polymorphism and HT can explain incongruences between host-associate phylogenies (Page 2003; Page & Charleston 1998; Fontdevila 2011).

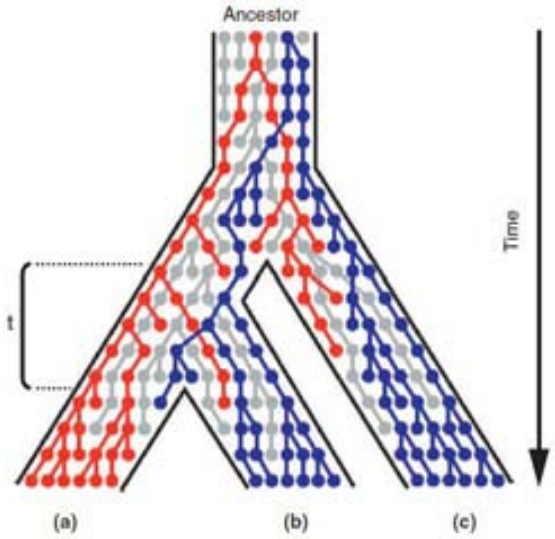

Figure I-3. Virus embedded in its host phylogenetic tree (taken from Sharp & Simmonds 2011). The descendants of two viruses present in the ancestor are shown in red and blue. Polymorphism persisted during the period (time t) between (i) the initial split of species *c* and (ii) the later split between *a* and *b*. so that a phylogeny for his virus differs from the true phylogeny for the three host species.

To determine the timescale of the origin, emergence and evolution of a TE is pivotal to understanding the long-term association with its host. To-date there is no record about comparisons of a DNA transposon and host phylogenies. However, the remarkable similarity between this and other natural associations like retrotransposons in their host (Sacristán et al. 2009), virus in their host (Jackson & Charleston 2004; Switzer et al. 2005; Arnaud et al. 2007) and bacterial endosymbiont in their host (Clark et al. 2000), that have been investigated using the cophylogenetic perspective, have led us to tackle this problem using the same strategy.

II. OBJECTIVES

II. OBJECTIVES

The main goal of this thesis is to determine the long-term evolutionary paths that Galileo transposon has taken with respect to *Drosophila* species at macro-evolutionary level. Given that this goal only could be addressed with robust phylogenetic inferences on both, the transposon and the *Drosophila* host species, a considerable effort was made to recover the most accurate evolutionary relationships on each one of these entities. Robust phylogenetic inference is the result of adequate assessing on: taxon sampling, characters selection and phylogenetic methods. Thus, this thesis has been divided in three subcategories corresponding to three chapters.

II.1. Species level─ Chapter 1

- To perform the formal description of a new species of Drosophila collected in Ecuador.
- To determine the features of paratypes, larvae, pupae and ecology of the new species.
- To select the methods and traits that enable to place the new species in the phylogeny of Drosophilidae.

II.2. Species group level─Chapter 2

- To determine the phylogenetic position of the *inca* species subgroup within the *repleta* radiation.
- To estimate the divergence time in subgroups of the *repleta*, *nannoptera* groups and the new species.
- To analyze the *repleta* radiation in a biogeographical context.

II.3. Genus level-Chapter 3

- To obtain a representative sample of Drosophilidae.
- To analyze the phylogenetic relationships of the *Drosophila* genus.
- To determine the presence of Galileo in Drosophilidae.
- To obtain the TPase sequences of Galileo in detected species.
- To infer a robust phylogenetic tree of Galileo.
- To analyze the sequences of Galileo in a biogeographical context.
- To compare the Galileo and host species phylogenies to determine its historical association.

III. MATERIAL AND METHODS

III.1. Drosophilid collections

Field trips were carried out from December 2010 to February 2011 in 12 localities of Ecuador, South America. The sampling localities were selected according to previous taxonomical reports of drosophilid diversity distribution in Ecuador (Acurio & Rafael 2009). On each locality daily collections were made over 3 days at each site. Drosophilid traps 25 x 5 cm were filled with ca. 110 ml of a 3:1 of fruit and Baker's yeast and were hung in vegetation. Baits were replaced daily after collection of trapped insects.

Trapped male drosophilids were identified by their terminalia and other morphological characters using own criteria and literature. Single inseminated females collected from the wild were allowed to oviposit and the larvae were reared to adults in order to analyze the terminalia of offspring males for species determination. Specimens collected and samples from other sources were stored using a code for each sample.

III.2. Molecular techniques

Procedure followed for DNA extraction, PCR and cloning, in order to generate the sequences used in phylogenetic analysis is detailed in Chapter 3 (Material and Methods). Laboratory protocols followed in this study are detailed in Appendix 1.

III.3. Sequence analysis

Sequence chromatograms were assembled using Geneious (Drummond et al. 2011). Multi sequence alignments were performed using MAFFT (Katoh et al. 2009), SATe (Liu et al. 2012), PRANK (Fletcher & Yang 2010) and CLUSTAL W (Larkin et al. 2007)

III.4. Dataset analysis

Recombination detection was approached with RDP software (Martin et al. 2010). Number of informative sites was calculated using MEGA 4. Model of nucleotide substitutions was selected using jModelTest (Posada 2008).

III.5. Phylogenetic analysis

Cladistic analysis of morphological characters were performed using TNT (Goloboff et al. 2006). Maximum Likelihood analysis were perfomed on SATe (Liu et al. 2012) and PhyML (Guindon et al. 2010). Bayesian Inference analysis using BEAST (Drummond & Rambaut 2007) and BEAUti (Drummond et al. 2012a). Several tools of phylogenetic analysis available on CIPRES (Miller et al. 2010) were used in this study.

III.6. Biogeographical analysis

Ancestral Reconstructions were performed on MESQUITE (Maddison & Maddison 2010). The historical biogeographic ranges of the *Drosophila* repleta group were reconstructed using BioGeoBEARS (Matzke, 2013) in R (R Core Team 2013).

III.7. Cophylogenetic analysis

Congruence between phylogenetic trees of Galileo and host species was assesed with TreeMap 3.0 (Charleston & Robertson 2002; Charleston & Page 2002).

IV. RESULTS AND DISCUSION

During the specimen collection performed in this study, a new species of Drosophila was discovered. The taxonomical description of *D. machalilla* is performed in Chapter 1. The evolutionary relationship of the closest related species group is assessed in the article "Radiation of the *Drosophila nannoptera* species group in Mexico" from Lang M, Polihronakis M, Acurio A, Markow T and Orgogozo V (Appendix 2). Also results of Chapter 1 are: a short popular scientific article (Appendix 3) and the scientific poster exhibit in the XXXII meeting of the Willi Henning Society (Appendix 4).

The phylogenetic and biogeographical analysis of the *repleta* species group that include for the first time the *inca* subgroup─collected in the specimen collection of this study─ is approached in Chapter 2 (*submitted*). Also result of Chapter 2 is the scientific poster exhibit in the Annual meeting of the Society for Molecular Biology and Evolution 2012 (Apendix 5).

The long-term evolutionary dynamics of the transposon Galileo transposon in the Drosophilidae is approached in Chapter 3. A partial result of this chapter is the scientific poster exhibit in the 2013 CSHL Meeting on Mobile Genetic Elements (Apendix 6).

Chapter 1.-Description of a New Spotted-Thorax Drosophila (Diptera: Drosophilidae) Species and Its Evolutionary Relationships Inferred by a Cladistic Analysis of Morphological Traits

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Description of a New Spotted-Thorax *Drosophila* (Diptera: Drosophilidae) Species and Its Evolutionary Relationships Inferred by a Cladistic Analysis of Morphological Traits

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ABSTRACT A phylogenetic approach based on morphological characters is the only alternative applicable in cases where molecular data are unavailable. During a taxonomic inventory of Drosophilidae in 12 localities of Ecuador (South America), we discovered a new species of cactophilic spotted-thorax *Drosophila* Fallen that here we formally describe as *Drosophila machalilla* Acurio 2013. To classify this new species, we analyzed the terminalia of male and female adults, finding similarities with flies of two neotropical spotted-thorax species groups of *Drosophila*, namely *repleta* and *peruensis*. Flies or DNA sequence data are unavailable for the latter species group, hindering a molecular approach. Thus, to accurately classify the new species, we carried out a maximum parsimony cladistic analysis using 52 morphological characters from nine representative taxa of *virilis*, *willistoni*, *repleta*, and *peruensis* species groups. The results indicate that *D. machalilla* sp. nov. belongs neither to the *repleta* group nor to the *peruensis* group and suggest that a new species group should be erected to house *D. machalilla* and *Drosophila atalaia* Vilela & Sene (1982, previously considered a member of the *peruensis* species group).

KEY WORDS *Drosophila*, cladistic analysis, *repleta* group, *peruensis* group

Given the striking advances in Molecular Systematics (Moritz and Hillis 1996, Felsenstein 2004), it may seem that there is not much point in reconstructing phylogenies using morphological data anymore. However, a phylogenetic approach based on morphological characters is the only possibility if no molecular material is available.

Taxonomic inventories or species censuses, the fundamental data in biogeography, macroecology, and conservation ecology (Mora et al. 2008), are important in the assessment of species richness, diversity patterns, and provide verifiable information when specimens are deposited in appropriate institutions (Wheeler 1995, 2010).

Systematics requires accurate data on distribution patterns of taxa provided by taxonomic inventories to resolve evolutionary relationships among species (Wheeler 2004, Wilson 2004, Agnarsson and Kuntner 2007). When previously unknown species are discovered, classifications may need revision to reflect their placement. This undoubted may have a large impact on existing classification schemes because, at this time, we cannot say how many more species exist on earth awaiting discovery (Lipscomb 1998).

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We are engaged in a taxonomic inventory of Drosophilidae in Ecuador (Rafael and Arcos 1989; Vela and Rafael 2004; Acurio and Rafael 2009a,b; Céspedes and Rafael 2012; Figuero et al. 2012). In December 2010, 12 localities of Central Coast, North and South of Ecuador (A. A. et al., unpublished data) led to the discovery of a new cactophilic spotted-thorax *Drosophila* species (Fig. 1A) described below. To classify the new species, we analyzed the external terminalia on male and female adults. We found similarities with two neotropical species groups of spotted-thorax flies: the *Drosophila repleta* species group with >100 described species (Brake and Bächli 2008) and the *Drosophila peruensis* species group with six species described so far (Ratcov and Vilela 2007, Döge et al. 2011). Although we have the new *Drosophila* species in culture and specimens of *repleta* group are available from our collections and *Drosophila* stock centers around the world, specimens of the *peruensis* group species maintained as culture in laboratory or preserved in alcohol are not available. Although several attempts have been made to collect *D. peruensis*, the first species described from the group, at the Urubamba River in Peru, not one specimen was captured (Ratcov and Vilela 2007, p.310). Therefore, a molecular analysis to find *D. machalilla* phylogenetic affinities to the *peruensis* group has not been possible. Nevertheless, we found an important source of reliable data on species descriptions made by specialists on taxonomy of *Drosophila* Fallen (Supp. Table 1

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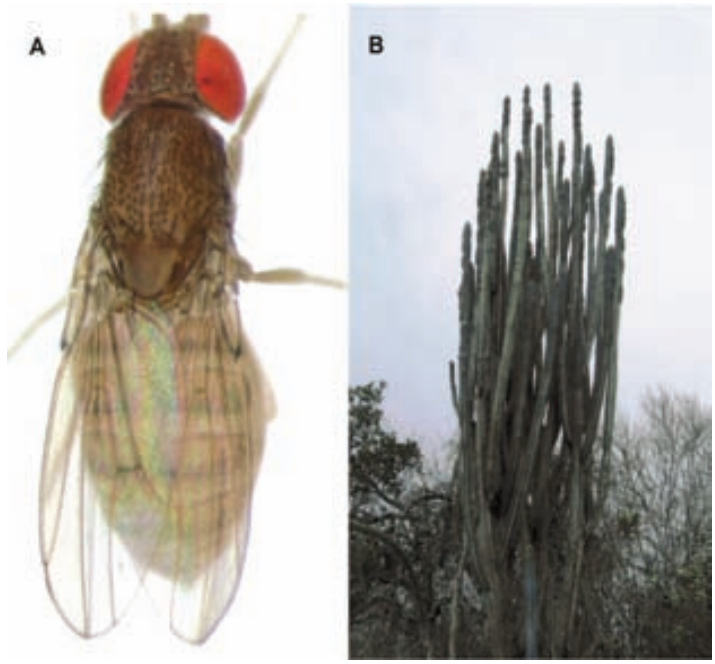

Fig. 1. *D. machalilla* sp. nov. and the substrate where it was collected. (A) Female specimen of *D. machalilla* sp. nov. (B) Columnar cactus *A. cartwrightianus*.

[online only]); this information provides not only description, illustration, and data on biological aspects, but also provides the standardized measures and diagnostic characters. This data source contains enough information to create a matrix and perform a cladistic analysis including species with no molecular data available, as those of the *peruensis* group. A cladistic analysis provides us with a solid framework to reconstruct phylogenetic relationships among taxa by looking for shared derived characters (Hennig 1966).

Here, we describe *Drosophila machalilla* Acurio 2013, and place it in the phylogeny of the genus *Drosophila* by performing a cladistic analysis using 52 morphological characters of male and female adults and immature stages with selected representatives of four species groups (*willistoni*, *virilis*, *peruensis*, and *repleta*) of subgenera *Sophophora* and *Drosophila*. The cladograms generated are the basis to propose a new species group (*atalaia*) and formulate a hypothesis of the evolutionary relationships between the spotted-thorax *Drosophila* species groups *repleta*, *peruensis*, and *atalaia*.

Materials and Methods

Taxon Sampling. *D. machalilla* sp. nov. was recorded only at 1 of 12 localities sampled in Ecuador in December 2010. Twenty individuals were collected in San José Beach (01° 13′ 46.4″ S, 80° 49′ 14.6″ W), located on the Central Coast of Ecuador, in Manabí Province. The site of collection is a coastal dry forest with a high density of cacti, particularly the giant columnar cactus ***Armatocereus cartwrightianus*** (Britton & Rose) Backeb. ex A.W. Hill (Fig. 1B). The sampling area is limiting with the northern border of the Machalilla National Park, one of the megadiverse areas of the world (Mast et al. 1997). This park was established in 1979 as World Biosphere Reserve because it harbors high levels of species richness and species endemism.

The method of collection has been described in previous works (Acurio et al. 2010). For terminalia preparation, we followed the method proposed by Bächli et al. (2005) with minor modifications. Once dissected, terminalia were mounted on glass slides using glycerine. The wings were mounted on glass slides using natural Canada balsam to obtain wing indices and measures. Morphological measurements and counts were taken on a Carl Zeiss DiscoveryV8 stereomicroscope equipped with a Zeiss AxioCam MRc (AFX Services, Quito, Ecuador). Genitalia indices were calculated on Zeiss ImagerA2 microscope using Zeiss AxioVision software release 4.8.2. Images of male and female genitalia, pupae, and eggs were processed using Adobe Illustrator CS to produce the figures.

Analyzed Taxa. Eight taxa of the *Drosophila* subgenus were selected because: 1) they are representatives of species groups that share morphological characters with ***D. machalilla*** sp. nov.; 2) they are representatives of monophyletic groups; their evolutionary relationships have been inferred by morphological or molecular data; and 3) they have a complete taxonomic description that contains standardized indices and ratios. ***Drosophila willistoni*** Sturtevant 1916 of subgenus ***Sophophora,*** was selected as outgroup. The eight taxa from the ***Drosophila*** subgenus include two represen-

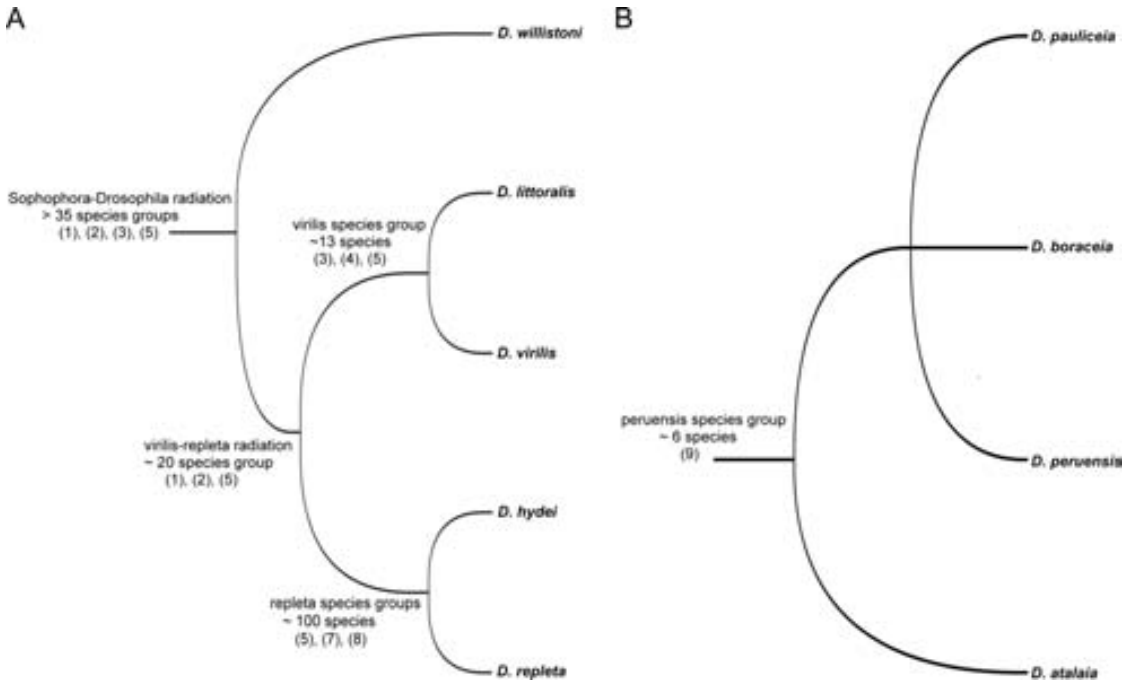

Fig. 2. Evolutionary landscape of the species possibly related to *D. machalilla* sp. nov., numbers in parenthesis on each node show phylogenetic studies supporting each evolutionary hypothesis: (A) *Sophophora–Drosophila* radiation hypothesis: (1) (Throckmorton 1975), (2) (Remsen and O'Grady 2002), (3) (Spicer and Bell 2002), (4) (Wang et al. 2006), (5) (Clark et al. 2007), (6) (Wasserman 1992), (7) (Tatarenkov and Ayala 2001), and (8) (Vilela 1983). (B) *peruensis* species group hypothesis: (9) (Ratcov and Vilela 2007).

tatives from the ***virilis group*** (***Drosophila virilis*** Sturtevant 1916, with a worldwide distribution, and ***Drosophila littoralis*** Meigen 1830, with a Holarctic distribution), two representatives from the ***D. repleta*** species group (***D. repleta*** and ***Drosophila hydei*** Sturtevant 1921), and four representatives from the ***peruensis*** species group (***D. peruensis***, ***Drosophila boraceia***, ***Drosophila pauliceia***, and ***Drosophila atalaia*** Vilela & Sene 1982). The selected species span a wide variety of evolutionary distances, from closely related pairs such as ***D. virilis*** and ***D. litoralis*** (8.6 myr) (Morales–Hojas et al. 2011), and ***D. repleta*** and ***D. hydei*** (16.3 myr) (Oliveira et al. 2012) to the distantly related species of the ***Drosophila*** and ***Sophophora*** subgenera (62.9 myr) (Tamura et al. 2004). Figure 2 provides a summary of the known phylogenetic relationships between the nine taxa.

Selection of Characters. We made a selection of the most informative characters on *Drosophila* imagines, pupae, and eggs (Throckmorton 1962, 1975; Bock 1976; Vilela and Bächli 1990; Bächli et al. 2005; and the authors' unpublished data). Because we were trying to detect a phylogenetic signal, we were interested only on heritable traits. As previously has been established by Grimaldi (1990) in a morphological systematic approach to Drosophilidae, when we are using morphological characters in a cladistic analysis, we are surveying the expressions of thousands of genes, for instance, quantitative trait locus (QTL) mapping studies (Laurie et al. 1997, Zeng et al. 2000) have identified a minimum of 20 loci underlying the morphological difference between ***Drosophila mauritiana*** Lemeunier and Ashburner 1976 and ***Drosophila simulans*** Sturtevant, 1919, closely related species of the ***Drosophila melanogaster*** species subgroup. Another recent study (Yassin 2013) confirms as well the strong phylogenetic signal that morphological characters provide at different phylogenetic scales.

The following criteria were used to select traits: 1) characters taxonomically informative, they should correlate well with taxonomic grouping; 2) independent characters, the measures should not correlate with specimen size. We used not only discrete characters (traditionally used for phylogenetic analyses) but also continuous characters that contain phylogenetic information and often support or reinforce the results generated by discrete characters (Goloboff et al. 2006). Our dataset contains 52 morphological characters, 27 discrete and 25 continuous (Supp. Table 2 [online only]). Two discrete traits pertain to immature stages, the rest to the imago: head (2), thorax (3), wing (4), leg (1), male genitalia (11), and female genitalia (4). All continuous traits belong to the adult: head (7), thorax (5), and wing (13). An almost complete date set was generated for nine taxa, all except *D. peruensis* (Supp. Table 3 [online only]). Only five of the nine taxa have missing data, usually very few (1, 2, 7, 17, and 1 in *D. littoralis*, *D. virilis*, *D. boraceia*, *D. atalaia*, and *D. hydei*, respectively). However, only 15 characters were recorded from the description of *D.*

peruensis. Because specimens of this species have been misidentified frequently (Ratcov and Vilela 2007), the taxon was omitted from analyses.

Cladistic Analysis

A maximum parsimony cladistic analysis was performed with the program TNT (Goloboff et al. 2008). Continuous characters were analyzed as such to avoid ad hoc methods for discretization. The analysis was carried out using the implied weighting method of Goloboff (1993), with k = 15. Continuous characters were optimized as additive by TNT, and discrete characters were considered as unordered, so an evolutionary change could hypothetically transform freely between any of the described states.

To measure concordance between datasets, two measures of group support—Jackknifing ($P = 0.36$) and Symmetric Resampling ($P = 0.33$)—were calculated under implied weighting, with 500 replications. Measures of raw frequency groups were calculated for both, the strict consensus tree obtained by discrete data set and the optimal tree obtained by the complete data set. Similarity on trees was estimated using subtree pruning and regrafting (SPR) distances implemented in TNT. The most parsimonious tree was obtained by implicit enumeration search using the branch-and-bound algorithm. Polarity on the characters was defined by using *D. willistoni* from *Sophophora* subgenus as outgroup. Character mapping and best tree diagnosis was produced in TNT with the option of common synapomorphies on the optimal tree obtained.

Taxonomic Description. We used the traditional morphological terms applied in taxonomic studies of Drosophilidae (Wheeler 1981, Grimaldi 1990, Vilela and Bächli 1990). Abbreviations are as follows: or1 = proclinate orbital seta; or2 = anterior reclined orbital seta; or3 = posterior reclinate orbital seta; vtm = medial vertical seta; vtl = lateral vertical seta; vi = vibrissa; h = postpronotal seta; dc = dorsocentral seta; C = costa; ac = acrostical setae; hb = wing heavy bristles. The indices and measures calculated are based mainly in Bächli et al. (2005).

Drosophila machalilla sp. nov.

Type Material. HOLOTYPE: ♂ QCAZ2519. PARATYPE: ♀ QCAZ2534. Remain in the Invertebrate Museum Collection of the Pontificia Universidad Católica del Ecuador (QCAZ). Labeled: "Ecuador: Manabí: San José Beach, 10–XII–2010, (01° 13′46.4″ S, 80° 49′14.6″ W). Acurio A. coll." Both specimens have microvials with terminalia preserved in glycerol. PARATYPES: ♂ QCAZ2520, ♀ QCAZ2535. Same data as holotype. Additional PARATYPES: 2 ♂♂ and 2 ♀♀ have been deposited in the American Museum of Natural History (AMNH).

Diagnosis. *D. machalilla* can be differentiated from closely related taxa by having a scutellum light brown, medially darker with brownish spots around scutellar setae, without prescutellar setae. Wing indices 4V = 1.83, 5x = 1.79. Aedeagus apically with one pair of short pointed spurs in the ventral margin, hypandrium with spurious disto-dorsal arms.

Male. Head (from live material). Frons yellowish with brownish patches, frontal length 0.43 mm; frontal index = 0.79, top to bottom width ratio 1.44. Frontal triangle narrow, pale brown, as long as frons, ocellar triangle almost completely yellow with dark brown spots around yellow ocellus, ≈45–48% of frontal length. Frontal vittae pale brown. Orbital plates narrow, pale brown with dark brown spots around or1, or2, or3, vtm, and vtl, ≈78–90% length. Orbital setae black, or2 slightly outside of or1, distance of or3 to or1 = 74–80% of or3 to vtm, or1/or3 ratio = 0.8, or2/or1 ratio = 0.5. Postocellar setae 44%, ocellar setae = 70% of frontal length; vibrissal index = 0.55. Face yellowish. Carina yellowish, prominent, nose like, broadened downward, dorsally slightly grooved longitudinally. Gena and postgena light brown. Cheek index ≈6–7. Eyes red bright, eye index 1.2. Occiput dark brown narrowly yellow along eye margins. Pedicel yellowish. Flagellomere one pale brown. Arista with 3–4 dorsal, 2 ventral, and ≈3 small inner branches, plus terminal fork. Proboscis light brown. Clypeus brown, palpus light brown with ≈3 setae and several setulae.

Thorax. Length 1.06 mm. Scutum yellowish with a pattern of dark brown spots around bases of most setae and setulae, eight rows of acrostical setulae. H index 1.6. Transverse distance of dorsocentral setae 170–200% of longitudinal distance; dc index = 0.77. No prescutellars. Scutellum light brown medially darker with brown spots around scutellar setae, distance between apical scutellar setae ≈75–80% of that between apical and basal one, basal setae convergent; scut index = 0.83. Pleura predominantly brown with a yellowish central area, subshining, sterno index 0.72, median katepisternal setae ≈36% of anterior one. Haltere brownish-yellow. Legs yellowish brown, preapical setae on all tibiae, apical seta on mesotibia.

Wings. Hyaline all veins yellowish with a yellowish shadow in the dorsal part of marginal and submarginal cells, costal section with heavy bristles, R1 + 2 and R3 + 4 slightly darker in older individuals, length 2.16 mm. Length to width radio = 1.92. Indices: C = 2.43, ac = 2.22, hb = 0.38, 4C = 0.98, 4v = 1.64, 5x = 1.72, M = 0.62, prox. x = 0.68.

Abdomen. Yellowish, with a narrow brown marginal band, reaching posterior margin of each tergite, subshining.

Terminalia (Fig. 3). Epandrium (Fig. 3A) mostly microtrichose, with seven lower setae and no upper setae; ventral lobe roundish at the tip, dorsally broad and ventrally narrow, microtrichose. Cercus anteriorly fused to epandrium, microtrichose and without ventral lobe. Surstylus microtrichose, with a slightly concave row of *ca.* 14 peg-like prensisetae, *ca.* four inner and seven outer setae. Hypandrium (Fig. 3B) slightly shorter than epandrium, anterior margin convex; posterior hypandrial process and hypandrium with spurious disto-dorsal arms; gonopod linked to paraphysis by membranous tissue, with one seta an-

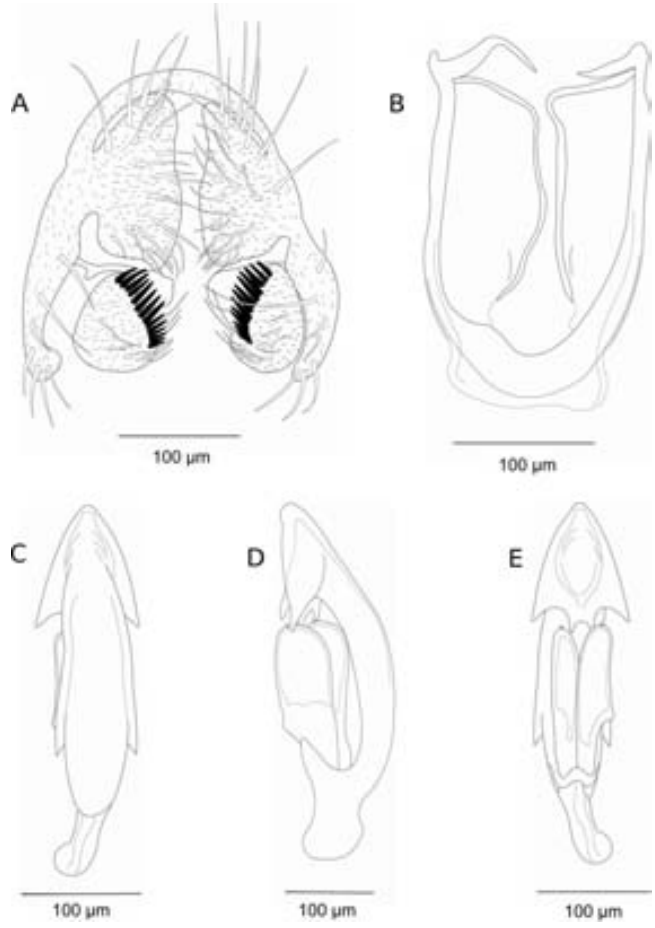

Fig. 3. Male terminalia of *D. machalilla* sp. nov.; (A) Epandrium, cerci and surstyli, and decasternum posterior view; (B) Hypandrium; (C–E) aedeagus, aedeagal apodeme, and paraphyses, several views dorsal, ventral, and right lateral, respectively.

teriorly near inner margin. Aedeagus (Fig. 3C–E) apically pointed, ventrally expanded with a pair of subapical pointed spurs and one pair of short pointed spurs in the center of the ventral margin. Aedeagal apodeme shorter than aedeagus anteriorly expanded dorsoventrally, laterally flattened. Ventral rod as long as gonopod, dorsoventrally flattened. Paraphysis linked both to ventrodistal margin of aedeagal apodeme and to gonopod by membranous tissue, medially with one setula near to dorsal margin.

Female. *Measurements.* Frontal length 0.44; frontal index = 0.79, top to bottom width radio = 1.43. Ocellar triangle ≈43–44% of frontal length. Orbital plates ≈80–90% of frontal length. Distance of or3 to or1 = 78–80% of or3 to vtm, postocellar setae = 45%, ocellar setae = 64% of frontal lenght; vibrissal index = 0.58. Cheek index ≈6.5. Eye index = 1.27. Thorax length 1.11 mm. H index = 1.4. Transverse distance of dorsocentral setae 180–206% of longitudinal distance; dc index = 0.6. Distance between apical scutelar setae ≈82% of that between apical and basal one; scut index = 0.71, sterno index = 0.69, median katerpisternal setae ≈34% of anterior one. Wing length 2.26 mm, length to width radio = 1.97. Indices: C = 2.37, ac = 2.48, hb = 0.46, 4C = 1.1, 4v = 1.83, 5x = 1.79, M = 0.64, prox. x = 0.77.

Terminalia (Fig. 4). Valve of oviscapt (Fig. 4A) brownish, distally rounded, ventrally slightly convex, with *ca.* two distal and *ca.* 11–12 marginal, peg-like outer ovisencilla, first ones roundish and latter ones sharp at tip; trichoid-like outer ovisencilla: three thin, distally positioned and one long curved subterminal.

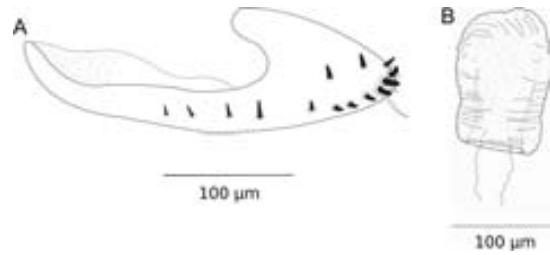

Fig. 4. Female terminalia of *D. machalilla* sp. nov.; (A) Left oviscapt valve, lateral view; (B) Spermathecae.

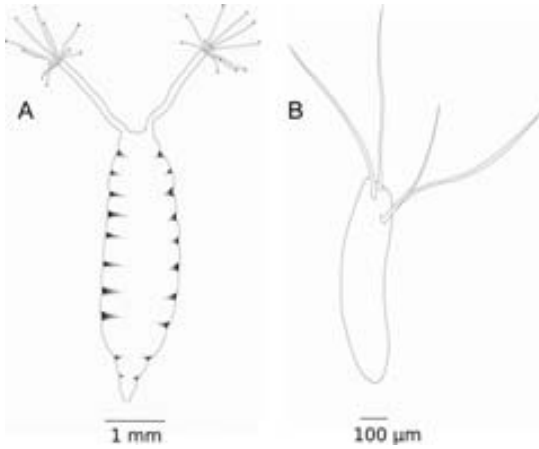

Fig. 5. Immature stages of *D. machalilla* sp. nov. (A) Egg; (B) Pupae.

Spermatecae (Fig. 4B) fingertip-shaped slightly invaginated, heavily sclerotized.

Biology. Puparia (Fig. 5A) yellowish; horn index ≈1.56; each anterior spiracle with ≈12 branches. Lifespan. At 24°C and 33% humidity: larvae hatches 3 d after the egg is fertilized. First, second, and third instar larvae take ≈ 6 d and pupae ≈ 6.5 d. The imagines reach maturity *ca.* 15.5 d. Eggs with four filaments (Fig. 5B).

Etymology. Named to honor the Machalilla culture; one of the most important early societies from Ecuadorian Coast and region where this new species was found. This culture inhabited southern Manabí and Santa Elena Peninsula in a period ranging between: 1400–850 B.C. The Machalilla culture is known by a characteristic pottery style and the practice of skull deformation (Meggers and Evans 1962).

Results

The implicit enumeration analysis of the 27 discrete characters alone, yielded two most parsimonious trees with six nodes, a total adjusted homoplasy of 0.56 and a length of 51 steps (Fig. 6A and B), the strict consensus cladogram of which is shown in Fig. 6C. The consensus tree has five nodes, a total adjusted homoplasy of 0.61, and 52 steps of length. The phylogenetic signal recovered with the discrete data alone is good enough to recover the evolutionary relationships from taxa of the same species group as the clades *virilis* and *repleta.* The addition of 25 continuous characters to the data matrix and an implicit enumeration search under the same parameters yielded the optimal tree of Fig. 6D; this tree has seven nodes, a length of 129 steps. Autapomorphic features distinguishing *D. machalilla* sp. nov. from other spotted-thorax *Drosophila* species (Table 1) are differences in the sterno index, wing indices 4V and 5X.

The minimum number of SPR moves from strict consensus tree obtained by discrete data set (Fig. 6C) to transforming in the best tree obtained analyzing discrete and continuous data set (Fig. 6D) is 0; no movements are necessary because both trees recover identical relationships. We find no pattern of increase or decrease of group support (Jackknifing or Symmetric Resampling) by addition of continuous characters. However, the additions of continuous characters increase the resolution of the phylogeny, as several synapomorphies belong to the class of continuous traits (Fig. 6C and D).

As is depicted in Fig. 6, *D. machalilla* sp. nov. is a sister taxon of *D. atalaia*, and together conform a separate clade of *peruensis* and *repleta* clades. The *atalaia* clade is recovered using both discrete alone and complete data set; this clade is supported by two synapomorphies (Table 2), character 32, presence of a dark costal lappet on the wing, and character 43, presence of disto-dorsal arms of the hypandrium; this structure and differences between taxa is easily distinguished in a graphical comparison of the male genitalia, the most used morphological structure in *Drosophila* taxonomy (Fig. 7).

One of the synapomorphies found in the *repleta–peruensis–atalaia* clade is the character 27, presence of spots at base of setae on mesonotum. Figure 7 shows this trait, shared by species of the *peruensis* group, *repleta* group, and *D. machalilla* sp. nov., mapped on the optimal tree obtained by implicit enumeration.

Discussion

The phylogenetic relationships retrieved in our reanalysis of the *peruensis* group mostly corroborated the previous work by Ratcov and Vilela (2007), which was based on a taxonomic analysis. The previous hypothesis and the results obtained in our cladistic analysis of 52 morphological characters are congruent in the respect that *D. pauliceia* is a sister species of *D. boraceia*, and both species conform a monophyletic group separate from *repleta* species group, despite the different taxa analyzed and methods applied on each study. However, our analysis is discordant with Ratcov and Vilela's (2007) in the phylogenetic relationships of *D. atalaia* because, according to our cladistic analysis, this species belongs to a separate clade outside the peruensis group. Ratcov and Vilela (2007) pointed out that *D. atalaia* was the only species from *peruensis* group that: 1) has no prescutellar setae on thorax; 2) has not both main crossveins darker on wing; 3) has a spurious dorsal arch on hypandrium; and 4) has a different disposition of sensilla in the oviscapt. However, they classified *D. atalaia* in the *peruensis* group based on morphological similarities on male and female terminalia, because at the time, those were the closely related species known. Also noteworthy is the difference in habitat and geographical distribution between the other three species that belong to the *peruensis* group and *D. atalaia* as reported by Ratcov and Vilela (2007 p. 310): "The triad of forest-dwelling species, namely *D. boraceia*, *D. pauliceia*, sp. nov, and *D. peruensis*, are more closely related to each other than they are to the xerophilous and probably cactophilic *D. atalaia.*" It is interesting that both species *D.*

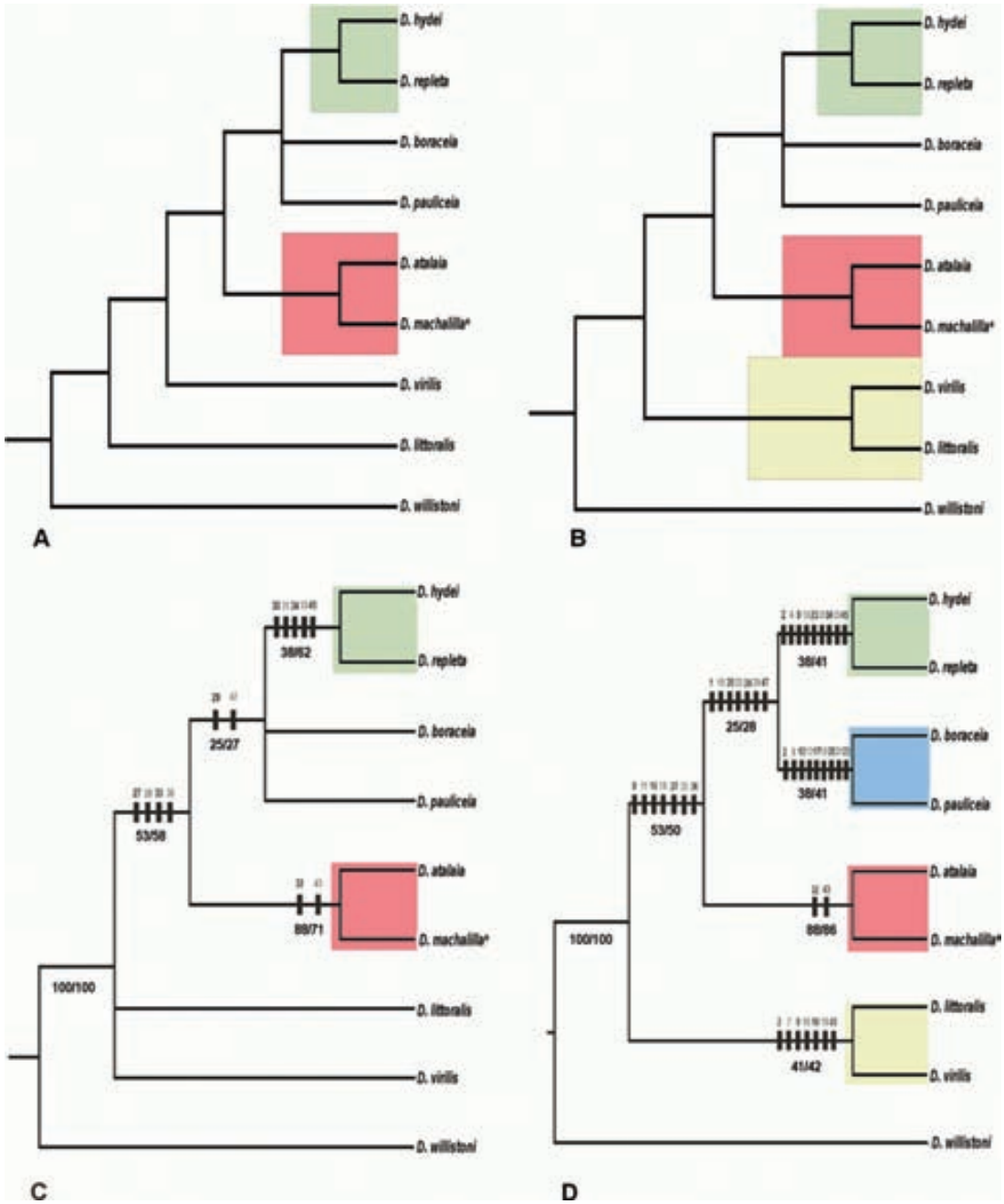

Fig. 6. Results of the cladistic analysis of 52 morphological traits in nine *Drosophila* species. (A and B) Two equally parsimonious trees found by implicit enumeration of the discrete data set (27 discrete morphological traits). (C) Strict consensus tree of two most parsimonious trees A and B found in the analysis of 27 discrete morphological traits. (D) Optimal tree obtained by implicit enumeration of the complete data set (27 discrete + 25 continuous morphological traits). In (C) and (D) synapomorphies (black rectangles) are mapped on trees; the numbers above rectangles refer to character numbers (Supp. Table 2 [online only]); the numbers beneath branchings indicate group support Jacknifing ($P = 36$) and Symmetric Resampling ($P = 33$). Colors denote *Drosophila* clades: *repleta* clade (green), *peruensis* clade (blue), *atalaia* clade (red), and *virilis* clade (yellow). Asterisks denote new species here described.

atalaia and *D. machalilla* sp. nov. occur on coastal dry forest with predominance of Cactaceae. Although we only can speculate about the area of distribution of both species because more collections are necessary, we know that the type locality of *D. atalaia* was Arraial do Cabo located at Brazilian coast of South Atlantic

Table 1. Autapomorphies of each taxa obtained in the cladistic analysis of discrete and continuous characters

littoralis	*virilis*	*boraceia*	*pauliceia*	*atalaia*	*hydei*	*repleta*	*machalilla*
1: 0.84→0.78	**5:** 0.85→0.92	**0:** 1.34→1.5	**2:** [7.40–10]→5	**14:** 2.16→1.8	**0:** 1.3→1.23	**1:** [0.86–0.92]→0.99	**10:** 0.70→0.69
2: 4.0→3.5	**6:** 0.54→0.58	**1:** 0.92→1.0	**3:** 0.6→0.75	**15:** 2.26→1.9	**2:** 6.0→4.5	**3:** 0.56→0.52	**21:** 1.69→1.89
3: [0.56–0.85]→0.55	**10:** 0.83→0.87	**4:** 1.19→[1.2–1.4]	**5:** 0.85→1.0	**19:** 2.43→2.2	**6:** 0.54→0.63	**4:** 1.17→1.16	**24:** 1.6→1.72
4: 1.20→1.14	**23:** [0.61–0.68]→0.72	**5:** 0.85→0.80	**8:** 1.44→1.55	**20:** 0.98→1.2	**9:** 1.32→1.41	**5:** 0.85→0.93	**48:** 3→2
5: 0.85→0.71	**24:** 1.30→1.0	**6:** [0.53–0.54]→0.70	**14:** 2.6→3.0	**21:** [1.69–1.89]→2.0	**10:** 0.81→0.82	**7:** 0.8→0.72	
7: 0.96→1.08		**7:** [0.80–0.83]→0.90	**16:** 2.1→2.13	**22:** 0.62→0.70	**11:** [33.0–34.0]→29.5	**11:** [33.0–34.0]→37.5	
9: 1.22→1.29		**11:** 33.00→27.00	**19:** 3.4→3.79	**47:** 3→2	**18:** [0.40–0.41]→0.48	**14:** 2.6→2.81	
12: 0.67→0.68		**12:** 0.63→0.60	**20:** 0.80→0.65		**22:** [0.50–0.51]→0.46	**17:** [2.04–2.22]→2.24	
13: 1.15→1.16		**13:** 0.88→0.80	**21:** 1.6→1.47		**23:** 0.79→0.80	**36:** 1→0	
15: 2.64→2.83		**16:** 2.1→2.0	**22:** 0.50→0.43		**24:** [1.18–1.27]→1.11	**41:** 2→4	
16: 2.22→2.25		**17:** 1.94→1.90	**32:** 0→1		**33:** 1→0		
17: 2.13→2.08		**23:** 0.60→0.40					
18: 0.59→0.61		**24:** 1.18→1.10					
19: 2.88→2.96		**41:** 2→9					
20: 0.94→0.77		**42:** 4→5					
21: [1.69–1.89]→1.57		**44:** 1→0					
22: 0.56→0.46							
23: [0.61–0.68]→0.51							
38: 2→15							

Numbers in bold denote the characters listed in (Supp. Table 2 [online only]), in brackets ranges of character variation.

Ocean and type locality of *D. machalilla* sp. nov. is San Jose beach located at Ecuadorian Pacific Coast. An analysis of male and female terminalia of both species also confirmed the evolutionary relationship recovered in our cladistic analysis (see above). Besides, the results found here are congruent with a molecular phylogenetic analysis of *D. machalilla* and representatives of six subgroups (*mulleri*, *fasciola*, *hydei*, *mercatorum*, *repleta*, and *inca*) of the *repleta* group and *nannoptera* group using sequences of five molecular markers: three mitochondrial and two nuclear genes (Acurio, Oliveira, Rafael, and Ruiz, unpublished data).

Classification

***Drosophila peruensis* Species Group.** As lineage of the subgenus *Drosophila* Patterson and Mainland, 1944 (or *Siphlodora* in Yassin 2013, classification scheme proposed). In the absence of a male specimen of *D. peruensis*, the phylogenetic position of this group is based on the female specimen.

Diagnosis. sensu lato Ratcov and Vilela (2007) Small flies, with most setae and setulae of the thorax and head arising from dark brown spots, which may be somewhat fused; wings with both main crossveins darker, hypandrium somewhat square-shaped, mostly fused to gonopods and devoid of dorsal arch.

Discussion. Previously, both the *peruensis* and *repleta* species groups were included in the *Drosophila* subgenus (Ratcov and Vilela 2007, O'Grady and Markow 2009). In the classification scheme proposed recently by Yassin (2013 p. 11), the *peruensis* group was placed in the reorganized *Drosophila* subgenus along with *Phloridosa*, *Chusqueophila*, and *Palmophila*, whereas the *repleta* group was transferred to the new Subgenus *Siphlodora.* However, this seems to be incorrect because there are no available molecular sequences for *peruensis* group and male genitalia of this group should place it in the subgenus *Siphlodora* (A. Yassin, personal communication). In addition, the bibliographic reference cited in the study of Yassin (2013) to classify the *peruensis* group is Vilela and Pereira (1985), which has been reported as a misidentification (Ratcov and Vilela 2007 p. 310). Our cladistic analysis corroborates that the *peruensis* species group is closely related to the *repleta* species group and therefore both should belong to the same subgenus.
Taxon content. Five extant species—*D. peruensis*, *D. boraceia*, *D. pauliceia*, *D. itacorubi*, and *D. paraitacorubi.*

Drosophila atalaia* new Species Group.** As lineage of the subgenus ***Drosophila Patterson and Mainland (or ***Siphlodora*** in Yassin 2013 scheme classification). Inside the ***virilis–repleta*** radiation, one of the three major radiations inside the subgenus ***Drosophila*** according to Throckmorton hypothesis (O'Grady and Markow, 2009).

Taxon content. Two extant species: ***D. atalaia*** and ***D. machalilla*** sp. nov.

Diagnosis. Small yellowish flies with dark brown spots on mesonotum, hypandrium with disto-dorsal

Table 2. Common synapomorphies found in each node of the most parsimonious tree obtained in the cladistic analysis of 27 discrete and 25 continuous characters

virilis node	*peruensis* node	*repleta* node	*atalaia* node
2: 6.50→4.00	**2:** 6.50→7.40	**2:** 6.50→6.00	**32:** 0→1
7: 0.80→0.83	**8:** [1.27–1.37]→1.44	**4:** 1.1–1.2→1.17	**43:** 0→1
9: 1.11→1.22	**12:** [0.66–0.67]→0.63	**9:** 1.11→1.32	
10: 0.70→0.80	**13:** 1.06→0.88	**10:** [0.79–0.80]→0.81	
16: 2.18→2.22	**17:** [2.04–2.22]→1.94	**23:** [0.61–0.68]→0.79	
18: 0.53→0.59	**19:** [3.12–3.25]→3.40	**31:** 1→0	
45: 1→2	**20:** [0.81–0.82]→0.8	**34:** 0→1	
	21: [1.69–1.72]→1.60	**35:** 1→0	
	23: [0.61–0.68]→0.60	**45:** 1→2	
peruensis–repleta clade		*atalaia–peruensis–repleta* clade	
15: [2.26–2.64]→[2.84–2.93]		**0:** [1.20–1.27] 0→1 [1.30–1.34]	
19: [2.43–2.88]→[3.12–3.25]		**11:** 35.5 0→140.00	
20: [0.94–0.98]→[0.81–0.82]		**16:** 2.18 0→12.10	
22: [0.56–0.62]→[0.50–0.51]		**18:** [0.53–0.40]→0.41	
24: [1.30–1.60]→[1.18–1.27]		**27:** 0→1	
29: 0→1		**33:** 0→1	
47: 3→4		**36:** 0→1	

Numbers in bold denote the characters listed in (Supp. Table 2 [online only]), in brackets ranges of character variation.

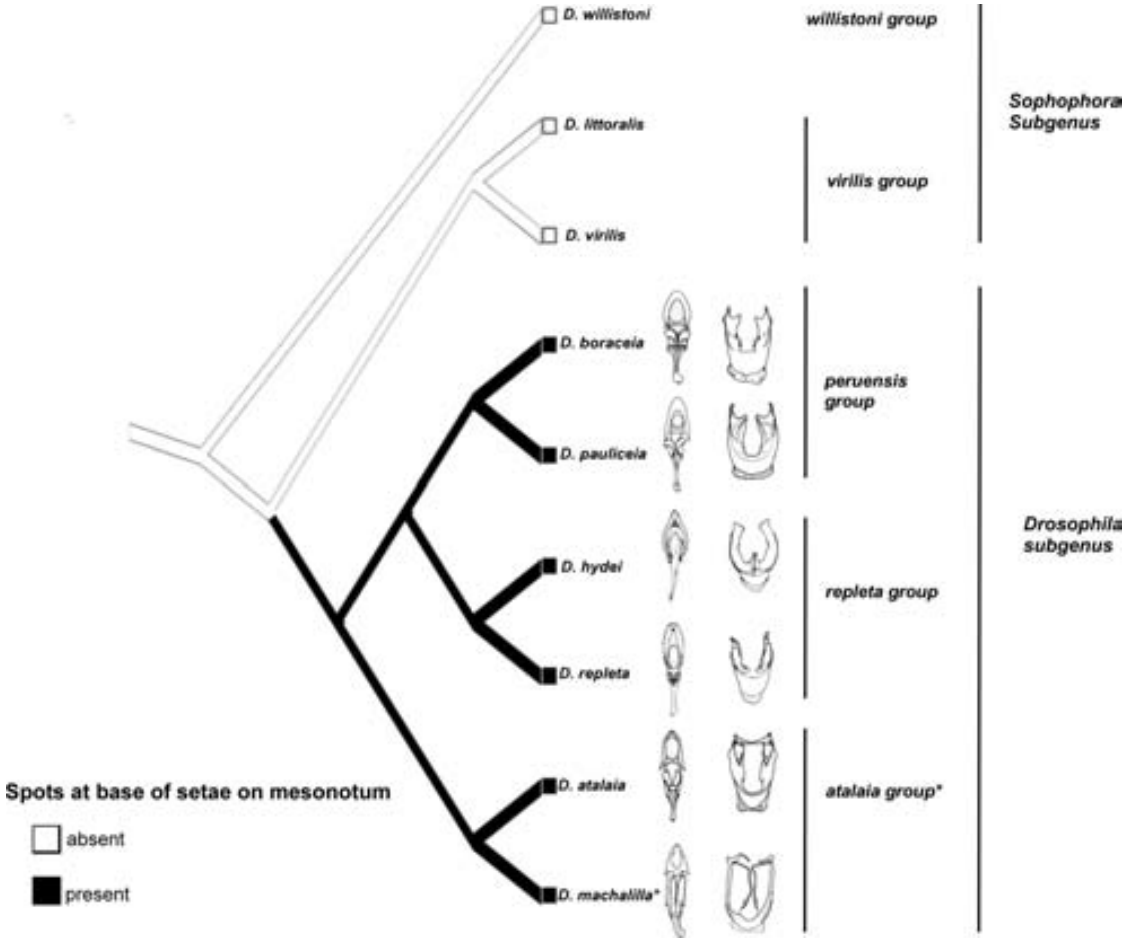

Fig. 7. Phylogenetic tree of *Drosophila* relationships based in the cladistic analysis of 52 morphological traits with spotted-thorax character mapped onto it. Draws show the aedeagus and hypandrium structures of male genitalia (taken and modified from [Vilela and Sene 1982, Vilela and Bächli 1990, Vilela and Val 2004, Bächli et al. 2005, Ratcov and Vilela 2007]). Asterisks denote new species and group species here proposed.

arms, females with a lower most-distal ovisensilla on oviscapt, and habitat preference for coastal dry forest with predominance of Cactaceae.

Discussion. D. atalaia, previously belonging to the *peruensis* species group (Ratcov and Vilela 2007), and *D. machalilla* sp. nov. are now grouped in the new species group *atalaia* on the basis of male and female genitalia, monophyly on a cladistic analysis, preference of substrate, and habitat ecology.

The Spotted-Thorax Character

Neotropical species of *Drosophila* with each hair and bristle arising from black or dark brown spot on mesonotum and a substrate preference for Cactaceae plants were, until few years ago, identified as belonging to the *repleta* species group. Species in this group have been studied in morphological and cytological detail (Wasserman 1982, Vilela 1983) and have served as a model system for evolutionary (Ewing and Miyan 1986; Wasserman 1992; Ruiz et al. 1997; Oliveira et al., 2008, 2012) and ecological studies (Markow 1981, Ruiz and Heed 1988, Krebs 1991, Etges 1993). In the light of our results, we recommend caution in the use of this morphological trait for identification at lower taxonomical levels such as species groups.

Currently it is unclear whether the *virilis–repleta* radiation can be defined as monophyletic (Grimaldi 1990, Tatarenkov and Ayala 2001, Remsen and O'Grady 2002, O'Grady and Markow 2009, Yassin 2013) in this context; high quality systematic research including both alpha-taxonomy and phylogenetically supported hypotheses becomes critical to better resolve the evolutionary relationships of a prime model system as *Drosophila.*

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Chapter 2.-Evidence of a South American origin for the *Drosophila repleta* lineage.

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Short running head: South American origin for the *Drosophila repleta* lineage

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CHAPTER 2 CONTENTS

ABSTRACT

Aim

The *Drosophila repleta* lineage is one of the most widely used model systems for studies of ecological adaptation and speciation. Five subgroups have been traditionally recognized: *fasciola*, *hydei*, *mercatorum*, *repleta* and *mulleri*. A sixth subgroup, *inca*, has recently been described. The *inca* species group includes three species, *Drosophila inca*, *Drosophila huancavilcae* and *Drosophila yangana*, all of them endemic to Ecuador and Peru. Previous molecular phylogenetic studies have been inconclusive regarding the geographic location, time and mode of diversification of lineages within the *repleta* group. We aim to: (1) determine the relationship of *inca* to the other five species groups within *repleta,* (2) improve unresolved branching and low support within the basal portion of the *repleta* phylogeny and (3) estimate the geographic and temporal context of the early divergence within the *repleta* group.

Location

North, Central and South America.

Methods

We collected and identified five endemic species from South America and conducted phylogenetic and biogeographical analyses of all six *repleta* species subgroups based on two nuclear and three mitochondrial gene regions.

Results

Our results confirm the *inca* subgroup's position as the most basal within the *repleta* group and indicate that early diversification occurred within South America.

Main conclusion

Based on the results of our analysis, we suggest that diversification of the *repleta* lineage is associated with the uplift of the Central Andes.

Keywords: Andes, *Drosophila inca* species subgroup, *Drosophila repleta* species group, Ecuador, Peru.

CHAPTER 2

INTRODUCTION

The *repleta* species group of the genus *Drosophila* Fallen 1823, has been used as a model system for studies of ecological adaptation, evolution and speciation for more than ninety years (Sturtevant, 1915; Wharton, 1942; Wasserman, 1982; Markow & O' Grady, 2006). It includes *ca*.100 species, many of which are cactophilic and live in the deserts and arid zones of North and South America. Six subgroups are recognized within the *repleta* species group: *fasciola*, *hydei*, *mercatorum*, *repleta*, *mulleri*, and the most recently defined, *inca* (Rafael & Arcos, 1989).

Recently, a revised phylogeny of the *repleta* group was proposed by Oliveira *et al*., (2012). In this study they provided support for a monophyletic origin of the *repleta* group and presented the first global dating of species divergence times, estimating that the diversification of the crown group began ca. 16 Million years ago (Mya). Historically, the Mexican Trans-Volcanic Region had been considered the center of diversification for the *repleta* group (Patterson & Stone, 1952, Throckmorton, 1975). Oliveira *et al*., (2012) suggested that the origin of the *repleta* group is in South America and is associated with the radiation of its cactus hosts, but could not provide statistical support for the hypothesis. A later study from Morales-Hojas & Vieira, (2012) analyzing the patterns of diversification across the subgenus *Drosophila* also supported the monophyly of the *repleta* lineage but also was not able to resolve the ancestral distribution of the *repleta* species group. Despite the significant contribution of these two recent studies, neither has been able to resolve the origin of this group as being either North America or South America.

One major problem in understanding the origin of diversity in the *repleta* group is that there is a significant bias in the geography of *Drosophila* collections. For decades, sampling effort to collect *Drosophila* specimens has been directed within

North and Central America with an emphasis on arid zones of Mexico (Sturtevant, 1921; Patterson & Mainland, 1944; Oliveira *et al*., 2005). In contrast, relatively little collecting has occurred within South America (Oliveira *et al*., 2012). As a result, the *Drosophila* fauna of North America is very well known while new species and new records are still being described from South America (Acurio & Rafael 2009; Acurio *et al*., 2013). Inclusion of new species from South America has the potential to change the results of biogeographic analyses – particularly if the new species are from basal lineages.

The relatively newly described *inca* species subgroup and several endemic South American species have never previously been included in phylogenetic or biogeographic analyses. Morphological and cytological evidence suggests that the *inca* subgroup occupies a basal position within the *repleta* group (Rafael & Arcos, 1989; Mafla & Romero, 2009), and is comprised of three species known only from northwestern South America. *Drosophila huancavilcae* Rafael & Arcos 1989 and *Drosophila yangana* Rafael & Vela 2003 are endemic from isolated valleys from Ecuadorian Andes. *Drosophila inca* Dobzhansky & Pavan 1943 has the least restricted distribution of the three, being found in Inter-Andean desertic valleys from Perú and Ecuador (Dobzhansky & Pavan 1943, Acurio & Rafael 2009a). Other members of the *repleta* lineage also have endemic representatives within South America, for example *D. huaylasi* Pla & Fontdevila 1990 from the *mulleri* subgroup, endemic from Ecuador and Peru. *Drosophila guayllabambae* Rafael & Arcos 1988, from *hydei* subgroup (Morán & Fontdevila 2005) and the newly discovered *D. machalilla* Acurio 2013 from *atalaia* species group are also endemic only from

Ecuador. We include all of these South American species for the first time in a phylogenetic and biogeographic analysis of the *repleta* species subgroup.

In this study we present an analysis including divergence time estimates for 54 *Drosophila* species using two nuclear (sinA, marf) and three mitochondrial gene regions (COI, COII, ND2). Maximum Likelihood, Maximum Parsimony and Bayesian approaches all infer a well-supported *inca* clade. Furthermore, these analyses show that the *inca* clade is sister to the remainder of the *repleta* group, indicating that it is the earliest diverging lineage in this radiation. Based on the evidence that we present here and the estimated divergence time from ours and previous studies, we propose that the radiation of the *repleta* lineage is associated with the formation of the Central Andes.

MATERIAL AND METHODS

Taxonomic sampling

We included representatives of all six subgroups within the *repleta* group along with four outgroup taxa (*D. virilis* Sturtevant 1919 and three *nannoptera* group species). Adult samples for five *Drosophila* species, *D. inca*, *D. huancavilcae*, *D. yangana*, *D. huaylasi* and *D. machalilla* were collected from the dry habitats of Northern, Central and Southern Ecuador. Within the cactophilic *inca* subgroup we included all three known members. The collecting method described in Acurio et al. (2010) was used, but rotting prickly pear (*Opuntia ficus-indica*) cladodes were substituted for banana in the baits. Once specimens were identified, isofemale strains were established adding a piece of fresh *Opuntia* cladode to the culture medium.

Individuals from isofemale strains sacrificed and stored in ethanol -20ºC. For details about taxa analyzed, refer Appendix S1.

DNA sequences and alignment

We studied three mitochondrial and two nuclear gene regions (Table 1). These markers were selected because they provide a good phylogenetic signal at the deepest taxonomic levels within the *Drosophila* (see references Table 1). Template DNA was extracted from three flies per isofemale strain using a modified Cetyl trimethyl Ammonium Bromide (CTAB) protocol (Wagner *et al*., 1987). Gene regions of interest were amplified using standard PCR protocols, DNA Taq polymerase (Roche). PCR products were purified using Nucleo Spin Extract II (Clontech Laboratories) and sent to Macrogen Inc. (Seoul, Korea) for Sanger sequencing. Chromatograms were compiled using Geneious version 5.0.4 (Biomatters). The sequences generated in this study were deposited in GenBank under accession numbers KC011819-KC011843. Identifiers for all sequences used in this study are given in Appendix S1.

To explore the variability within estimated alignments, we compared the alignment quality scores obtained with the programs PRANK (Fletcher & Yang 2010), MAFFT (Katoh et al. 2009) and CLUSTAL W (Larkin et al. 2007). The three programs produced nearly the same high scores for four of the five genes analyzed. To determine positional homology of introns or intergenic regions, we used the visual interface implemented on Suite MSA (Anderson *et al*., 2011). Columns with low quality scores were removed prior to phylogenetic analysis. The concatenated alignment comprised 2,462 aligned sites, including 147 constant characters, 468 parsimony-uninformative characters, and 1847 parsimony informative (75%) sites.

Phylogenetic analysis

Maximum Parsimony (MP) analysis was performed in MESQUITE 2.74 (Maddison & Maddison, 2010). A search of the most parsimonious tree was conducted based on tree-length criterion, using SPR (Subtree Pruning and Regrafting). A consensus tree was obtained from the trees using a Majority Rule Consensus, considering tree weights with a frequency of clades of 0.5 in unrooted trees.

Maximum Likelihood (ML) analysis was performed on Saté software (Liu *et al*., 2012), set as a multi-locus analysis. The model of nucleotide evolution chosen to best fit our data set was General Time Reversible (GTR). The alignment and merger steps were done separately for each locus and tree inference was made on a single tree for all loci.

The Bayesian Inference (BS) analysis was performed in BEAST (Drummond *et al*., 2012). We set locus specific substitution models and molecular clocks for a nuclear and a mitochondrial partition, using the best-fit models calculated in jModelTest 2.1.3. The nucleotide substitution model for the mitochondrial partition (including *ND2*, *COI*, *COII*) was (GTR), with empirical base frequencies plus Gamma model of site heterogeneity (four categories). The nuclear partition (*SinA*, *Marf*) had the same settings but without codon partition. The same concatenated dataset was used in all three (MP, ML, BS) analyses described here.

Divergence time and diversification analysis

There have been a variety of divergence time estimates proposed for the time to the most recent common ancestor (TMRCA) of the *virilis-repleta* radiation in previous work, which vary according the model used and number of points chosen to calibrate the molecular clock. Obbard *at al*., (2012) estimated the ancestor of the *repleta* group and related species groups split at approximately 12±3 Mya. However,

most estimates are significantly older and are in general agreement: Oliveira *et al*., (2012) proposed a date of 26± 6 million years ago (Mya), Morales-Hojas & Vieira (2012) provided two estimates based on different calibration strategies of 23±4 and 31±4 Mya, and Russo *et al.* (2013) estimated the split to be 27± 5 Mya.

To estimate the divergence time of the *inca* clade, calibration points were chosen from Oliveira *et al*., 2012: the TMRCA of the split between *D. mojavensis* and *D. arizonae* (1.83 Mya) and the TMRCA of the *repleta* group (16.3 Mya). Priors were assumed to follow a normal distribution with the mean and a standard deviation according to Oliveira et al. (2012). Due to differences in rate variation between mitochondrial and nuclear genes (Moriyama & Powell, 1997), the analysis was run on a concatenated data set with two partitions, nuclear and mitochondrial. Clock models were linked, and a common strict clock rate was assumed for all partitions. A starting tree was randomly generated under the Yule process. Four independent runs, using Markov Chain Monte Carlo (MCMC) chains with 10 million generations were performed and sampled every 1000 generations. The resulting output file was processed by using Tree Annotator 1.5 with a burn-in parameter setting of 1000. Effective sample sizes were reviewed with Tracer v. 1.4 to ensure that they were greater than 500 for each parameter. Independent runs were compared to ensure they converged on the same posterior distributions and reached stationarity.

Biogeography

The historical biogeographic ranges of the *Drosophila repleta* group were reconstructed using BioGeoBEARS (Matzke, 2013) in R (R Core Team 2013). First, a three-state presence-absence matrix was constructed that represented the known distribution of each species in North, Central and/or South America. Then, the

historical ranges were estimated under two different unconstrained models (1) Dispersal-Extinction-Cladogenesis (DEC) (first implemented in Ree and Smith, 2008) and (2) Dispersal-Extinction-Cladogenesis-Jump (DEC+*j*) (Matzke, 2013) using maximum likelihood. Comparison of these two models allowed an assessment of the relative roles of range expansion, range extinction and founder events (defined in this model as the acquisition of a new range without the parent lineage having already expanded into it) in the evolution of ranges in this group (Matzke, 2013). Model performance was assessed using a likelihood ratio test. Reconstructions were conditioned in absolute time with the chronogram from BEAST.

RESULTS AND DISCUSSION

Evolutionary relationships of the *inca* species subgroup

The topology of the phylogenetic trees generated using MP, ML and BS analyses are quite similar. The *inca* subgroup is monophyletic and well-supported (Appendix S2) in all of them, as suggested by previous morphological and cytological analyses (Rafael & Arcos, 1989; Rafael & Vela, 2003; Mafla & Romero, 2009). Within the *inca* species subgroup, *Drosophila huancavilcae* is a sister taxon of *D. inca* and both species are closely related to *D. yangana* (Fig. 1).The *inca* clade is the first diverging lineage inside the *repleta* species group. Other early-divergent clades within the *repleta* radiation are *eremophila*, *fasciola*, and *hydei*, and all are well-supported across analyses (Fig. 1, Appendix S2).

Drosophila machalilla from the *atalaia* species group is closely related to the *nannoptera* species group. Lang *et al*., 2014 estimate that the *nannoptera* group diverged from *D. machalilla* around 16.9 - 7.4 Mya. This time period corresponds to

the closure of the Panama isthmus (Montes *et al.*, 2012) which suggests that the ancestor of the *nannoptera* species group may have migrated over the isthmus from South America (Lang *et al*., 2014).

Biogeography of the *repleta* lineage

Overall likelihood scores, *d*, *e* and *j* parameters for the two biogeographic models were as follows: (1) DEC= LnL=-87.8, *d*=0.02, *e*=0, *j*=0 and (2) DEC+j=LnL=-81, *d*=0.014, *e*=0, *j*=0.076. The DEC+*j* model performed significantly better than the DEC (LRT *pval*=0.0002). The difference between the two biogeographic models tested is that in addition to allowing range expansions and range extinctions (*d* & *e*), the DEC+*j* model also allows for founding events (*j*). Both models support zero role for range extinction, but the addition of the *j* parameter in the DEC+*j* model appears to create a better fit to these data.

Our analyses indicate that the *repleta* group formed 17 (95% HPD 16.35-17.85) million years ago in South America (prob=0.66; Fig. 2, Appendix S3). There is relatively only a very small amount of support for the origin of the group in North America (prob=0.17) or both North and South America (prob =0.17; Fig. 2). We place the divergence of the *inca* species subgroup at 13.11 (95% HPD 11.53-14.63) Mya, also in South America (prob=1.0; Fig. 2, Appendix S3). Oliveira *et al*., (2012) earlier hypothesized that switches among major cactus host lineages promoted the radiation of the *repleta* species group. Host plant switches likely did play a role in the diversification of this group, but based on our timing, biogeographic reconstructions and distribution data, we suggest that the larger context for diversification of the basal lineages was the uplift of the Andes.

Geological changes can result in barriers and filters affecting biotic migration. Andean uplift dated in the mid-Miocene (Gregory-Wodzicki, 2000; Capitanio *et al*., 2011) has been proposed to play a major role in species distributions of a variety of animal species groups, for example: rodents (Reig, 1986), butterflies (Descimon, 1986), and amphibians (Duellman & Wild, 1993). The distribution of the *inca* clade, as well on the *repleta* lineage (Fig. 2) is restricted to isolated desertic Inter-Andean Valleys of Northwestern South America. The range distribution of *D. inca* and *D. yangana* is the Huancabamba region, which has been identified as an Andean center of endemism and species richness (Young & Reynel, 1997). In studies of birds and amphibians, Vuilleumier (1969) and Duellman & Wild (1993), respectively, proposed that the high level of endemism and species richness observed corresponded to the dynamic and changing environment presented by the growing Andes as they rose to their current elevation.

The orogenic sequence of the Andes proceeded in a south-to-north fashion (Gregory-Wodzicki, 2000) allowing dispersion of southern species to the north through Central America. Patterns in the endemic bird species to the trans-Andean region (Weir & Momoko 2011) suggest that Andean uplift promoted the build-up of biodiversity in lowland Neotropical faunas both through vicariance-based speciation during uplift and through dispersal-based speciation following uplift. This pattern may hold for the *Drosophila repleta* group as well, as there are several species within this group endemic to the lowland tropics east of the Andes (for example, *Drosophila vicentinae*, *Drosophila peninsularis* and members in the *fasciola* species subgroup). More collecting within South America is necessary to address questions about how the colonization of the trans-Andean region occurred and the impact of the Andean uplift on speciation and diversification of the *repleta* species group.

Our findings are consistent with the pattern of diversification of drosophilids proposed by Russo *et al*., (2013). According to the authors, the radiation of the family Drosophilidae began during the Palaeogene, peaked during the Miocene and was fuelled by the flies' exploitation of the newly diversified fleshy fruits of Angiosperms. Members of the *repleta* group are known to occupy a great diversity of habitats ranging from wet, tropical forests to temperate environments (Vilela, 1983, Acurio & Rafael, 2009b), but the majority of the species are specialized on cacti. The biogeography of cacti also appears to have been influenced by Andean uplift. According to Arakaki *et al*., (2011), most of the extant diversity in cacti was generated throughout the mid to late Miocene and into Pliocene, resulting in three main centers of cactus diversity and endemism: Mexico, the central Andes, and Brazil. The temporal concordance between major diversification events within cacti and the crown diversification of the *repleta* lineage are dated to the same period as Andean uplift in the middle Miocene (Oliveira *et al*., 2012; Morales-Hojas & Vieira, 2012, and results from this study).

CONCLUSION

For the first time we included several endemic South American species, including the *inca* species subgroup, in a phylogenetic analysis of the *Drosophila repleta* group. Our results support the hypothesis that *inca* is the most basal lineage within the *repleta* group. Our phylogenetic analyses, combined with divergence time estimates and biogeographic analysis indicate that the oldest diversification events in the *Drosophila repleta* lineage occurred in the mid-late Miocene in South America.

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BIOSKETCH

Andrea Acurio is an evolutionary biologist who has a long-term interest in understanding the underlying mechanisms that promote biodiversity in nature. Her research interest includes alpha-taxonomy, systematics, ecology and biogeography using *Drosophila* as a model organism. The interdisciplinary research group involved in this study has different fields of expertise ranging from systematics, comparative genomics and phylogeography of *Drosophila*, a premier model system.

AUTHOR CONTRIBUTIONS

A.A. performed the specimens collections and molecular analysis; A.A. and V.R., identified the specimens; A.A. and D.C.S.G.O., performed the phylogenetic analysis; A.A and K.G., performed the biogeographical analysis; A.A., K.G. and A.R. wrote the manuscript and conceived the ideas.

TABLES

Table 1. Summary of the different gene regions used in this study and reference of each primer.

	Abbreviation	Length	Primer design reference
Mitochondrial genes			
Cytochrome C oxidase subunit I	COI	367	(Oliveira et al. 2005)
Cytochrome C oxdase subunit II	COII	706	(Beckenbach et al. 1992)
Mitochondrial-ubiquinone oxidoreductase chain	NADH	782	(Oliveira et al. 2005)
Nuclear genes			
Mitochondrial assembly regulatory factor	Marf	552	(Bonacum et al. 2001)
Seven in Absentia	SinA	397	(Bonacum et al. 2001)

Table 2 . Estimates (with 95% Credibility Interval, CI) of divergence times (MY) for the main nodes recovered in our phylogenetic analysis of the *repleta* lineage on BEAST.

Clade	Mean node age (My) +95%CI
inca crown	13.11(11.53-14.63)
fasciola crown	10.73 (9.28-12.29)
eremophila crown	7.8 (6.87-8.99)
hydei crown	10.61(9.32-11.93)
anceps crown	12.82(11.7-13.95)
repleta crown	12.12(11.07-10.10)
mulleri crown	11.38(12.32-14.08)
repleta group radiation crown	17.00(16.35-17.85)

FIGURE LEGENDS

Figure 1. ***repleta* group phylogeny: Chronogram showing divergence dates for 54 *Drosophila* species analyzed in this study, in red the *inca* species subgroup.** The relationships depicted among taxa, and the divergence dates on the chronogram were estimated using BEAST by analysis of 5 loci.

Figure 2**. Divergence time estimation and historical range reconstructions for the *Drosophila repleta* subgroup.** Reconstructions were performed using the DEC+*j* model in BioGeoBears (Matzke in revision), conditioned on a cladogram generated in BEAST (Drummond et al. 2012). N=North America, C=Central America, S=South America. "*" indicates the species is endemic to Northwestern South America.

Figures

Figure 1.

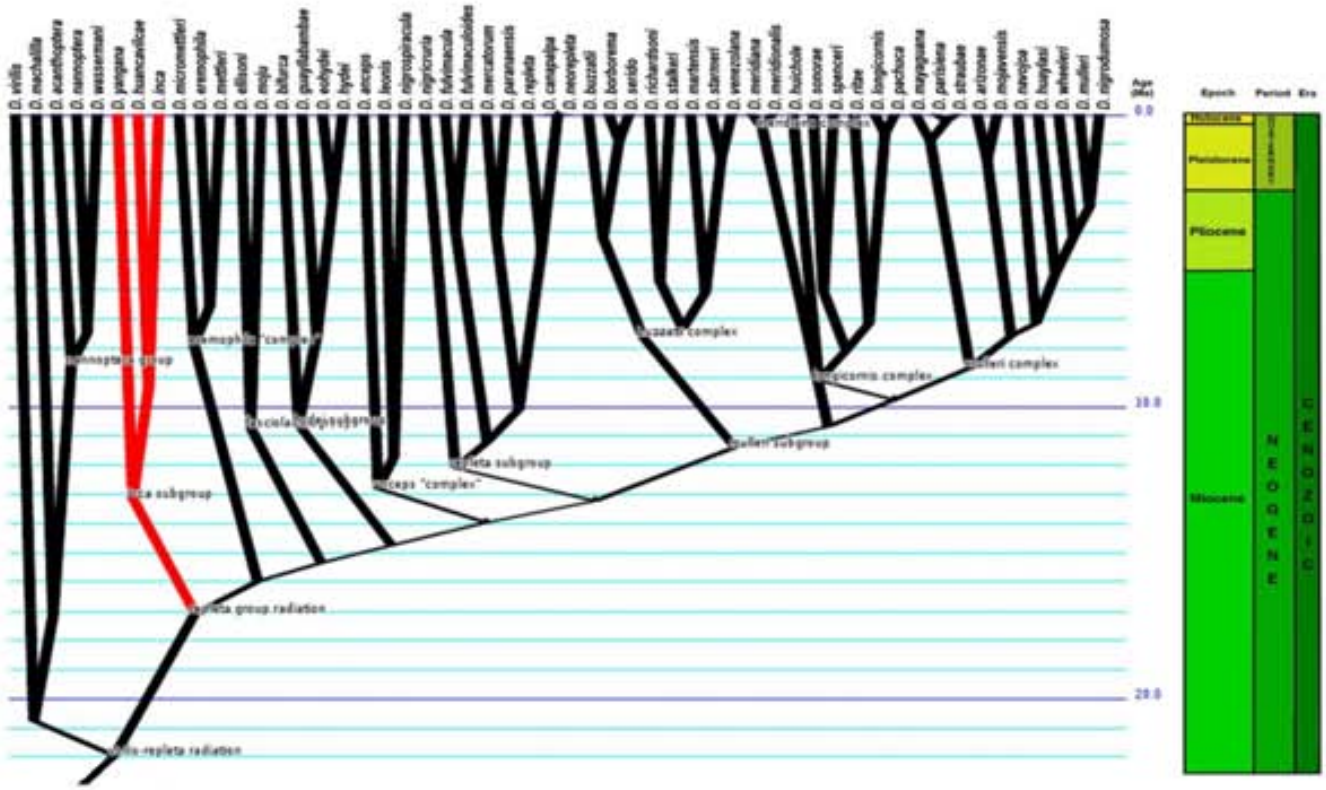
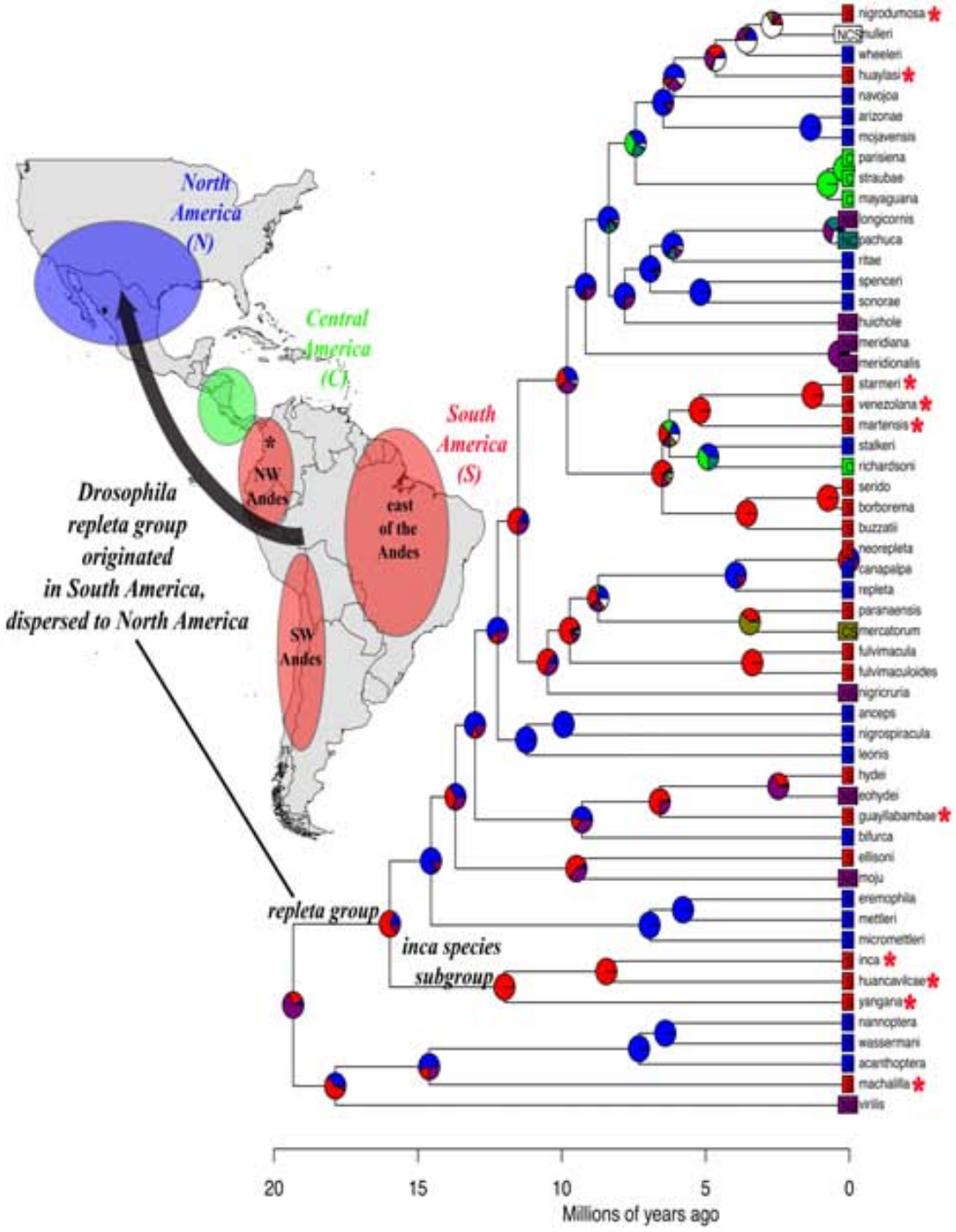


Figure 2.

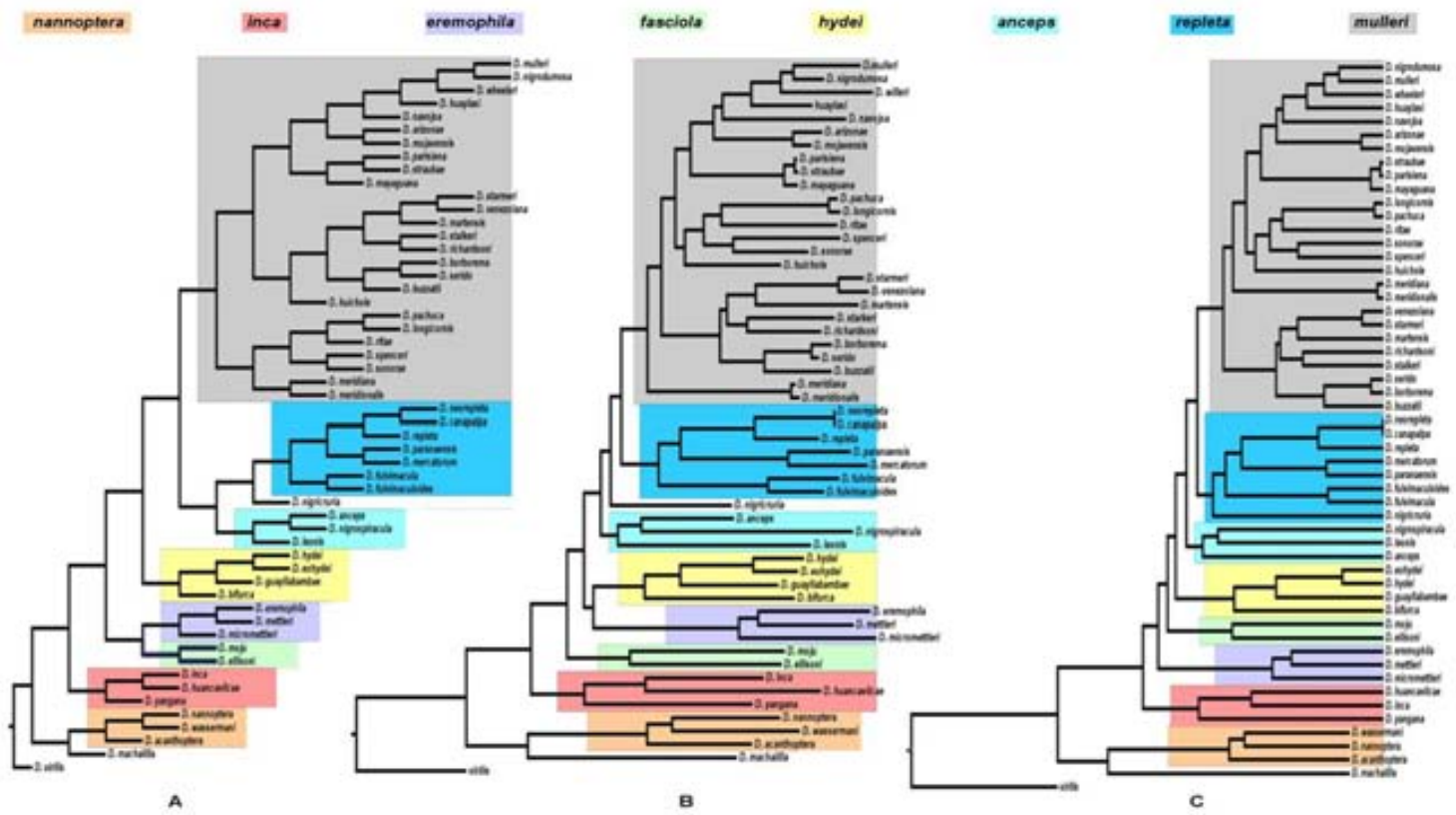

North America (N)
Central America (C)
South America (S)
NW Andes
east of the Andes
SW Andes
Drosophila repleta group originated in South America, dispersed to North America
repleta group
inca species subgroup
nigrodumosa
nullieri
wheeleri
huaylasi
navojoa
arizonae
mojavensis
parisiena
straubae
mayaguana
longicornis
pachuca
ritae
spenceri
sonorae
huichole
meridiana
meridionalis
starmeri
venezolana
martensis
stalkeri
richardsoni
seridо
borborema
buzzatii
neorepleta
canapalpa
repleta
paranaensis
mercatorum
fulvimacula
fulvimaculoides
nigricruria
anceps
nigrospiracula
leonis
hydei
eohydei
guayllabambae
bifurca
ellisoni
moju
eremophila
mettleri
micromettleri
inca
huancavilcae
yangana
nannoptera
wassermani
acanthoptera
machalilla
virilis
20
15
10
5
0
Millions of years ago

APPENDICES

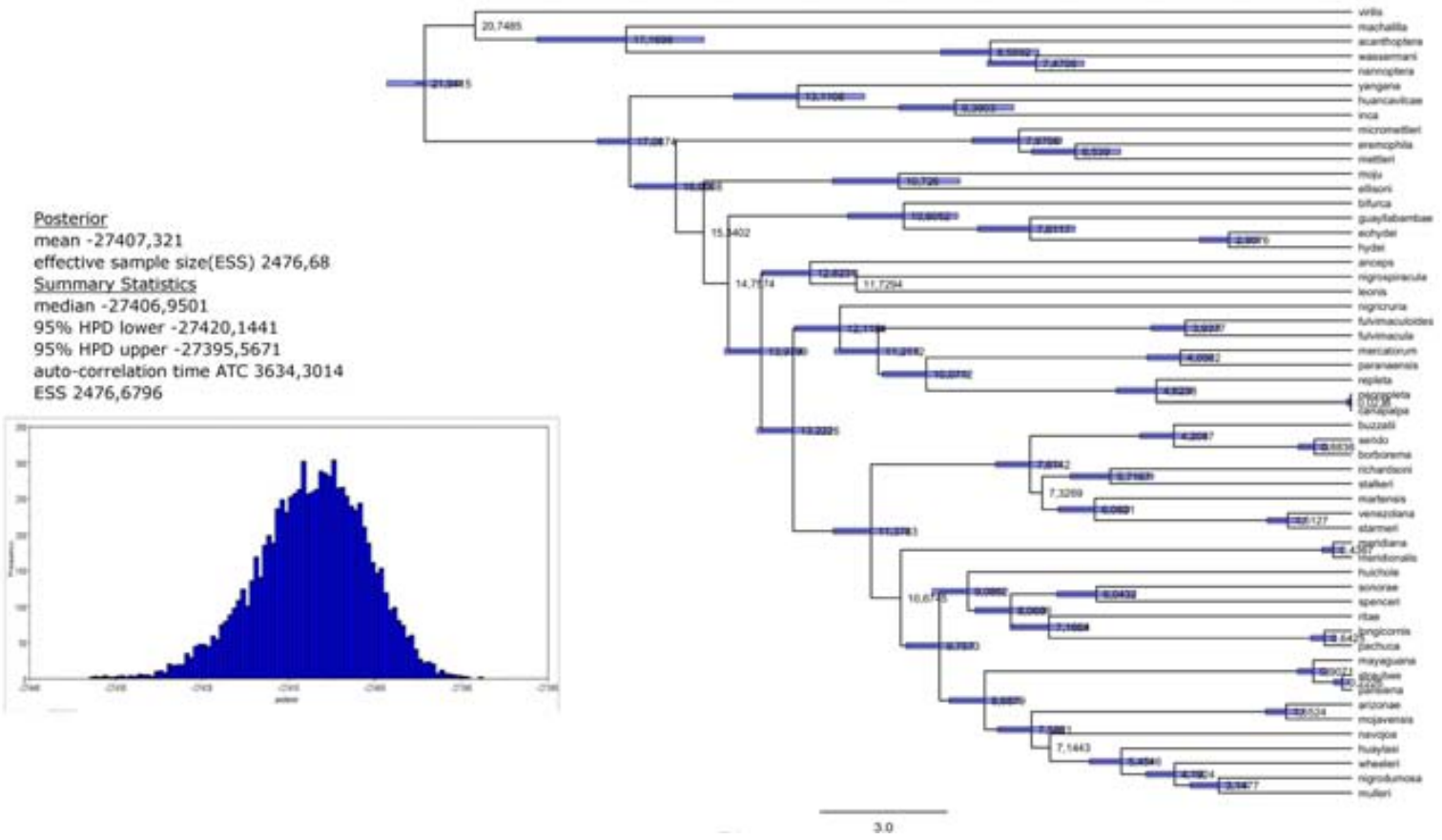
Appendix S1. Complete list of *Drosophila* species used in this study and corresponding GenBank entries. Accession numbers for newly generated sequencing data are highlighted in boldface type.

Drosophila species	ND2	COII	COI	Marf	SinA
acanthoptera	DQ202090	DQ202010	DQ202050	EU3416293	EU341611
anceps	JF736133	JF736093	JF736059	JF736208	JF736324
arizonae	EU341707	JF736122	EU341676	EU341636	EU341620
bifurca	JF736166	JF736130	JF736090	JF736256	JF736378
borborema	JF736157	JF736121	JF736081	JF736241	JF736362
buzzatii	DQ202091	DQ202011	DQ202051	EU341631	EU341621
canapalpa	JF736162	JF736126	JF736086	JF736248	JF736369
ellisoni	DQ202092	DQ202012	DQ202052	JF736235	JF736356
eohydei	JF736159	JF736124	JF736083	JF736245	JF736366
eremophila	DQ202093	DQ202013	DQ202053	JF736249	JF736370
fulvimacula	JF736156	JF736120	JF736080	JF736240	JF736361
fulvimaculoides	JF736134	JF736094	JF736060	JF736209	JF736325
guayllabambae	JF736167	JF736131	JF736091	JF736258	JF736380
huancavilcae	**KC011834**	**KC011824**	**KC011819**	**KC011829**	**KC011839**
huaylasi	**KC011835**	**KC011825**	**KC011820**	**KC011830**	**KC011840**
huichole	DQ202098	DQ202018	DQ202058	JF736257	JF736379
hydei	DQ202100	DQ202020	DQ202060	JF736212	JF736328
inca	**KC011836**	**KC011826**	**KC011821**	**KC011831**	**KC011841**
leonis	JF736136	JF736096	JF736062	JF736214	JF736330
longicornis	DQ202101	DQ202021	DQ202061	JF736232	JF736353
machalilla	**KC011837**	**KC011827**	**KC011822**	**KC011832**	**KC011842**
martensis	JF736160	JF736125	JF736084	JF736247	JF736368
mayaguana	DQ202107	DQ202027	DQ202067	EU341634	EU341623
mercatorum	JF736155	EU493737	EU493607	JF736239	JF736360
meridiana	JF736153	JF736118	JF736078	JF736236	JF736357
meridionalis	DQ202110	DQ202030	DQ202070	JF736250	JF736372
mettleri	JF736137	JF736097	JF736063	JF736215	JF736331
micromettleri	JF736138	JF736098	JF736064	JF736216	JF736332
nannoptera	JF736140	JF736100	JF736066	JF736218	JF736334
navojoa	EU341709	EU493739	EU341678	EU341635	EU341626
neorepleta	DQ202113	DQ202033	DQ202073	JF736219	JF736335
nigricruria	JF736141	JF736101	JF736067	JF736220	JF736336
nigrodumosa	EU341710	JF736102	EU341679	EU341633	EU341627
nigrospiracula	DQ202114	DQ202034	DQ202074	JF736221	JF736337
pachuca	DQ202118	DQ202038	DQ202078	JF736251	JF736373
paranaensis	JF736164	JF736128	JF736088	JF736252	JF736374
parisiena	JF736142	JF736103	JF736068	JF736222	JF736338
pavani	EU493474	JF736115	EU4935832	JF736231	JF736350
repleta	EU341711	JF736105	EU341680	EU341630	EU341628
richardsoni	JF736144	JF736106	JF736070	JF736224	JF736340
ritae	DQ202122	DQ202042	DQ202082	JF736233	JF736354
serido	JF736165	JF736129	JF736089	JF736254	JF736376
sonorae	DQ202124	DQ202044	DQ202084	JF736225	JF736341
spenceri	DQ202127	DQ202047	DQ202087	JF736255	JF736377
stalkeri	DQ202128	DQ202048	DQ202088	JF736226	JF736342
starmeri	JF736145	JF736107	JF736071	JF736227	JF736343
straubae	JF736146	JF736108	JF736072	JF736228	JF736344
venezolana	DQ202129	DQ202049	DQ202089	JF736243	JF736364
virilis	EU493510	EU493751	EU493622	JF736234	JF736355
wassermani	JF736147	JF736109	JF736073	JF736229	JF736345
wheeleri	EU341705	JF736110	EU341685	EU341656	EU341616
yangana	**KC011838**	**KC011828**	**KC011823**	**KC011833**	**KC011843**

Appendix S2. *repleta* group phylogeny: Phylogenetic trees showing topology based on the concatenated dataset of 54 *Drosophila* species analyzed in this study. (A) MP analysis performed on MESQUITE; (B) ML analysis performed on SATé; (C) BS analysis performed on BEAST. Colors denote nodes with: Bootstrap values >0.75 on ML, Shimodaira-Hasegawa values >0.90 on MP and Posterior Probabilities = 1 on BS.



Appendix S3. Chronogram of the *Drosophila* species analyzed in this study, bars denote 95% HPD bars for divergence times. Square in the inferior left corner shows the results of BEAST analysis summarized on TRACER.



Chapter 3.-Long-term evolutionary dynamic of a DNA transposon, the case of Galileo in Drosophilidae

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CHAPTER 3 CONTENTS

ABSTRACT

Host-parasite assemblages offer exciting possibilities for the comparative study of rates of speciation and evolution. The basis for such studies can only be approached from a phylogenetic analysis of host-parasite association. Transposable elements are short DNA sequences that behave as intragenomic parasites. They are vertically transmitted through many generations, although horizontal transfer has been proposed as an essential step in their long-term evolutionary dynamics. Galileo is a member of the P superfamily of DNA transposons. It was initially discovered in *Drosophila buzzatii*, where it is responsible for the generation of three chromosomal inversions, and subsequently reported in closely related species and in six *Drosophila* genomes sequenced.

Here in a thorough search of the Galileo transposon has been carried out in 234 samples of 133 species from the genera *Drosophila*, *Scaptodrosophila*, *Scaptomyza* and *Zaprionus*. The samples come from eight zoo-geographical regions. In order to detect Galileo, *in silico* BLAST searches and experimental searches by PCR + cloning of the most conserved region of the TPase were performed. Galileo was unequivocally detected in 152 samples of 51 *Drosophila* species from the subgenera *Sophophora*, *Drosophila* and *Siphlodora*. Simultaneously, the phylogeny of 174 Drosophilid species (including all taxa in which Galileo was searched) was inferred from partial coding sequences of four genes: SinA, ND2, COI and COII.

The results are consistent with an ancient coevolution of Galileo in the genus *Drosophila*. Galileo has been found in species of the subgenera *Sophophora*, *Drosophila* and *Siphlodora*, that diverged *ca*. 40-57 million years ago. An interesting fact is that Galileo was detected in several populations of the subgenus *Sophophora* from Asia, where it is thought the ancestor of *Sophophora* has its origin. In comparisons of both, the *Drosophila* species and Galileo transposon phylogenies, it was found: 1) discontinuous occurrence of Galileo across 31 species groups (patchy distribution), 2) incongruence between host and TE tree topologies, 3) in the latter case, divergence between Galileo sequences was smaller than between genes of the host species, and 4) a bio-geographical signal in the Galileo phylogeny.

These results found herein suggest that the Galileo transposon was present in the most recent common ancestor of the *Sophophora* subgenus. The invasion of Galileo in the subgenera *Drosophila* and *Siphlodora* could be dated at *ca*. 40-56 Mya, when this clades split. Inside its host, Galileo has been mostly vertically transmitted with stochastic losses and occasional ancient horizontal spreads.

RESUMEN

La asociación entre un parásito y un hospedador ofrece una excelente oportunidad para el estudio de tazas de especiación y evolución. La base para dichos estudios sólo puede ser enfocada mediante el análisis de filogenético de la asociación parásito-hospedador. Los elementos transponibles son secuencias cortas de ADN que se comportan como parásitos intragenómicos. Ellos son transmitidos verticalmente a través de las generaciones, aunque la transferencia horizontal ha sido propuesta como un paso esencial en su dinámica evolutiva a largo plazo. Galileo es un miembro de la Superfamilia P de transposones de ADN. Galileo fue inicialmente descubierto en *Drosophila buzzatii*, en donde es responsable de la generación de tres inversiones cromosómicas y subsecuentemente reportado en especies cercanas y en seis genomas secuenciados de *Drosophila*.

En este estudio se ha ejecutado una búsqueda exhaustiva del transposon Galileo en 234 muestras de 133 especies de los géneros *Drosophila*, *Scaptodrosophila*, *Scaptomyza* and *Zaprionus* con muestras provenientes de ocho regiones zoo-geográficas. Para detectar Galileo se realizaron búsquedas bioinformáticas y experimentales mediante PCR + clonación de la región más conservada de la transposasa. Galileo fue detectado en 152 muestras de 51 especies de Drosophila de los subgéneros *Sophophora*, *Drosophila* y *Siphlodora*. Simultáneamente, la filogenia de 174 especies de Drosophilidae (que incluye todas las especies en las que se realizó la búsqueda de Galileo) se construyó con secuencias parciales de cuatro genes: SinA, ND2, COI y COII.

Los resultados son consistentes con una antigua coevolución de Galileo en el género *Drosophila*. Galileo ha sido encontrado en especies de los subgéneros *Sophophora*, *Drosophila* and *Siphlodora*, que divergieron hace *ca*. 40-57 millones de años. Un hecho interesante es que Galileo fue detectado en varias poblaciones del subgénero *Sophophora* de Asia, en donde se piensa ha tenido su origen el ancestro de dicho subgénero. En comparaciones de ambas filogenias, de las especies y Galileo se han encontrado: 1) ocurrencia discontinua (distribución parcheada) entre 31 grupos de especies, 2) incongruencias entre las topologías de los árboles filogenéticos de las especies hospedadoras y Galileo, 3) en el último caso, la divergencia las secuencias de Galileo fue más pequeña entre los genes de las especies hospedadoras, y 4) una señal biogeográfica en la filogenia de Galileo.

Los resultados encontrados en este estudio sugieren que el transposon Galileo estuvo presente en el ancestro común más reciente del subgénero *Sophophora*. La invasión de Galileo en el subgénero *Drosophila* y *Siphlodora* puede ser datada en *ca*. 40-56 Ma, cuando estos clados se separaron. Dentro de su hospedador, Galileo ha sido mayoritariamente transmitido verticalmente con pérdidas estocásticas y propagaciones horizontales antiguas.

INTRODUCTION

The term "ecology of the genome" was initially proposed by Kidwell & Lisch (2001) to illustrate, from an evolutionary perspective, the complexity of interactions occurring between TEs and their hosts. The concept was originally hypothesized using interactions of two types of TEs that co-exist occupying different "niches" within the same genome. The analogy of a genome as an ecological community, further developed by several authors (e.g. Venner et al. 2009; Brookfield 2005; Le Rouzic et al. 2007), has provided the conceptual framework to understand the evolutionary dynamic of TEs.

Theoretical approaches using the genomic ecology concept (Leonardo & Nuzhdin 2002; Le Rouzic & Capy 2006; Abrusán & Krambeck 2006), has shown that interactions between TEs can be of parasitic, competitive or cooperative nature. Studies of interactions at the level of transposons and their hosts have applied considerably less this analogy. However it has also been demonstrated that evolutionary forces acting at the level of the host species can influence TE distribution and maintenance (Rouzic & Deceliere 2005; Lynch & Conery 2003).

Galileo is a DNA transposon, initially described in *D. buzzatii,* where it is the causative agent of chromosomal inversions (Cáceres et al. 1999; Casals et al. 2003; Delprat et al. 2009). Previous screenings of this transposable element determined its presence in some closely related species of the *repleta* group (Casals et al. 2005) and six of the 12 *Drosophila* genomes sequenced (Marzo et al. 2008; Gonçalves et al. 2014; Casals et al. 2005). From *in silico* searches it is known that several subfamilies of Galileo can co-exist inside the same *Drosophila* host genome (Marzo et al. 2008; Marzo et al. 2013a; Gonçalves et al. 2014). The most conspicuous features of this

element are its TIRs that have variable length/structure and its transposase (TPase) that is similar to those of P and 1360 elements (Marzo et al. 2008). All Galileo copies described so far carry premature codon stops or/or frameshift mutations and thus do not encode a full length TPase.

In this study a thorough screening of the DNA transposon Galileo has been performed in 234 samples from 133 species of Drosophilidae, using the most conserved region of Galileo TPase. A phylogeny of the element was built with the Galileo sequences generated. Simultaneously, the evolutionary relationships of 174 species of Drosophilidae were inferred using four molecular markers (COI, COII, ND2, SinA). Both phylogenies have been compared. The results of this study give insights about the long-term evolutionary dynamics of a DNA transposon.

MATERIAL AND METHODS

Drosophilidae phylogeny

Taxon sampling

In order to build the species phylogeny, taxa were selected based on two main criteria, species where Galileo was screened (133 species) and sister taxa for those species (41 species). In total 174 taxa from *Drosophila*, *Scaptodrosophila*, *Zaprionus* and Hawaiian *Drosophila* were analyzed.

Sequence data from partial genomic regions of the mitochondrial cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II (COII), NADH ubiquinone oxidoreductase chan 2 (ND2) and the nuclear seven in absentia (SinA) genes were retrieved from the sources detailed on Table S1 (Supplementary Material). The generated data set contained the homologous genomic regions in 174 species. Multi-sequence alignment was performed using MAFFT software (Katoh et al. 2009).

Phylogenetic inference

Two methods of phylogenetic inference were employed to retrieve phylogenetic trees: Neighbor-Joining (NJ) and Bayesian Inference (BI).

NJ phylogenetic tree was inferred using CIPRES (Miller et al. 2010). The best fit model of nucleotide substitution was selected according to the Akaike information criterion (AIC). Statistical support for the tree inferred was evaluated using the Bootstrap test (Felsenstein 2004) with 500 replicates.

BI phylogenetic tree was obtained using BEAST 1.7.5 (Drummond et al. 2012b). Markov Chain Monte Carlo (MCMC) sampling was conducted in the dataset.

Best fit model to the dataset was selected according AIC. One cold chain and tree heated chains were run simultaneously for one million generations, and one tree per 100 generations was sampled. The first 100 trees were discarded as burn-in, and Bayesian posterior probabilities were estimated on the 70% majority rule consensus of the remaining 9900 trees.

Galileo phylogeny

Taxon sampling

The species used to test for Galileo presence and to buid the Galileo phylogeny were chosen to maximize phylogenetic representation across Drosophilidae. Therefore, in addition to laboratory strains, specimen collections were carried out using the methods detailed in Acurio et al. (2010). Taxonomic identification was made as is described in Acurio et al. (2013). Isofemale strains were established for each taxon and preserved in pure ethanol to be stored at -20ºC.

DNA extraction

The source of each sample used to test for Galileo presence and to build the Galileo phylogeny is shown in Table S2 (Supplementary Material). A total of 234 samples from 110 species were analyzed. Template DNA was extracted using 3 flies per isofemale strain using a modified Cetyl trimethyl Ammonium Bromide (CTAB) protocol (Appendix 1). Quality of DNA samples were later checked using a 0.7% agarose gels using Agarose D1 EEO (Conda Laboratory). Template DNA was labeled using an ID code and distributed on DNA plates.

Detection and amplification

Six pairs of primers were designed in the most conserved region of the gene encoding the TPase of Galileo (Figure 1). Primer 3 Plus web interfase was used for primer design; details of each primer pair used in this study are shown in Table 1.

Template DNA was transferred to a multiwell PCR plate: a master plate of DNA on which 92 samples and 4 negative controls were arrayed in a 12 x 8 format, columns on plate were labeled with numbers 1-12 and rows with letters A-H. Thus, each sample had a coordinate in the plate for the subsequent confirmation by PCR using the primers designed to amplify Galileo TPase. PCR master mix was prepared by combining 2442 µl water, 330 µl 10x Taq buffer (Roche), 66 µl 20mM dNTP's, 110 µl primer forward "F", 110 µl primer reverse “R” and 22.5 µl Taq DNA Polymerase (Roche) in a tray on ice. Initial and final concentrations used on PCR mix are shown in Table 2.

PCR master mix was distributed into the wells using a multi-channel pipette (Eppendorf). Two microliters of DNA template were added to the PCR plate using a 12-channel pipette (Eppendorf). The 96-well plate was centrifuged during 5 seconds on a Centrifuge 5810R (Eppendorf). PCR cycling conditions were settled in a Peltier Thermal Cycler PTC-100 (Bio-Rad) as follows: initial denaturation 3 min at 94°C; 35 cycles of : 45 seconds at 94°C, 30 seconds at 50°C and a final elongation of 30 seconds at 72°C. Five microliters of 10x loading buffer (30% glycerol, 50mM EDTA, 0.25% bromo-phenol blue) were added to the 10 µl PCR reactions.

Fifteen microliters of the mix obtained in the previous step was charged on a 0.7% agarose gel. The agarose gel was electrophoresed at 70V for 1hr and then transferred into a plastic dish. 500 ml of Ethidium Bromide staining solution (500 ml water, 43 µl EtBr) was added to cover the gel. Finally, the gel was kept in the dark for

30-40 minutes. The banding pattern of DNA was recorded through the gel by photography using a camera and a transilluminators with 300-nm UV light. Software from AlphaDigidoc (Alpha Innotech. Corp, USA) was used in this step.

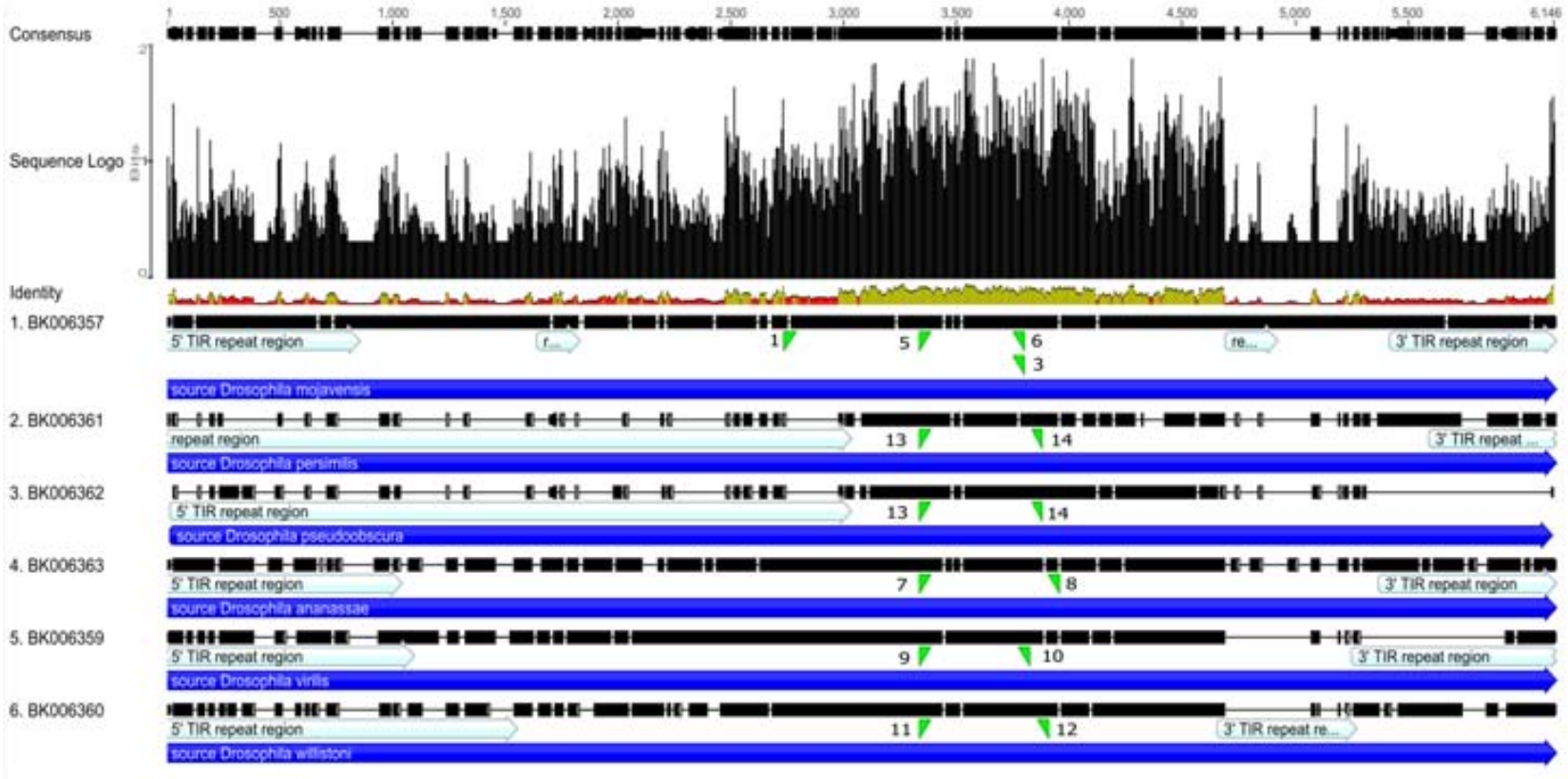

Figure 1. Sequence alignment of the most complete copies of Galileo reported on six *Drosophila* genomes using MAFFT software. On top consensus sequence, genbank accession numbers on the left side. Graph height (in pixels) denote most conserved region on alignment. Numbered green triangles show primers designed in this study; light blue arrows denote TIRs on Galileo TE. Species source is shown on blue arrows.

Table 1. Primer pairs designed to search for Galileo transposon in the Drosophila genus.

Sequence	Genebank ID	Primers pair	Amplicon length	Sense	Sequence	Primer length
Dbuz/GALILEO	EU334685	1-3	~ 1 kb	forward	TTATAATAGTGCTGAAAGGGT	21 bp
				reverse	GAAAATARTCTCTCATTTCCT	21 bp
Dmoj/GALILEO	BK006357	5-6	~ 0.5 kb	forward	TGCACCGCATCTWGTWAAATCC	22 bp
				reverse	AAATAATCACGCATTTCCWGAAG	23 bp
Dana/GALILEO	*BK006363*	7-8	~ 0.6 kb	forward	ATGCCCCACATCTCATAAAATY	22 bp
				reverse	AGGTTTTCTAAGGGATCTTGATTY	24 pb
Dvir/GALILEO	BK006359	9-10	~ 0.5 kb	forward	GACTTAATCAAATGAGGAACATCR	24 bp
				reverse	GTTTTGGGATAACGACATTTCAY	23 bp
Dwil/GALILEO	BK006360	11-12	~ 0.6 kb	forward	ATGTCCCCCACCTCATAAAATY	22 bp
				reverse	ACCTTCTCCTTGACTCCAAATATY	24 bp
Dpse/GALILEO	BK006362	13-14	~ 0.5 kb	forward	GCGATTTAATCAAATGTGGAACR	23 bp
				reverse	GGCCAATGAAAGTATGGAGTTR	22 p

Table 2. PCR mix concentrations used for 96-well plates and single reactions performed in this study.

	volume	initial concentration	final concentration
DNA	2 µl	---	----
Water	22,2 µl	---	---
Taq buffer	3 µl	10 x	10X
dNTP's	0,6 µl	20mM	0.2 mM
primer "F"	1 µl	10 mM	1µM
Primer "R"	1 µl	10 mM	1 µM
Taq Polymerase	0.2 µl	2.5 U/µl	1.25 U
Total	30 µl		

Galileo cloning

Positive detection of Galileo was determined based on the signal intensity and length of the fragment amplified. Samples with positive detection were later cleaned using the PCR Clean-up kit (NucleoSpin), and then cloned using the PCR Cloning kit from Stratagene (Agilent Technologies, CA, USA) (Appendix 1). Under sterile conditions, four clones of Galileo where selected from each cloning plate using plastic tips, each clone was later isolated in a Eppendorf tube containing 50 µl of sterile water and kept for 10 minutes. After that, tubes were placed at 100°C on a heater block SB-200D (Stuart) for five minutes. Finally, the samples where centrifuged for 10 seconds.

To recover the Galileo insert cloned, a second PCR was carried out using the universal primers T3 and T7, same concentrations and volumes per reactions than in the

96-well plate PCR were used. The PCR cycling conditions in the Personal Thermal Cycler MJ-Mini (Bio-Rad Laboratories, CA, USA) were settled as follows: initial denaturation 2 minutes at 94°C; 35 cycles of: 30 seconds at 94°C, 45 seconds at 49°C and a final elongation of 45 seconds at 72°C. Same procedure than Agarose gel electrophoresis for 96-well plate PCR was then performed. From gel results one clone was selected according to their intensity of signal and length. The Galileo clone selected was then cleaned to eliminate primer dimers using the PCR Clean-up kit (Machery Nagel, Düren, Germany) and sent for sequencing to Macrogen, Korea. Chromatograms were compiled using the Geneious software (Drummond et al. 2011). Identity of the sequences obtained was corroborated through BLASTN searches against NCBI database using E-value $\leq 10^{-20}$ as significance threshold.

Galileo in silico searches

Nucleotide BLAST searches were performed against ten newly released genomes of *Drosophila* available on NCBI and Flybase (Table 3). The significance threshold used for searches was E-value $\leq 10^{-3}$.

Multi-sequence analysis

Sequences obtained through PCR and retrieved by *in silico* searches were aligned using MAFFT software (Katoh et al. 2009). Patterns of nucleotide substitution, transition/transversion rate ratios k^1 (purines), k^2 (pyrimidines) and overall transition/transversion bias were calculated with MEGA 4 (Tamura et al. 2007). The complete data set was scanned in the search of recombinant events using the software RDP4 (Martin et al. 2010).

Table 3. Taxa and queries used in the BLAST searches of Galileo TE.

Species (Genbank ID)	Species group	Sequence used as query
1. D. bipectinata (42026)	*ananassae**	*Dana\Galileo*
2. D. kikkawai (30023)	*montium**	*Dana\Galileo*
3. D. rhopaloa (1041025)	*melanogaster*	*Dana\Galileo*
4. D. elegans (30023)	*melanogaster*	*Dana\Galileo*
5. D. biarmipes (125945)	*melanogaster*	*Dana\Galileo*
6. D. takahashii (29030)	*melanogaster*	*Dana\Galileo*
7. D. ficusphila (30025)	*melanogaster*	*Dana\Galileo*
8. D. eugracilis(29029)	*melanogaster*	*Dana\Galileo*
9. D. americana (40366) ■	*virilis*	*Dvir\Galileo*
10. D. miranda (7229)	*obscura*	*Dper\Galileo*

* Taxonomic classification based on phylogenetic analysis from Da Lage et al. (2007); Russo et al.(2013) and Yassin (2013).

■ The *Drosophila americana* genome is not available on NCBI database. Thus for the Galileo screening, the BLAST tool implemented in the *D. americana* genome webpage (Schlötterer et al. 2013) was used.

Phylogenetic inference

The NJ phylogenetic tree was inferred using CIPRES (Miller et al. 2010). Model of nucleotide evolution was selected according to AIC using JModelTest 2.1.5 (Posada 2008; Darriba et al. 2012). Statistical support for tree inferred was evaluated using the Bootstrap test (Felsenstein 2004) with 500 replicates.

The maximum likelihood (ML) phylogenetic tree was performed on PhyML 3.0 (Guindon et al. 2010). The Bio NJ algorithm was used to compute a full initial tree. The model of nucleotide substitution was selected according to the AIC. Both, transition/transversion ratio and proportion of invariable sites were estimated with PhyML. Tree topology and branch length were optimized using the NNI algorithm (Guindon et al. 2010). Node support on the inferred tree was calculated using the approximate likelihood ratio Shimodaira-Hasegawa test (a-LTR SH) (Anisimova & Gascuel 2006).

BI phylogenetic tree was inferred using BEAST 1.7.5 (Drummond et al. 2012b). The graphical user interface (BEAUti) was used to generate the XML input file. Coalescence was assumed as a prior in the phylogenetic reconstruction. The length of the MCMC chain was determinate using TRACER and Effective sample sizes (ESS) of each parameter generated by BEAUti were analyzed using TRACER. A target tree was selected using TreeAnotator with a burn in of 1000 trees on each run, a posterior probability limit of 0.80 and using the Maximum Clade credibility option. Target tree was visualized using Fig Tree v1.4.0.

Ancestral reconstructions of Galileo in the Drosophilidae

To examine the presence of Galileo in the Drosophilidae, Maximum Likelihood Ancestral Reconstruction (MLAR) and Parsimony Ancestral Reconstruction (PAR) both implemented on MESQUITE (Maddison & Maddison 2010) were undertaken using the BI phylogenetic tree previously recovered with four molecular markers and 174 species of Drosophilidae. Presence/ absence data obtained from PCR and *in silico* screening of Galileo was treated as a qualitative trait. The model of evolution assumed was Markov k-state 1 parameter model (Mk1), where the single parameter is the rate of change (Lewis 2001).

The world-wide distribution of Galileo was examined using the Ancestral Range Reconstructio (ARR) analysis also performed in MESQUITE. Data of *Drosophila* samples where Galileo was detected, shown on Table S3 (Supplementary Material), has been treated as categorical characters. The locality of each *Drosophila* sample was assigned to a zoo-geographical realm as reported by Holt et al. (2013). Thus, each sample was ascribed to one of the following regions: Nearctic, Neotropic, Paleartic, Oriental, Australia, Afrotropic and Madagascar.

Cophylogenetic analysis of Galileo in its host *Drosophila* species

In order to determine the historical association of Galileo with its host *Drosophila* species, the BI tree recovered from a highly conserved region of Galileo TPase and the BI tree of *Drosophila* inferred from four molecular markers were compared. The

MESQUITE software was used to prune the branches of the host phylogeny were Galileo was not detected and to prune the outgroup clade of Galileo (1360 element).

Cophylogenetic analysis were performed on TreeMap version 3 (Charleston & Robertson 2002). Congruence between trees was evaluated through Z statistic value calculated for each node in the Galileo tree to find the corresponding subtree from the *Drosophila* phylogeny. Because in most of the cases several copies of Galileo are harbored in one genome of *Drosophila* (this is not a branch to branch association), the "cherry-picking" test (CPT) (Jackson & Charleston 2004) was used to evaluate changes in phylogenetic significance.

Horizontal Transfer test

In order to test putative HT events, the average number of synonymous nucleotide differences per synonymous site (d_S) was calculated using MEGA 4 (Tamura et al. 2007) on each dataset analyzed (host species and Galileo). The transition/transversion bias assumed in the modified Nei-Gojobori method was estimated using the same software, gaps and missing data were eliminated by pairwise deletion option. Standard errors on d_S values were obtained by bootstrap procedure with 500 replicates.

RESULTS

Drosophilidae phylogeny

The dataset built with the sequences of partial coding-regions of COI, COII, ND2 and SinA genes from 174 drosophilid species comprised 1901 aligned positions, of them, 929 are conserved positions, 972 are single variable positions, and 844 are parsimony-informative positions (Table 4). No recombination events were detected using the software RDP4. Across 174 taxa, differences in nucleotide composition between mitochondrial and nuclear loci were found. The first are A+T rich with an overall average ranging from 72.9% to 78.7%. Third codon position has AT content of 90% on COI, 91.7% on COII and 91.5% on ND2. The SinA locus has most equally nucleotide composition with GC content of 57.1%.

Table 4. Number of sites that are invariable, polymorphic, parsimony informative (parsimony inf.) and singletons in the data set.

	Locus (lengths on base-pairs)				Concatenated Dataset
Sites	COI	COII	ND2	SinA	COI-COII-ND2-SinA
Length (bp)	367	658	479	397	1901
Invariable	166	326	183	254	929
Polymorphic	201	332	296	143	972
Parsimony-inf.	165	275	271	133	844
Singletons	36	57	25	10	128

The best fit model of nucleotide evolution estimated for mitochondrial and nuclear loci according to the AIC is GTR (Table 5).

Table 5. Best model selected according the AIC for each data set analyzed. Abbreviation are as follows: -lnL: negative log likelihood, K: number of estimated parameters, p-inv= proportion of invariable sites, α=Gamma distribution shape parameter.

Mitochondrial loci			Nuclear Loci
ND2	COI	COII	SinA
-lnL = 22879.6465	-lnL = 10952.4581	-lnL = 20588.1025	-lnL = 11175.0896
K = 324	K =330	K =356	K = 319
Model-averaged estimates			
GTR+I+G			GTR+G
freqA = 0.3831	freqA = 0.3782	freqA = 0.2691	freqA = 0.2137
freqC = 0.1183	freqC = 0.0832	freqC = 0.1387	freqC = 0.3064
freqG = 0.0836	freqG = 0.0301	freqG = 0.0348	freqG = 0.2682
freqT = 0.4150	freqT = 0.5085	freqT = 0.5574	freqT = 0.2117
R(a) [AC] = 0.8001	R(a) [AC] = 0.1972	R(a) [AC] = 1.0351	R(a) [AC] = 2.4706
R(b) [AG] = 8.0507	R(b) [AG] = 7.9241	R(b) [AG] = 22.6578	R(b) [AG] = 6.0678
R(c) [AT] = 2.0521	R(c) [AT] = 0.3575	R(c) [AT] = 2.5042	R(c) [AT] = 1.3367
R(d) [CG] = 2.3124	R(d) [CG] = 0.8248	R(d) [CG] = 2.4391	R(d) [CG] = 1.7499
R(e) [CT] = 9.2937	R(e) [CT] = 6.2099	R(e) [CT] = 14.2411	R(e) [CT] = 7.3433
R(f) [GT] = 1.0000	R(f) [GT] = 1.0000	R(f) [GT] = 1.0000	R(f) [GT] = 1.0000
p-inv = 0.08	p-inv = 0.2960	p-inv = 0.4350	p-inv=0.0000
α= 0.6670	α = 0.2770	α = 0.5530	α = 0.6850

The phylogenetic analysis of the combined dataset from three mitochondrial (COI, COII, ND2) and one nuclear (SinA) genes using Neighbor-Joining (NJ) (Figure 2) and Bayesian Inference (BI) (Figure 3) approaches resulted in highly congruent tree topologies. In the phylogenetic analysis six main clades were retrieved:

- Clade I encompasses *Scaptomyza* genus (BI: 1, NJ: 100).
- Clade II encompasses five species groups: *ananassae*, *melanogaster*, *montium*, *willistoni* and *saltans* (BI: 1, NJ: 100).
- Clade III encompasses eleven species groups: *immigrans*, *guttifera*, *quinaria*, *putrida*, *funebris*, *macroptera*, *cardini*, *calloptera*, *tripunctata*, *guarani* and *polychaeta* (BI: 1, NJ: 54).
- Clade IV encompasses the genus Scaptomyza and five species groups: *antopocerus*, *modified tarsus*, *ciliated tarsus*, *halekalae* and *modified mouthpart* (BI: 1, NJ: 56).
- Clade V encompasses the genus *Zaprionus* and *polychaeta* species group (BI: 1, NJ: 20).
- Clade VI encompasses eight species groups: *virilis*, *robusta*, *melanica*, *annulimana*, *atalaia*, *nannoptera*, *canalinea* and *repleta* (BI: 0.97, NJ: 39).

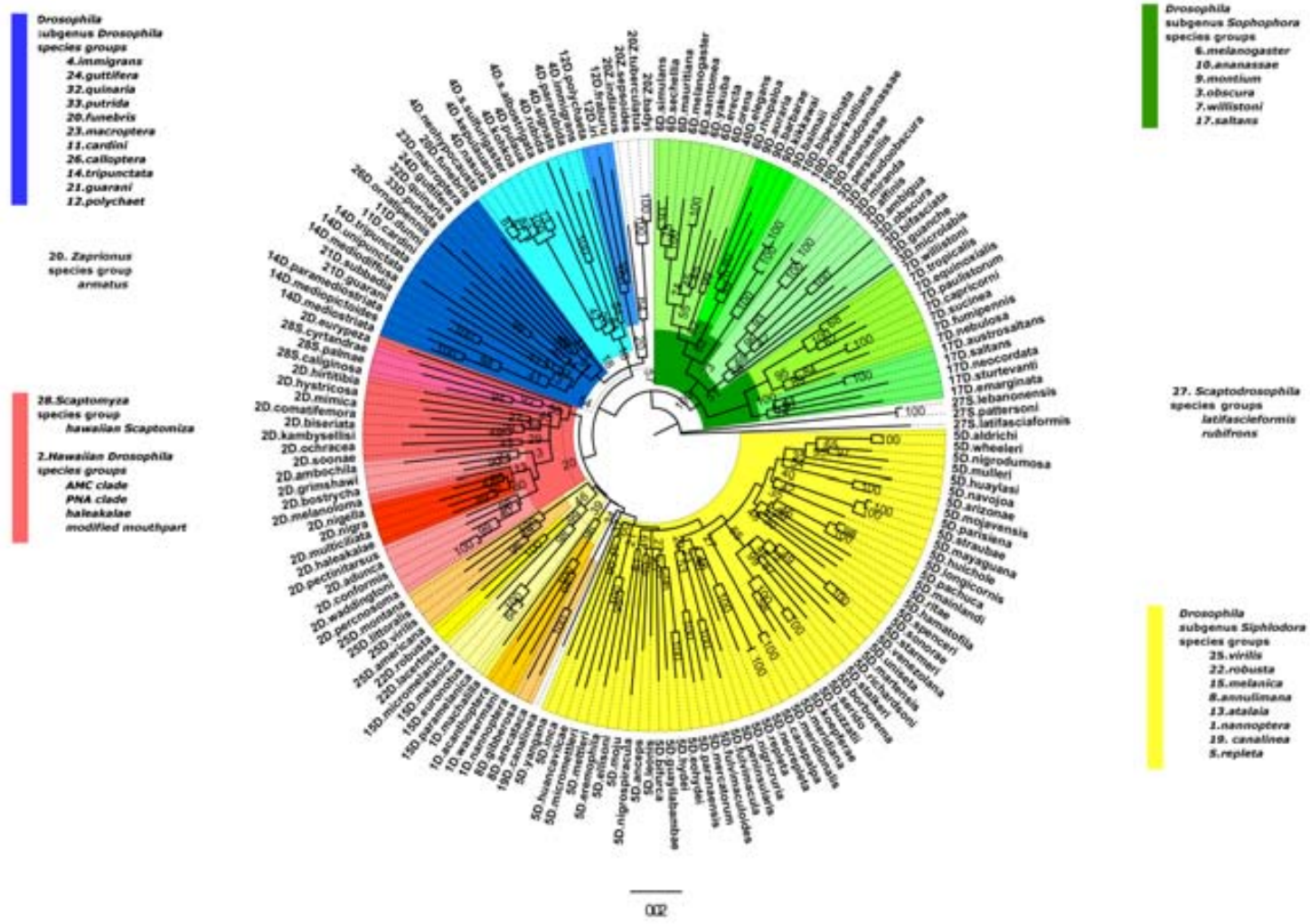

Figure 2. Neighbor-Joining phylogenetic tree based on the combined analysis of three mitochondrial (COI, COII, ND2) and one nuclear (SinA) genes (1901 bp) of 174 taxa from *Drosophila*, *Hawaiian Drosophila*, *Scaptodrosophila*, *Scaptomyza* and *Zaprionus*. Numbers at nodes indicates bootstrap value. The scale bar represents substitutions per site. Color tones denote different species groups within each subgenus.

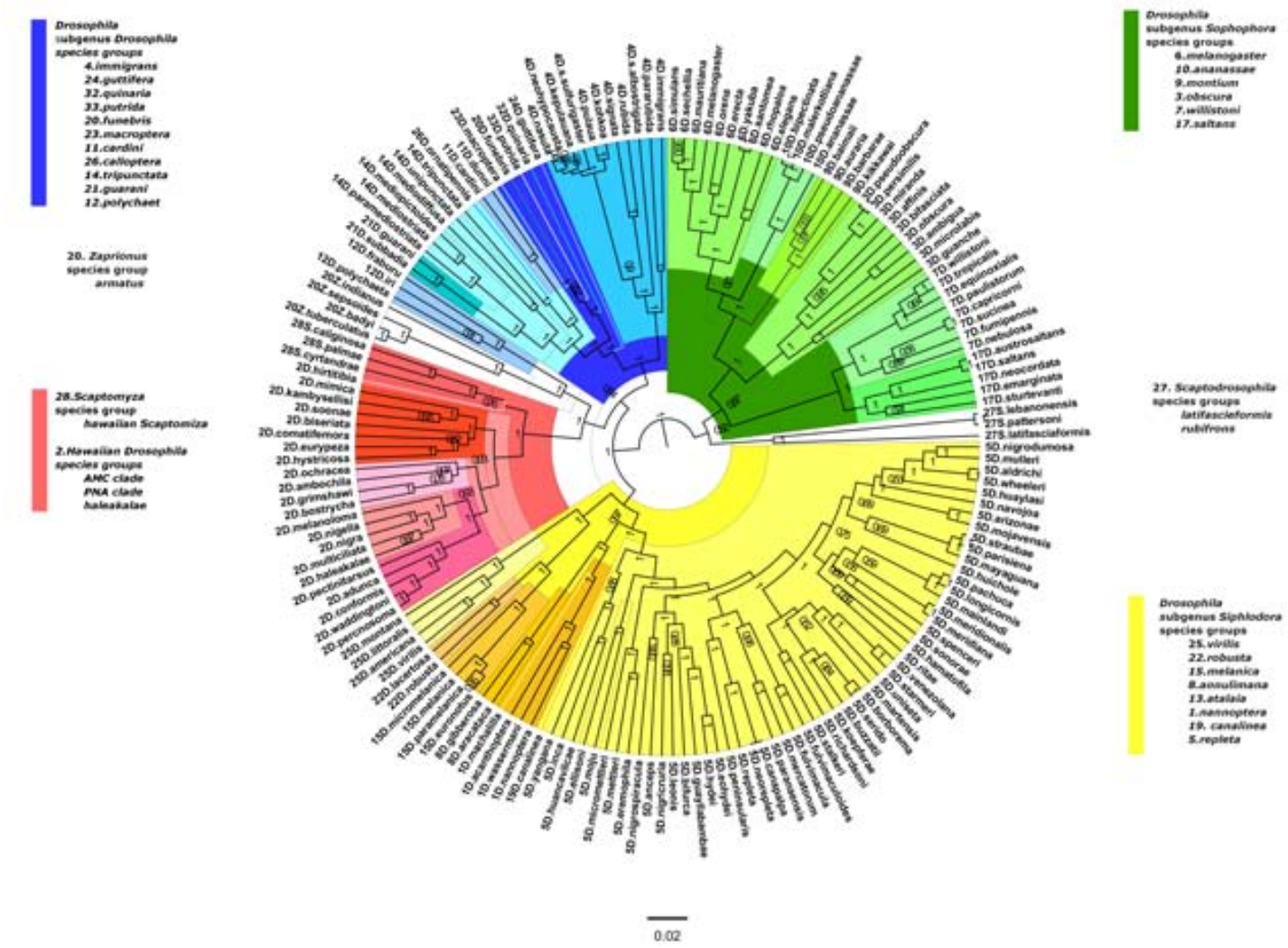

Figure 3. Bayesian Inference phylogenetic tree based on the combined analysis of three mitochondrial (COI, COII, ND2) and one nuclear genes (1901 bp) of 174 taxa from *Drosophila*, Hawaiian Drosophila, *Scaptodrosophila*, *Scaptomyza* and *Zaprionus*. Best tree using mitochondrial and nuclear partitions with the GTR+I+G model of nucleotide substitutions. Statistical support is shown on nodes (Posterior Probabilities). Clades labeled with colors according to their genera taxonomic classification. Branch lengths in the tree are in substitutions per site. Color tones denote different species groups within each subgenus.

Galileo transposon

Detection

The results of the Galileo search in 234 samples from 110 drosophilid species are given in Table S3 (Supplementary Material). Galileo was unequivocally detected on 51 taxa from ten species groups: *ananassae*, *montium*, *melanogaster*, *willistoni*, *tripunctata*, *guarani*, *saltans*, *obscura*, *virilis* and *repleta*. Significant hits retrieved in the BLASTN searches carried out in the recently sequenced *Drosophila* genomes are show in Table S4 (Supplementary Material). Through *in silico* screening, Galileo was detected in six species: *D. bipectinata*, *D. kikkawai*, *D. elegans*, *D. rhopaloa*, *D. miranda* and *D. americana*. The homologous region of Galileo TPase retrieved from these species was included in the data set to infer the phylogenetic tree. In the case of *D. elegans*, non-autonomous copies of Galileo were only found. Thus this species was labeled as positive in the detection test and mapped in the host phylogeny but not used to build the Galileo phylogeny.

The data set analyzed include 152 sequences, of them 125 were obtained by PCR and cloning, 14 sequences have been obtained through *in silico* searches and 13 are GenBank sequences reported by Marzo et al. (2008). The transposable element 1360 from the P superfamily, was selected as outgroup. Galileo dataset comprised 426 aligned positions, between them 316 are parsimony-informative. In the dataset 33 sites are conserved, 364 sites are variable and 52 sites are singletons. No recombination events were detected using RDP4 software.

From a dataset of 426 sites, 62 sites (14.55%) are without variation. According to the AIC, the best fit model to Galileo data set is GTR+I+G (-lnL = 10076.9552). Table 6 shows the parameter estimates that characterize the molecular evolution of

Galileo. The proportion of invariable sites, p-inv, is 0.1595, the most frequent nucleotide is A (0.3826), the most common substitution is between C and T (3.6484), around 6% of the sites have not changed (p-inv=0.056), and moderate rate variation among residues (α=1.4644).

Table 6. Parameter importance and model averaged estimates for 152 sequences of Galileo. Abbreviations are as follows: f = frequencies, r = ratio, I = proportion of invariable sites, G = shape parameter of the gamma distribution. Values are averaged for: I (considers only +I models), G (considers only +G models), IG (considers only +I+G models).

Parameter	Importance	Model averaged estimates
fA	1.0000	0.3826
fC	1.0000	0.1541
fG	1.0000	0.1723
fT	1.0000	0.2910
kappa	0.1378	3.0809
Titv	0.1378	1.3743
rAC	0.8622	0.9384
rAG	0.8622	2.7237
rAT	0.8622	0.9690
rCG	0.8622	1.2416
rCT	0.8622	3.6486
rGT	0.8622	1.0000
pinv(I)	0.0000	0.0560
alpha(G)	0.0133	1.4804
pinv(IG)	0.9867	--
alpha(IG)	0.9867	1.4644

The segment of Galileo TPase analyzed is A+T rich with an overall average of 65.6 %. First codon position has an AT content of 68.6 %, second codon position 65.5% and third codon position 62.7 %. Pairwise identity between Galileo copies in the same species is ≥ 93% while copies from same species groups are ≥ 83% identical.

The estimated instantaneous substitution rate matrix (Q matrix) from the general time-reversible model (GTR) is shown in Table 7. This matrix provides the description of the substitution process assumed to build the ML phylogeny of Galileo, The optimal tree inferred is depicted on Figure 5 (logL -10164.0777).

Table 7. PhyML estimated instantaneous rate matrix Q for GTR model. Each entry in the matrix represents the instantaneous substitution rate from nucleotide to nucleotide (rows and columns follow the order A, C, G, T).

$$Q = \begin{array}{c} \begin{matrix} \mathbf{A} & \mathbf{C} & \mathbf{G} & \mathbf{T} \end{matrix} \\ \begin{pmatrix} -0.80644 & 0.13230 & 0.41701 & 0.25713 \\ 0.30913 & -134.035 & 0.17541 & 0.85580 \\ 0.81619 & 0.14693 & -118.851 & 0.22540 \\ 0.32674 & 0.46541 & 0.14634 & -0.93849 \end{pmatrix} \end{array}$$

In the Bayesian Inference analysis, the two models tested (GTR and Yang 96) were congruent regarding tree topology and ESS values for posterior probabilities (Table 8). Using Bayes factor criteria (1000 bootstrap replicates), Yang 96 model (ln-10321.0220) better fits the data than GTR model (ln-10591.672).

Table 8. Posterior Statistics of GTR and Yang 96 models tested in the Bayesian phylogenetic reconstruction.

Posterior Statistics	GTR model	Yang 96 model
Mean	-10705.0033	-10485.4557
Standard error of mean	0.3059	0.3454
Median	-10704.5811	-10485.1124
95% HPD lower	-10726.2221	-10511.4379
95% HPD upper	-10683.3468	-10461.6693
Auto-correlation time (ACT)	6992.7199	6534.5783
Effective sample size (ESS)	1287.1958	1377.4416

Galileo phylogeny

The phylogenetic analysis of 152 sequences of Galileo TPase from 51 *Drosophila* species using Neighbor Joining (NJ) (Figure 4), Maximum Likelihood (Figure 5) (ML) and Bayesian Inference (BI) (Figure 6) approaches resulted in highly congruent tree topologies (Figure 7). In the phylogenetic analysis five main clades were retrieved:

Clade I (NJ: 97, ML: 1, BI: 1) encompasses element 1360 sequences (outgroup).

Clade II (NJ: 96, ML: 1, BI: 1) encompasses Galileo copies from species that belong to three different subgenera: from *Sophophora* subgenus, the *willistoni* and *saltans* species groups; from *Drosophila* subgenus, the *tripunctata* and *guarani* species groups and from *Siphlodora* subgenus, the *virilis* species group.

Clade III (NJ: 97, ML: 1, BI: 1) encompasses Galileo copies from the *ananassae*, *montium* and *melanogaster* species groups. The three species groups belong to the *Sophophora* subgenus.

Clade IV (NJ: 100, ML: 1, BI: 1) encompasses Galileo sequences from *obscura* species group that belongs to the *Sophophora* subgenus.

Clade V (NJ: 100, ML: 1, BI: 1) encompasses sequences from *repleta* species group in *Siphlodora* subgenus.

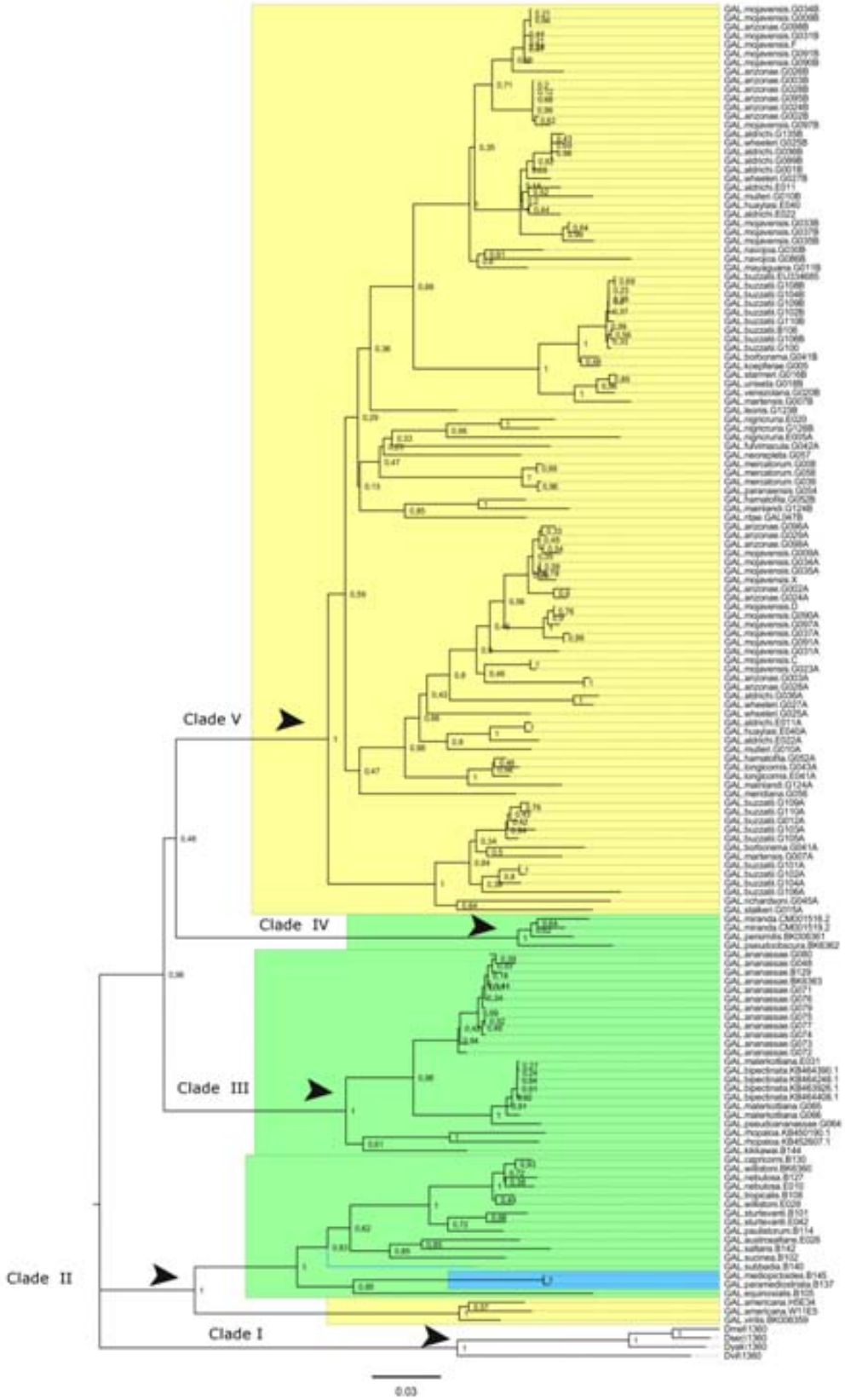

Figure 4. Neighbor-Joining phylogenetic tree inferred for 152 sequences of Galileo and 1360 element TPases in 51 Drosophila species. Bootstrap values are shown on nodes. Colors denote subgenera of the host as follows: yellow=*Siphlodora*, green=*Sophophora*, blue=Drosophila.

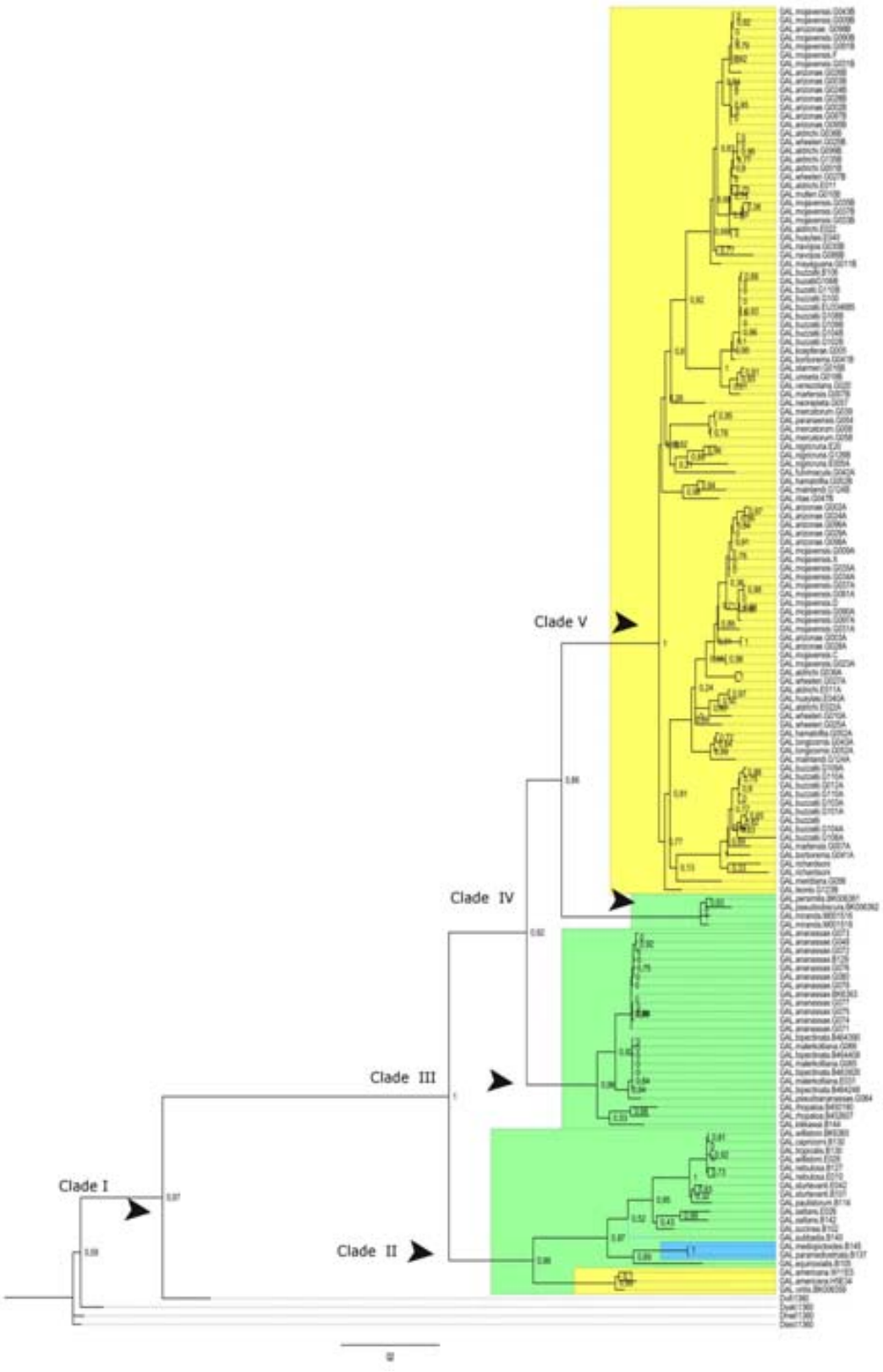

Figure 5. Maximum Likelihood phylogenetic tree inferred for 152 sequences from Galileo and 1360 element TPases in 51 *Drosophila* species. The aLTR-SH statistical support is shown on each node. Colors denote subgenera of the host as follows: yellow=*Siphlodora*, green=*Sophophora*, blue=*Drosophila*.

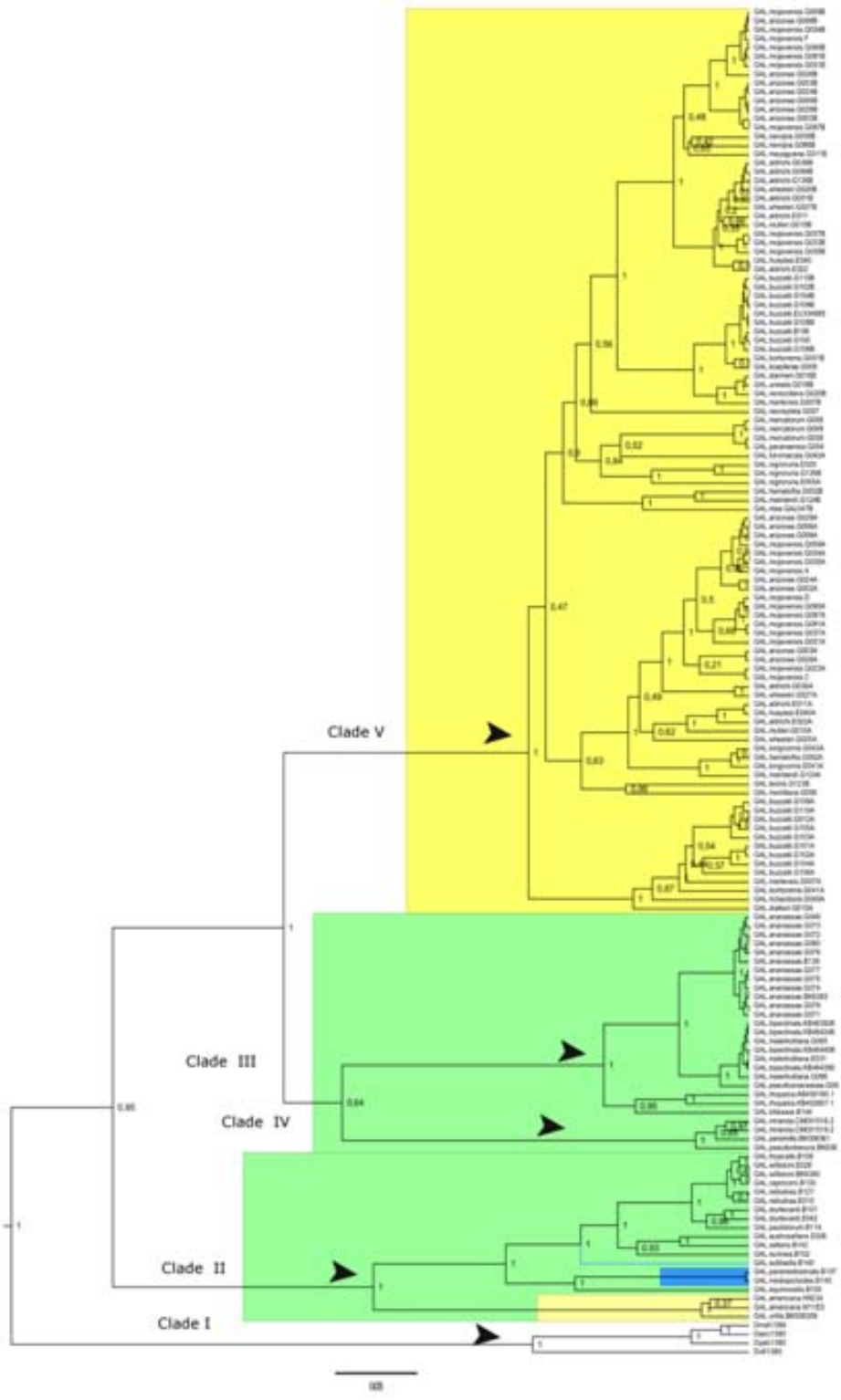

Figure 6. Bayesian Inference phylogenetic tree for 152 sequences of Galileo and 1360 element TPases in 51 *Drosophila* species. Posterior probability values are shown on each node. Colors denote subgenera of the host as follows: yellow=*Siphlodora*, green=*Sophophora*, blue=*Drosophila*.

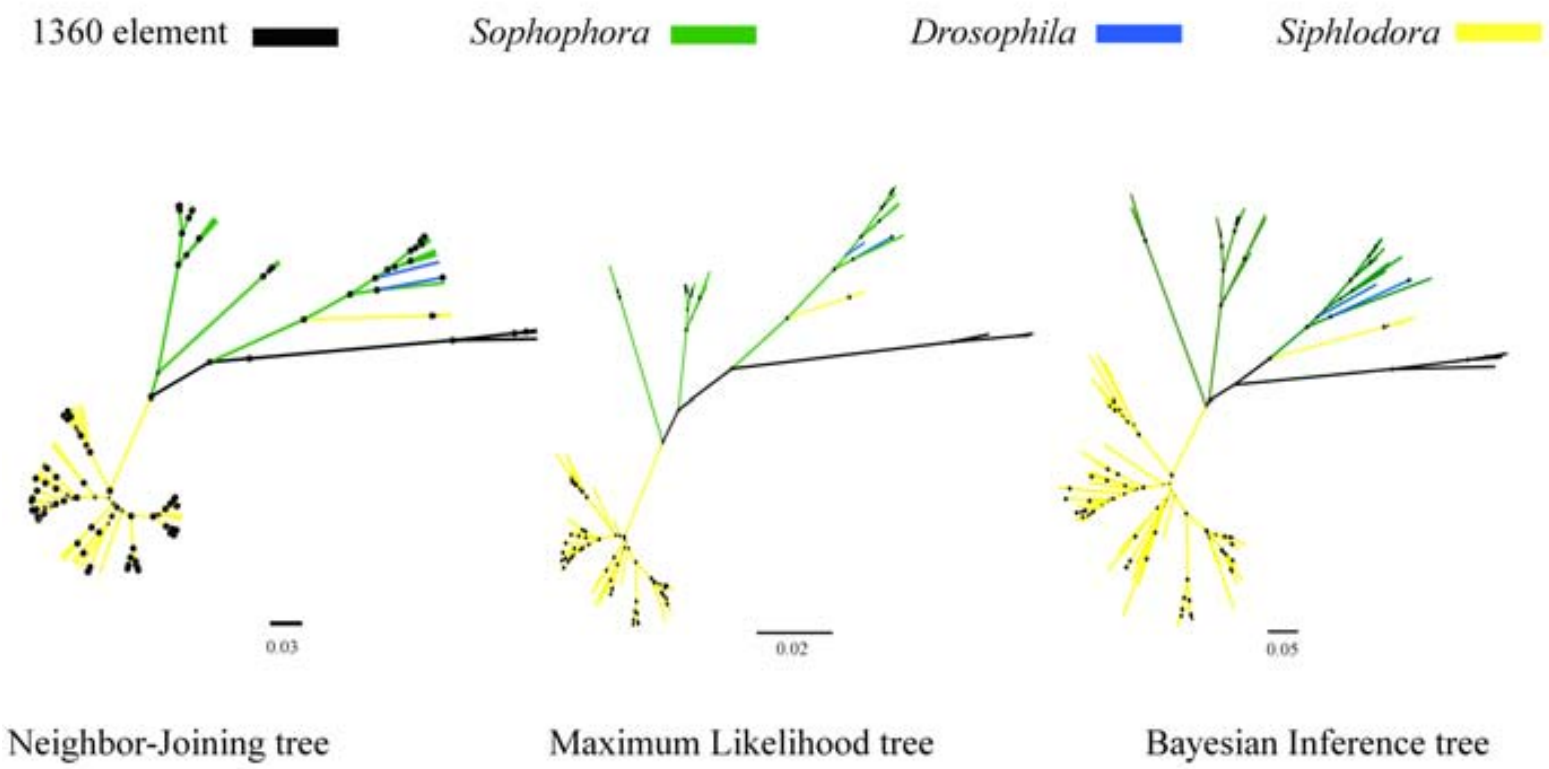

Figure 7. Graphical comparison of radial trees inferred for 152 sequences of the transposon Galileo and element 1360 TPases in 51 *Drosophila* species. Unrooted phylograms on scale, black dots denote statistical support for each phylogenetic method, maximum size of black dots is show high statistical support on each method on A: Bayesian posterior probabilities = 1, on B: aLRT non-parametric Shimodaira-Hasegawa values = 1, on C=Bootstrap values =100.

Galileo Ancestral Range Reconstruction

The current observed distributional pattern of Galileo has been used to reconstruct its ancestral distribution under the Mk1 model using a Maximum Likelihood approach. The results of this analysis are shown in Figure 8. The Ancestral Range Recolnstruction (ARR) of 152 sequences of Galileo in 51 *Drosophila* species has an overall likelihood score of -lnL=154.19.

The ARR shows that Galileo sequences from *willistoni-tripunctata-guarani* species groups more probably were originated in the Neotropics (prob=0.87 $p < 0.05$). The clade encompassing *virilis-tripunctata-guarani* and *willistoni* species groups from *Sophophora-Drosophila-Siphlodora* subgenus is more probably originated in the Neotropic (prob= 0.43) than in the Neartic (prob = 0.23). Galileo sequences from *ananassae*, *montium*, *melanogaster* and *obscura* species groups likely have an Oriental origin (prob=0.95 $p < 0.05$).

Galileo sequences from the *repleta* species group have a strong biogeographical signal. Clades can perfectly be distinguished at the level of species complexes. For instance, the *buzzatii* complex (*D. starmeri, D. uniseta*, *D. borborema*, *D. koepferae*, *D. buzzatii, D. martensis* and *D. borborema*) species has a Neotropical origin (prob=0.99 $p < 0.05$) and the *mulleri* complex (*D. arizonae*, *D. mojavensis* and *D. wheeleri)* has a Neartic origin (prob=0.98 $p < 0.05$).

Based on ecological, molecular and biogeographical evidence it has been proposed that the ancestor of the *Sophophora* subgenus has a Eurasian origin (Russo et al. 2013; Lachaise et al. 1988; Throckmorton 1975).

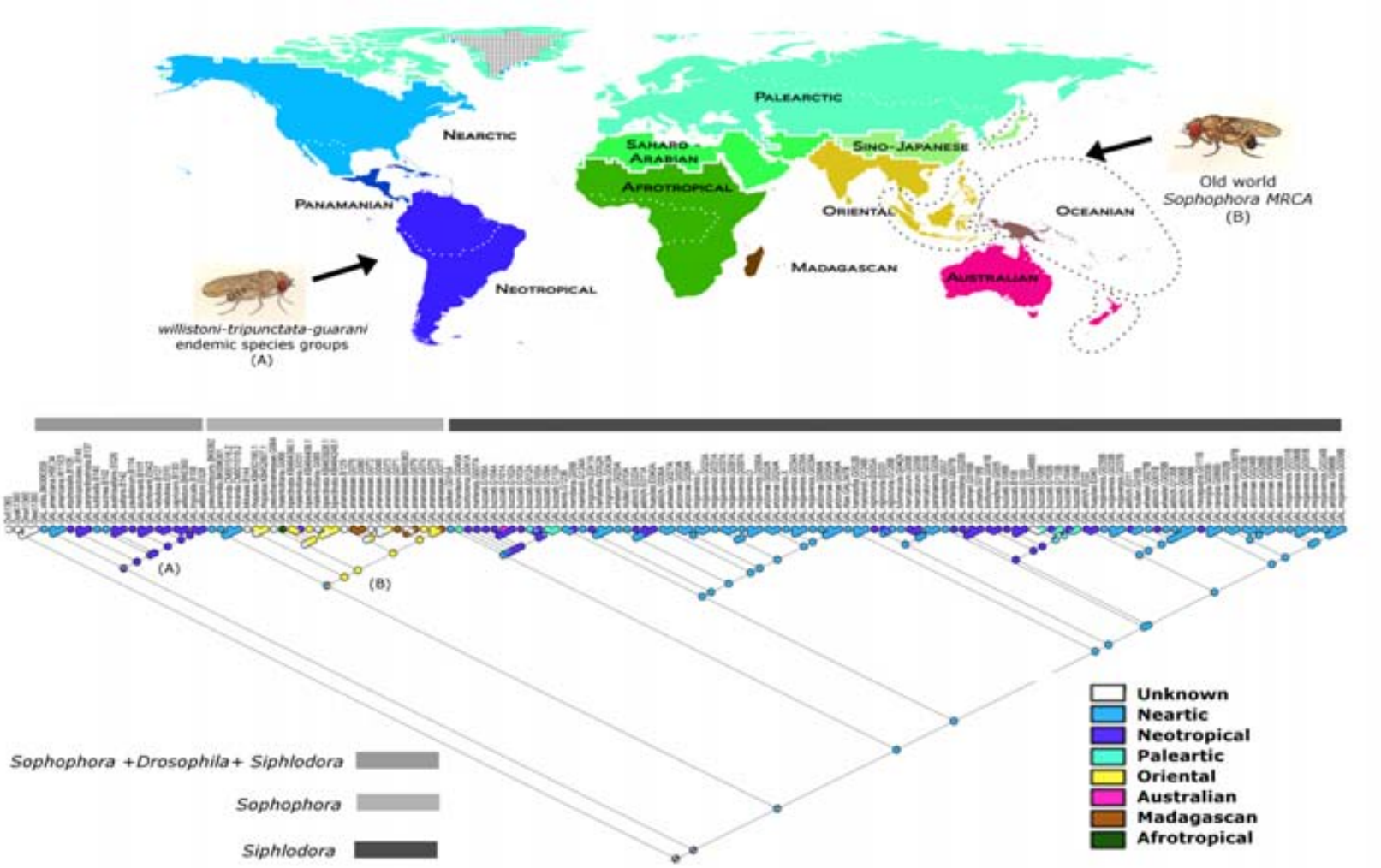

Figure 8. Ancestral Range Reconstruction of 152 sequences of Galileo in 51 *Drosophila* species. Zoo-geographical map modified from Holt et al. (2013). ML ancestral reconstruction mapped on the BI tree using the MK1 model. Proportional likelihoods are shown on nodes.

Galileo in Drosophilidae

Presence of Galileo detected through PCR and *in silico* screening is mapped in the phylogenetic tree of Drosophilidae (Figure 9). It is remarkable a discontinuous pattern of distribution across 31 species groups of the genera *Drosophila*, *Scaptodrosophila*, *Zaprionus* and *Hawaiian Drosophila*.

A patchy distribution of Galileo is also observed at the taxonomic level of subgenus. For example, in the *Drosophila* subgenus, Galileo was detected in the *guarani* and *tripunctata* species groups, sister taxa of the *cardini* species group, in which Galileo was not detected. Likewise, in the *Siphlodora* subgenus, Galileo was detected by *in silico* searches in the *virilis* group, but its sister clades *calloptera*, *annulimana*, *atalaia* and *nannoptera* groups, screened using PCR, seem devoid of Galileo in their genomes. In the *repleta* species group, a special sampling effort was made since this clade encompasses *D. buzzatti*, the taxon where Galileo was initially described, however this element was no detected in basal clades such as *inca*, *fasciola* and *hydei* subgroups.

The ancestral reconstruction analysis carried out with the data of Galileo presence/ absence in 133 species of Drosophilidae mapped in the BI phylogeny gave similar results with the two approaches used. Maximum Likelihood (MLAR) (Figure 10) and Parsimony (PAR) (Figure 11) show three more likely points of Galileo introduction in Drosophilidae. (i) the *Sophophora* subgenus, (ii) the *Drosophila* subgenus and (iii) the *Siphlodora* subgenus.

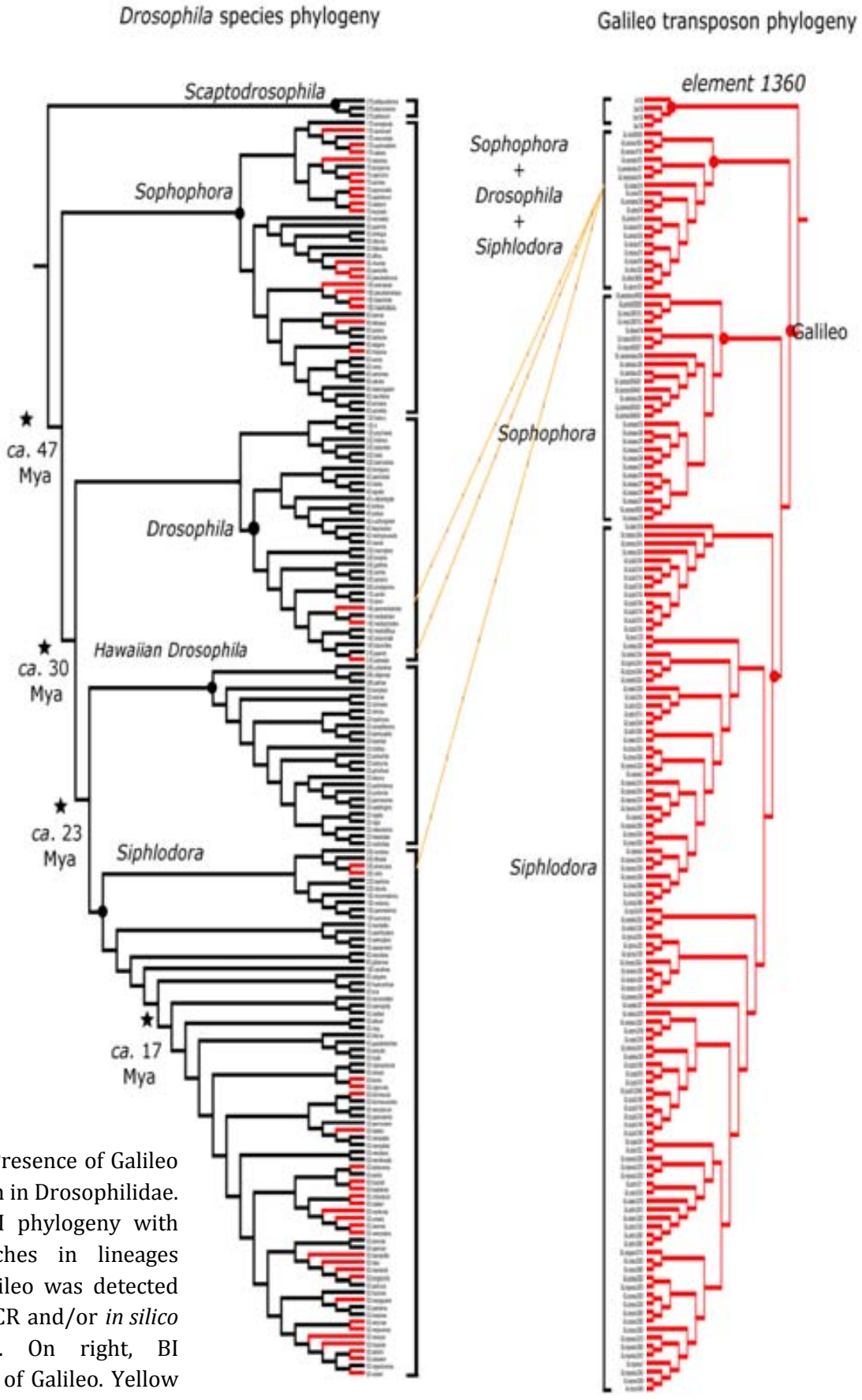

Figure 9. Presence of Galileo transposon in Drosophilidae. On left, BI phylogeny with red branches in lineages where Galileo was detected through PCR and/or *in silico* screenings. On right, BI phylogeny of Galileo. Yellow lines between trees denote phylogenetic incongruence, stars show estimated divergence dates from literature.

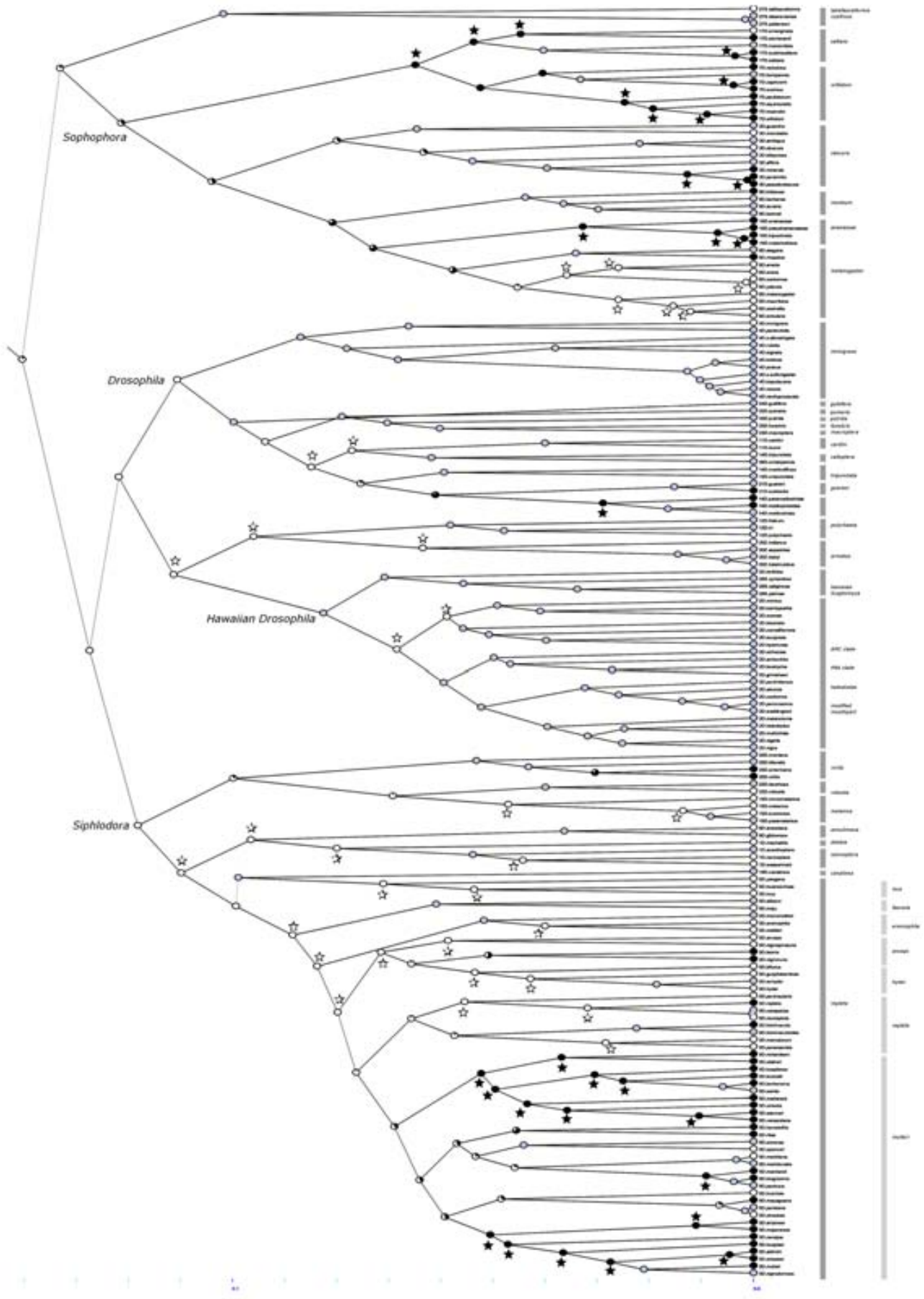

Figure 10. MLAR inferred for Galileo across 174 species of Drosophilidae. Terminal nodes are shown in: black (Galileo detected), gray (presence inferred) and white (undetected). Internal nodes denote proportional likelihoods of ancestral reconstructions.Stars denote statistical singnificance ($p < 0.05$).

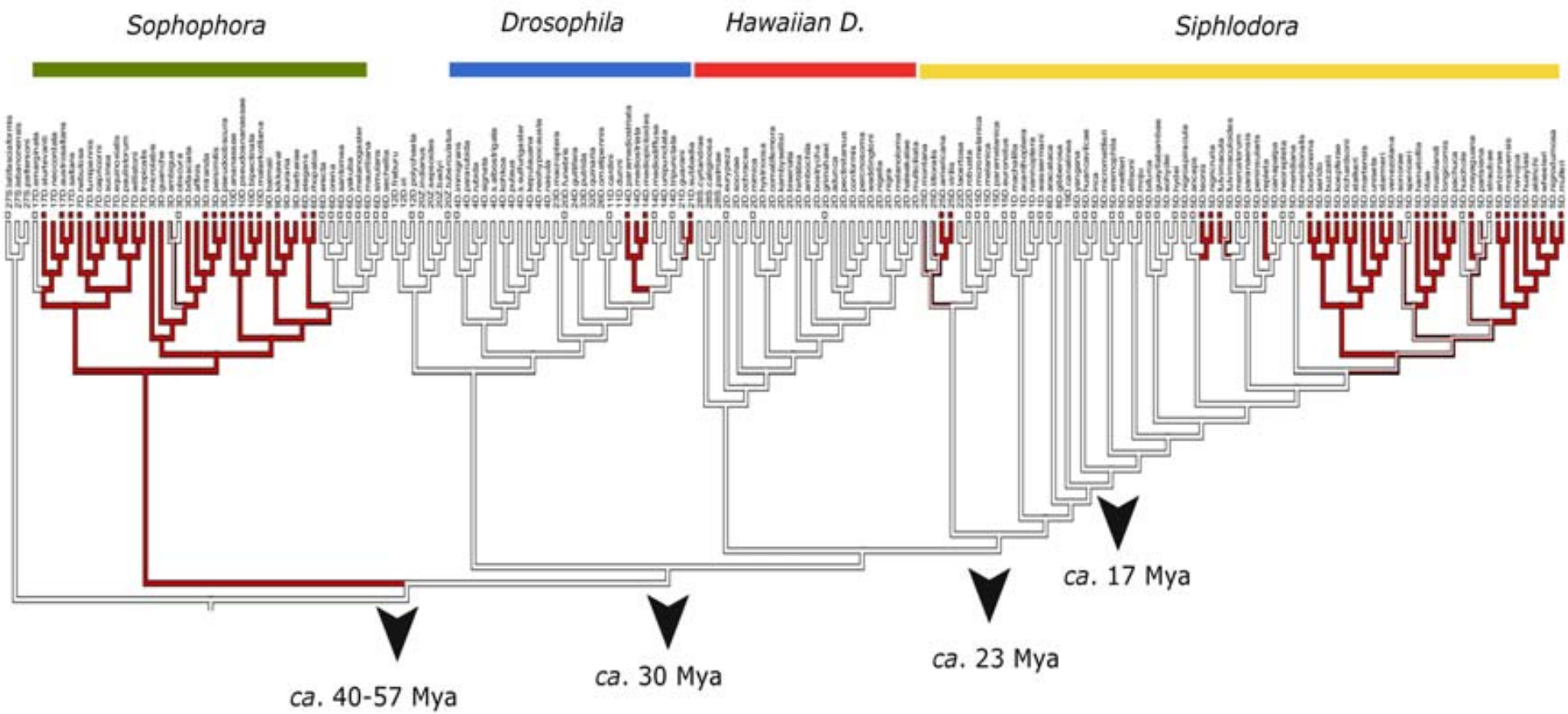

Figure 11. PAR inferred for Galileo transposon mapped in the BI phylogeny of 174 species of Drosophilidae. Red branches denote inferred presence of Galileo. Squares above taxa are in red when Galileo was detected, in white when Galileo was not detected Branches through PCR and *in silico* methods. Estimated divergence dates from Russo et al. (2013); Clark et al. (2007); Oliveira et al. (2012) and Acurio et al in preparation.

Cophylogenetic analysis

The graphical comparison of the ultrametric trees (tanglegram) for Galileo and its host species is shown in Figure 12. The phylogenetic tree inferred from four molecular markers (COI, COII, ND2 and SinA) and the phylogenetic tree inferred from 152 sequences of Galileo TPases in 51 *Drosophila* species were compared. The Galileo phylogeny resembles that of its host species with three exceptions:

1. *D. virilis* and *D. americana* that belong to the *virilis* species group, from *Siphlodora* subgenus.
2. *D. subbadia* from the *guarani* species groups from the *Drosophila* subgenus.
3. *D. mediopictoides* and *D. paramediostriata* from *tripunctata* species group of *Drosophila* subgenus.

These five lineages are nested within the Galileo clade from the *Sophophora* subgenus. Specifically, these lineages intermingle in the same clade than *willistoni* and *saltans* species groups.

The number of synonymous substitutions per synonymous site (d_s) from averaging over all sequence pairs across species groups estimated on each dataset analyzed (COI, COII, ND2, SinA and Galileo are shown in Tables S5-S9 (Supplementary Material). When the lineages involved in phylogenetic incongruences are compared, significant differences in d_s values from host genes and Galileo were found in *guarani*, *tripunctata* and *virilis* species groups (Figure 13).

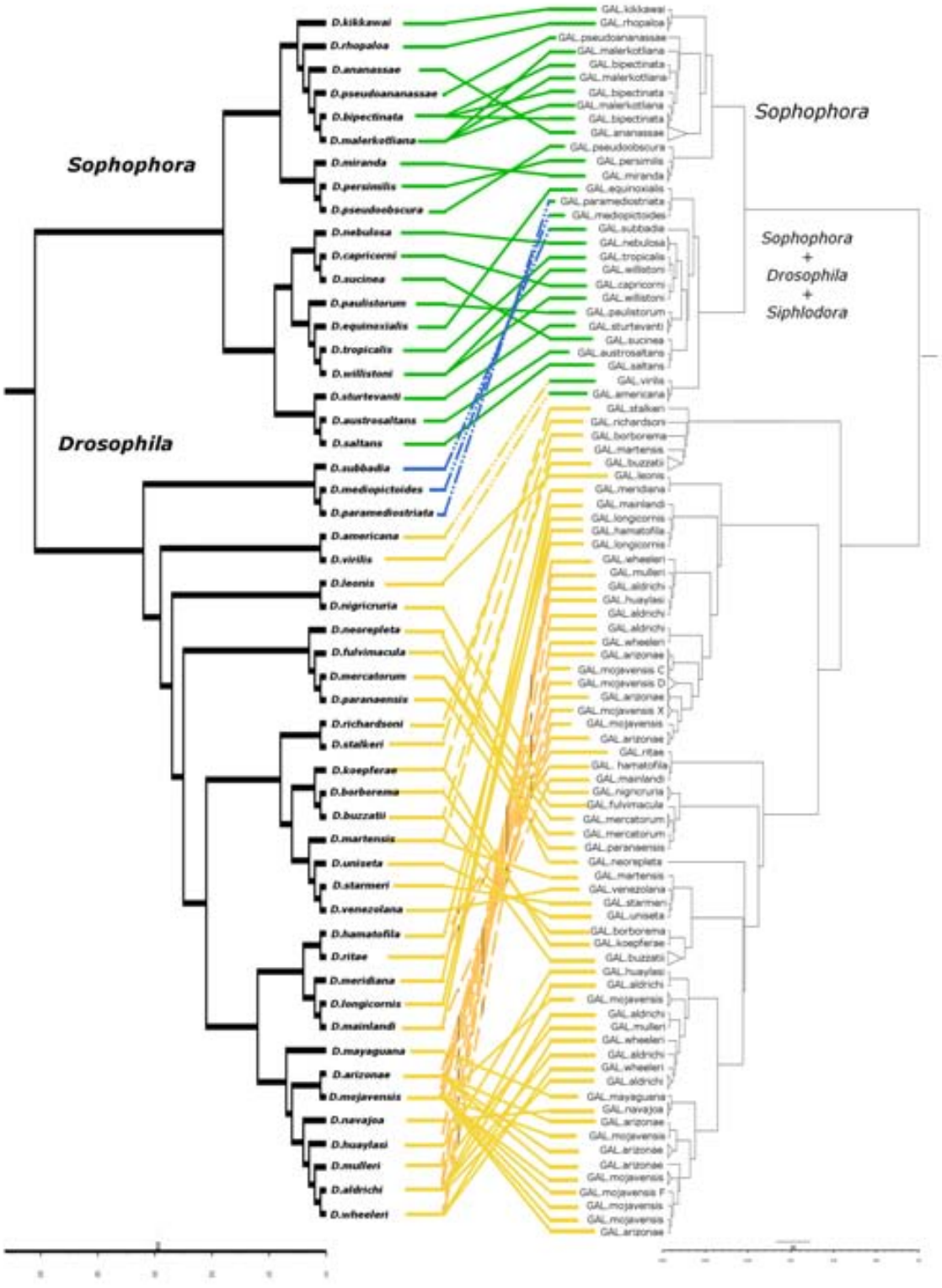

Figure 12. *Drosophila*-Galileo transposon tanglegram. Ultrametic trees inferred using BI methods for 51 host *Drosophila* species (left) and Galileo copies (right) found in their genomes. Line colors denote subgeneric level on both phylogenies, green for *Sophophora*, blue for *Drosophila*, yellow for *Siphlodora*. Taxa where Galileo was not detected have been trimmed using MESQUITE in the host phylogeny and clades collapsed using FigTree in the transposon phylogeny.

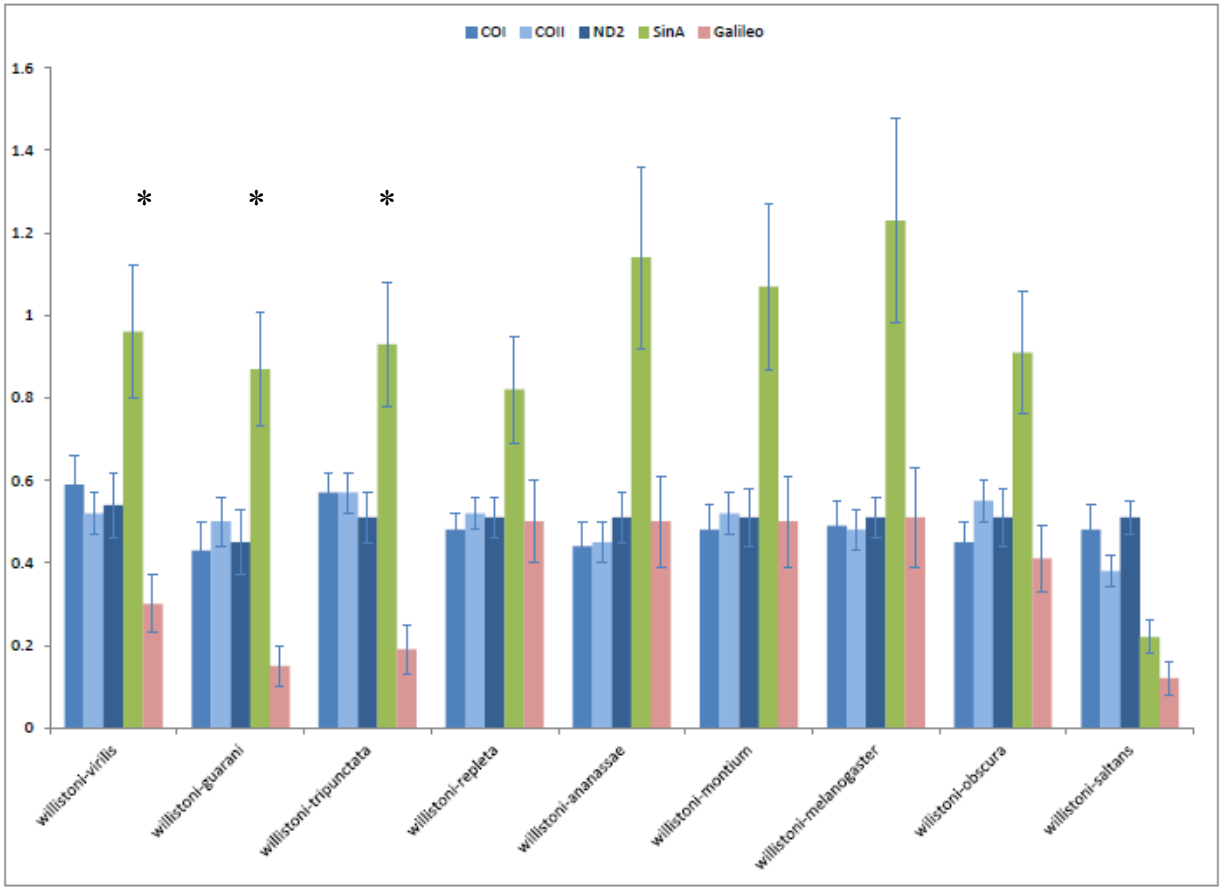

Figure 13. Number of synonymous substitutions per synonymous site (d_s) from averaging over all sequence pairs across 10 species groups of *Drosophila*. Values estimated using MEGA 4. Asterisks denote significant differences on d_S values from host species genes (COI, COII, ND2, SinA) and Galileo transposon (TPase).

Cophylogenetic analysis of the ultrametric tree of Galileo, inferred from TPase and the ultrametric tree of *Drosophila* host species, inferred from four molecular markers, carried out in TreeMap V.3.0, is show in Figure 14. The high level of congruence between the phylogenies of Galileo and its host *Drosophila* species is evident and denoted by the *z* statistic value from the randomized subtrees. According *z* statistic test, the incongruent clades of the host species phylogeny were:

- The *willistoni* clade that encompass seven lineages: *D. nebulosa, D. capricorni, D. sucinea, D. paulistorum, D. equinoxialis, D. tropicalis* and *D. willistoni*.
- The clade of the *Drosophila* subgenus that encompass tree lineages: *D. subbadia*, *D. paramediostriata* and *D. mediopictoides*.
- The *obscura* clade that encompass three lineages: *D. miranda*, *D. persimilis* and *D. pseudoobscura.*
- The *virilis* clade that encompass two lineages: *D. virilis* and *D. americana*.
- In the *repleta* species group: two lineages of the *mercatorum* subgroup (*D. mercatorum* and *D. paranaensis*), six lineages of the *buzzatii* complex (*D. martensis*, *D. uniseta*, *D. starmeri*, *D. venezolana, D richarsoni* and *D. stalkeri*), four lineages of the *mulleri* complex (*D. arizonae*, *D. mojavensis*, *D. mayaguana* and *D. navojoa*) and five lineages of the *longicornis* complex (*D. hamatofila*, *D. ritae*, *D. meridiana*, *D. mainland* and *D. longicornis*).

The overall result that these two phylogenetic trees are significant similar remained even when associated terminal taxa was eliminated through the “cherry picking” test (CPT) (Figure 15).

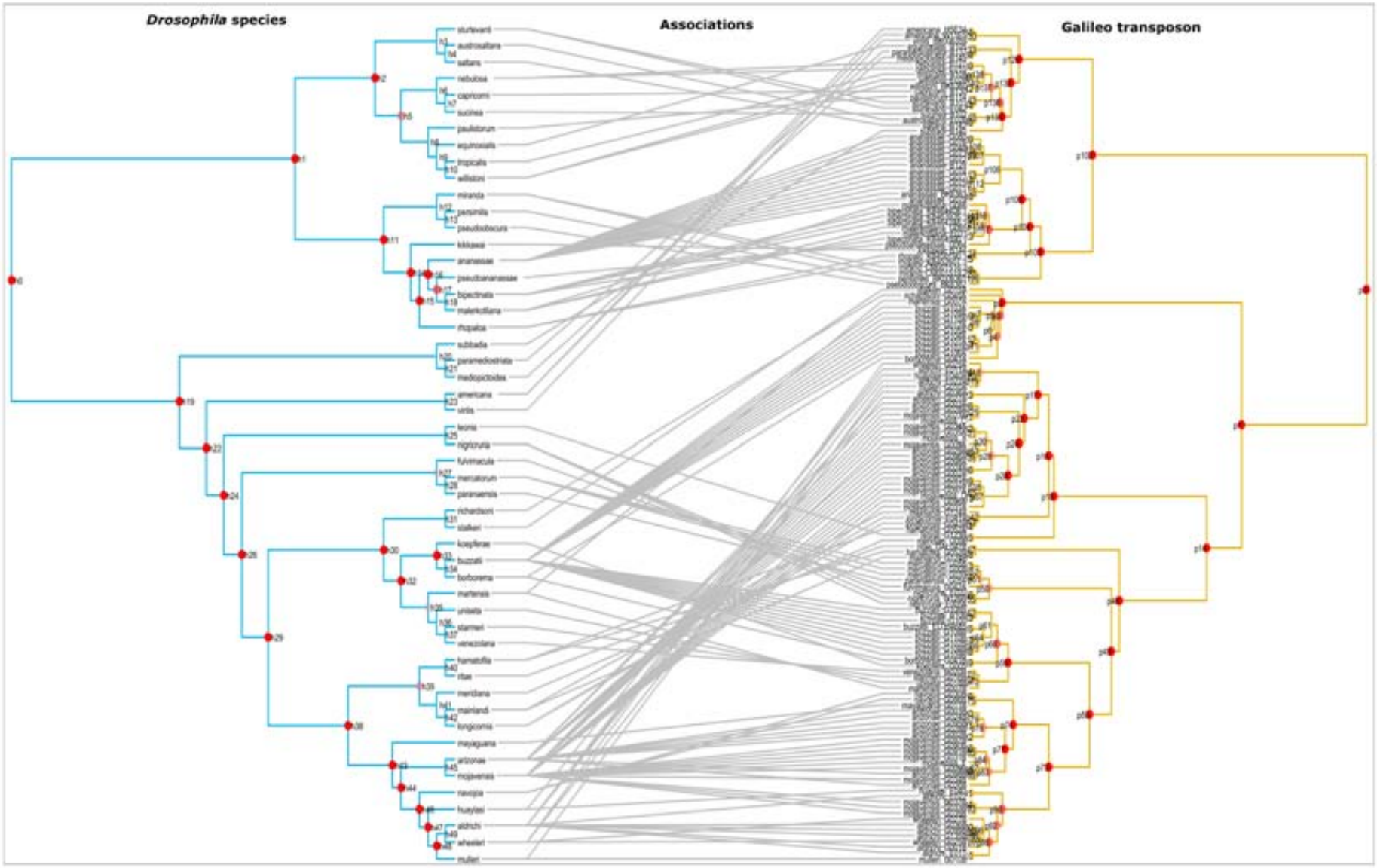

Figure 14. Cophylogenetic analysis of the ultrametric tree of Galileo (on yellow, inferred from TPase) and the phylogenetic tree of 51 *Drosophila* host species (in blue, inferred from COI, COII, ND2, SinA genes). Red dots denote congruence between transposon and host genome tested through *z* statistic value from the randomized subtrees.

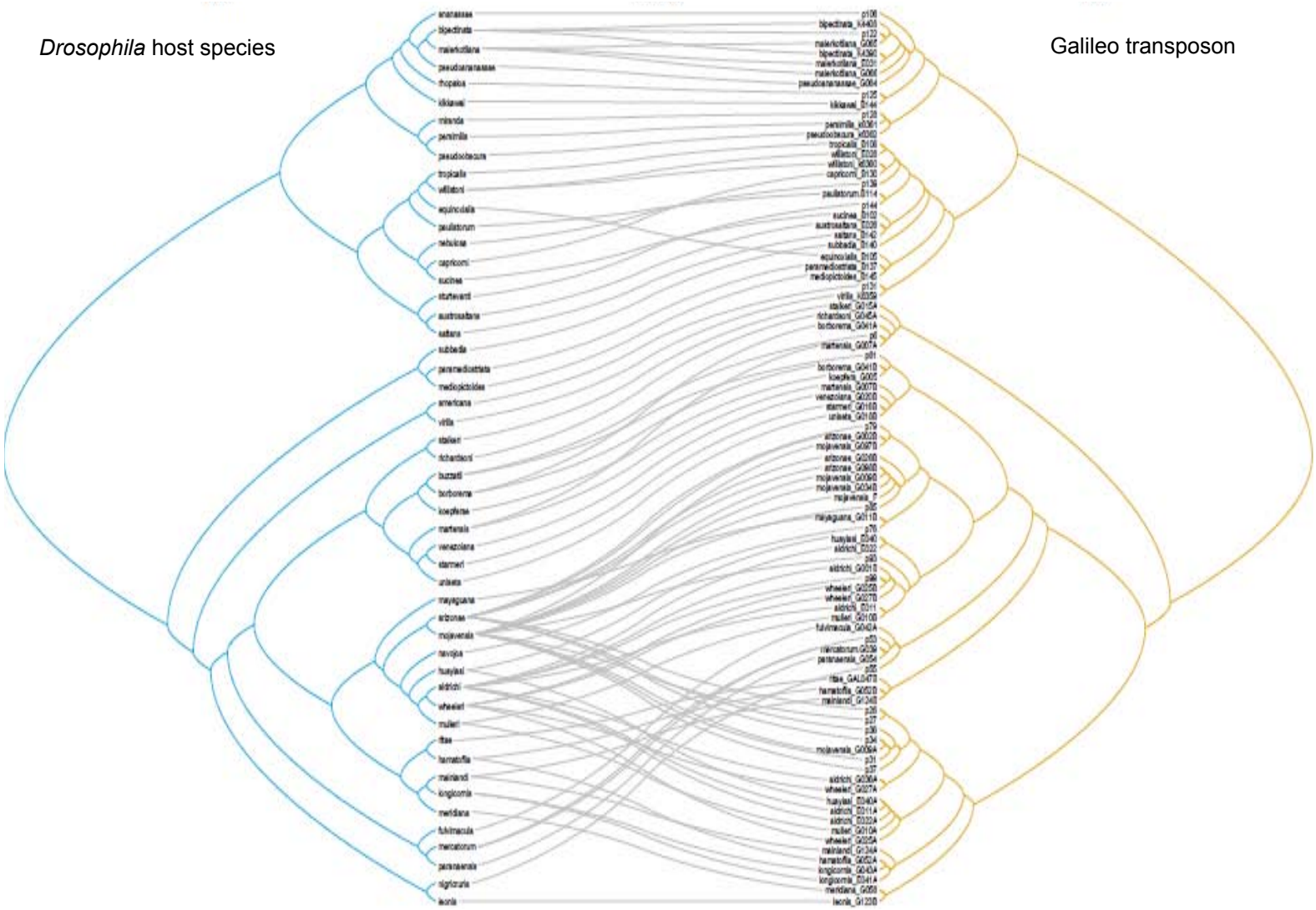

Figure 15. Cophylogenetic analysis of the ultrametric tree of Galileo (on yellow, inferred from TPase) and the phylogenetic tree of 51 *Drosophila* host species (in blue, inferred from COI, COII, ND2, SinA genes) after the removal of associated terminal taxa by CPT.

DISCUSSION

Drosophilidae phylogeny

The phylogenetic analysis carried out using four molecular markers (COI, COII, ND2, SinA) from 174 taxa of Drosophilidae classified in 31 species groups recovered well resolved phylogenies under BI and NJ methods (Figures 2 and 3). The results obtained in this analysis is in good agreement with two recent phylogenetic analyses of Drosophilidae by Yassin (2013) and Russo et al. (2013), which encompass most of the currently known *Drosophila* diversity. In spite of using different molecular markers and taxa, the same evolutionary relationships have been recovered here and in by previous phylogenetic approaches in the following clades:

Clade I (in this study) belongs to *Scaptodrosophila* Duda 1923. Monophyly of this clade has been previously reported by Bock & Parsons (1978).

Clade II (in this study) belongs to *Sophophora* Sturtevant 1939. Previous phylogenetic analyses in agreement with the monophyly of *Sophophora* are: Yassin (2013); Russo et al. (2013); Remsen & O'Grady (2002); Clark et al. (2007).

Clade III (in this study) is the newly diagnosed subgenus *Drosophila sensu stricto* Yassin 2013 or *tripunctata-immigrans* radiation according to Throckmorton (1975).

Clade IV (in this study) encompasses *Scaptomyza* genus Hardy 1849 and the Hawaiian *Drosophila sensu stricto* O'Grady 2011, which encompass five species groups: *antopocerus*, *modified tarsus*, *ciliated tarsus*, *halekalae* and *modified mouthpart.* Previous phylogenetic analyses supporting monophyly of Hawaiian Drosophila are: O'Grady & Desalle (2008); O'Grady & Markow (2009).

Clade V (in this study) encompasses *Zaprionus* Coquillett 1901 and *polychaeta* as its sister taxa. Monophyly of *Zaprionus* has been previously supported by several studies (Yassin et al. 2008; Russo et al. 2013). In this study however, *Zaprionus* is the sister clade of the *polychaeta* species group. The phylogenetic positions of Clade V was recovered with very low support in NJ method.

Clade VI (in this study) encompasses the newly diagnosed *Siphlodora* subgenus *sensu stricto* Yassin 2013 or *virilis-repleta* radiation according to Throckmorton (1962). This clade is also recovered by Russo et al. (2013) phylogenetic approach.

Pattern of distribution of Galileo in Drosophilidae

The most used experimental methods for DNA transposons detection are PCR and Southern/Dot blot techniques. PCR method allows the gathering of sequences of TEs homologous regions while Southern/Dot Blot techniques are preferred to estimate the copy number of TEs. Although there is no perfect experimental method for TEs detection, it is known that PCR amplification using degenerate primers from highly conserved regions may detect elements in species that are apparently devoid of them according Southern/Dot Blot techniques (Capy et al. 1998).

Efficiency of the PCR approach for screening of TEs in distantly related taxa has been demonstrated with other DNA transposons like mariner, which was originally described in *D. mauritania* (Drosophilidae), and has been detected using the homology PCR approach on distantly related species from different orders of Arthropods (Robertson 1993; Robertson & Lampe 1995). Efficiency of PCR method

also has been confirmed in this study because it was able to detect Galileo in distantly related species such as the *Sophophora* subgenus and different subgroups within the *repleta* lineage. For example, Galileo detections in the *mulleri* and *repleta* subgroups, where no previous signal of Galileo was detected using the Southern Blot method (Casals et al. 2005).

Similar discontinuous pattern or patchy distribution has been previously reported in Drosophilidae when a broad spectrum of species is screened. For example mariner-like elements (Brunet et al. 1994; Maruyama & Hartl 1991) and hobo-like elements (Daniels et al. 1990). It is difficult to prove that an element is not present in a species, no matter what technique of TEs detection has been used. The possibility that a lineage contains homologous sequences with strong divergence from the primers or probes used in the screening may cause that the element is undetected. Uncertainty regarding presence of Galileo in Drosophilidae was examined with MLAR (Figure 10) and PAR (Figure 11). Both methods inferred same discontinuous pattern of distribution when presence/absence of Galileo is mapped in the species phylogeny (Figure 9).

Galileo is detected on specific points of the host phylogeny and after such point of entrance presence or absence of the element is related to cladogenesis of host lineages. Exemplifications of these are the patterns of distribution of Galileo in the *montium-ananassae-melanogaster* clade and in the *repleta* species group.

The *montium-ananassae-melanogaster* clade

The presence of Galileo through PCR and *in silico* (when genomes were available) screening was determined for several species within the *montium* and *ananassae* species groups. However their closely related *melanogaster* group gave

negative results for Galileo presence with two exceptions: *Drosophila elegans* and *Drosophila rhopaloa*, species from the *elegans* and *rhopaloa* subgroups, respectively.

According to several studies (Da Lage et al. 2007; Russo et al. 2013; Yassin 2013), and the species phylogeny recovered here, the *melanogaster* group includes the *melanogaster* subgroup with an Afrotropical origin and other species subgroups with an Oriental origin. The *elegans* and *rhopaloa* lineages seem to have diverged first from other Oriental subgroups (Kopp 2006; Goto & Kimura 2001; Da Lage et al. 2007).

The fact that Galileo is present on several taxa from *ananassae* and *montium* groups, that are considered early diverging lineages within the *ananassae-montium-melanogaster* clade (Kopp 2006; Goto & Kimura 2001), strongly suggest that the most recent common ancestor (MRCA) harbored an autonomous copy of Galileo in its genome. The presence of Galileo in the MRCA of the *ananassae-montium-melanogaster* clade was inferred by MLAR and PAR. Interestingly, the ARR of Galileo (Figure 8) recovers the same geographical range proposed to the MRCA of Old World *Sophophora*.

With the data analyzed in this study we are not able to determinate if the splits of the *melanogaster* subgroup from its sibling taxa had an important effect on the Galileo, but it seems that cladogenesis in the melanogaster subgroups could have an effect in the element causing either, divergence in the TPase that avoid detection or loss of autonomous Galileo copies.

The *repleta* clade

Since Galileo was originally described in *D. buzzatii*, a taxon of the *repleta* species group, special sampling effort was made in this lineage. Six subgroups were sampled (*inca*, *mercatorum*, *fasciola*, *hydei*, *mulleri* and *repleta*). Galileo was detected in three of the six subgroups (*repleta*, *mercatorum* and *mulleri*) only.

The *inca*, *fasciola* and *hydei* subgroups represent basal lineages within the *repleta* radiation (Acurio et al. in preparation). At least two possible reasons could explain the absence of the element in *repleta* basal clades; one reason might be that there is a high degree of divergence in the element, whereby it is not detectable with the methods employed in this study, the second reason might be that the introduction of Galileo in *repleta* took place after the split of *repleta*, *mercatorum* and *mulleri* subgroups, divergence time for the split of these subgroups has been estimated in *ca.* 14 Mya (Acurio *et al*. *in preparation*, Oliveira *et al*.2012). The fact that Galileo has been detected in other nine species groups of the *Drosophila* radiation, but also that the phylogeny of the element mirrors that of host species, are evidences of the long-term association of Galileo in *Drosophila*. Therefore, the second reason looks unrealistic. Two scenarios can be proposed to explain the extant discontinuous distribution of Galileo in the *repleta, mercatorum* and *mulleri* subgroups (Figure 9).

The first scenario implies stochastic losses in several lineages. The second scenario implies reactivation of autonomous copies. It has been proposed (Venner et al. 2009) that during their evolution, non-autonomous copies of TEs can be dormant entities that persist in the genome as long as the environment remains unfavorable for its development, for example copies inactivated by methylation or epigenetic processes that can be reactivated when methylation is removed. TEs dynamic that

experience different periods of grow rate and periods of dormancy has been reported in *Drosophila* (Vieira et al. 1999).

Galileo subfamilies

It is important distinguish different levels of diversification of TEs. One level of diversification could be considered a genome (Venner et al. 2009). Cut-and-paste TEs, such as Galileo, may have transcriptional active copies (autonomous) and defective copies (non-autonomous) unable to encode a functional protein. Non-autonomous copies are presumably derived from autonomous copies by mutation and/or deletion (Feschotte & Pritham 2007). A second level of diversification on TEs could be considered the macro-evolutionary level, in which, factors affecting host species divergence may also affect divergence of TEs.

Several studies have tackled the intraspecific variation of Galileo using bioinformatics screenings of *Drosophila* genomes (Marzo et al. 2008; Marzo et al. 2013; Gonçalves et al. 2014). From these approaches it is currently known that *D. buzzatii* harbor three Galileo subfamilies (G, K, N), *D. virilis* harbor two subfamilies (A, B), *D. willistoni* harbor two subfamilies (V, W) and *D. mojavensis* harbor five subfamilies (F, C, D, X and E). Some of the subfamilies harbor only non-autonomous copies without significant TPase encoding segments. Most of such studies have analyzed the homologous TIR region from Galileo copies. The comparison of phylogenies build with TIR segments and TPase sequences led in some cases to congruent results (Gonçalves et al. 2014). However, in other cases (Marzo et al. 2013a), discrepancies were noticed that can be due to different evolutionary histories but also to phylogenetic uncertainty.

The results of the phylogenetic analysis performed here across 51 *Drosophila* species using a segment of the Galileo TPase support most of the classification at subfamily level proposed so far, although some subfamilies appear to be the result of intraspecific diversification (Figure 13). For instance, the F subfamily of Galileo, initially detected in *D. mojavensis* is recovered with high statistical support (BI: 1, ML: 0.99, NJ: 1) in other seven species from the *mulleri* complex such as: *D. arizonae*, *D. navojoa*, *D. huaylasi*, *D. mayaguana*, *D. aldrichi*, *D. wheeleri* and *D. mulleri*. Hence, it is highly probable that the MRCA of this species complex harbored active copies from the F subfamily.

On the other hand, the C, D and X subfamilies, initially characterized in *D. mojavensis,* were found in samples of *D. arizonae* (*D. mojavensis* close relative). This subfamilies were recovered as a single clade with quite good statistical support (BI: 1, ML: 0.95, NJ: 0. 90). Thus, it is apparent that autonomous copies of these three subfamilies were in the genome of D. *mojavensis* and *D. arizona* ancestor. The same could be applied to the E subfamily which is has been characterized only in non-autonomous copies of Galileo.

Two distantly related groups with high statistical support were found in the *buzzatii* species complex. The first clade (BI: 1, ML: 1, NJ: 1) encompass Galileo copies from 5 species, *D. buzzatii*, *D. martensis*, *D. borborema*, *D. richarsoni* and *D. stalkeri*. The second clade (BI: 1, ML: 1, NJ: 1) enclose Galileo copies from seven species: *D. buzzatii*, *D. martensis*, *D. borborema*, *D. starmeri, D. uniseta D. koepferae* and *D. venezolana*.

It is noteworthy that previous screenings of Galileo in *D. buzzatii* have reported three different subfamilies, namely Galileo, Kepler and Newton or G, K and

N subfamilies (Cáceres et al. 2001). All copies found so far in the latter two subfamilies (K and N) are non-autonomous copies lacking significant TPase-encoding segments. The TIRs of the tree subfamilies have the most terminal 40 bp almost identical and generate upon insertion a TSD of a palindromic 7 bp sequence (Casals et al. 2005; Casals et al. 2003). However, K and N subfamilies seem more clasely related than each of them is to the third subfamily G (Casals et al. 2005). The fact that described copies in N and K subfamilies lack the TPase-encoding segment precludes comparison with the results of this work. It could be that the two Galileo lineages detected here in the buzzatii species complex correspond to the G subfamily or that one of them represents the undescribed TPase of the K or N subfamilies. Further work is needed to clarify this issue.

From a previous study testing the ability of the TIRs of copies of N and K subfamilies to bind the THAP domain of Galileo TPase (Marzo et al. 2013b) it is known that cross-reactivity exist between Galileo TPase and K subfamily TIRs. Taking all this into account, it is possible speculate that one of the two subfamilies recovered in the phylogenetic analysis of the TPase motif correspond to autonomous copies from Newton subfamily in the *Drosophila buzzatii* complex.

The general pattern found in subfamilies of Galileo across *Drosophila* species is that subfamilies classified using the TIRs are shared at level of species complex and subgroups, which have short periods of time divergence ranging from 9 to 0 Mya as is illustrated in Acurio *et al*. *in preparation*. TPase motifs are good features in classification of TEs at level of superfamilies because of their conservation across different phyla (Capy et al. 1998; Yuan & Wessler 2011) while TIRs, highly variable on structure and length in Galileo (Marzo et al. 2013), are the only feature useful to classify non-autonomous copies.

Base composition of nuclear, mitochondrial and TPase genes

The strong bias toward A + T content (ca. 90%) at third codon position on mitochondrial genes of *Drosophila*, previously reported in several studies (Satta et al. 1987; DeSalle et al. 1987; Tamura 1992; Montooth et al. 2009), also found here in the analysis of three mitochondrial loci of 174 species, is hypothesized to be generated by mutation pressures that would oppose weak selection for codon-anticodon matching (Montooth et al. 2009). The nuclear locus analyzed on this study has a GC content of 57.1%. Biases toward G + C content on nuclear genes of *Drosophila* has been proposed to be due to C-ending codon preference (Moriyama & Hartl 1993). Differences in synonymous substitutions rates between nuclear and mitochondrial genes of *Drosophila* are attributed to elevated transition rates in mitochondrial genes and selective constrains associated with codon usage bias in nuclear genes (Moriyama & Powell 1997).

The analysis of 152 sequences from 51 *Drosophila* species revealed an average A + T content of 65.6 % on Galileo TPase. Tendency for TEs to be AT-rich has been previously reported on both, GC-rich genomes such as *D. melanogaster and H. sapiens* and AT-rich genomes like *A. thaliana, S. cerevisae* and *C. elegans* (Lerat et al. 2000; Lerat et al. 2002), suggesting that AT content is a specific characteristic of all TEs and independent from host genomes. In fact, Lerat et al. (2002) distinguished between two groups of TEs according the nucleotide composition at the third codon position, the rich-A and the rich-T-ending codons. The sequence comparison of Galileo showed that A (37%) is the more frequent nucleotide at third codon position; high AT values at this position is thought to be due to selective constraints acting on this third codon base (Grantham et al. 1980).

In spite that only a motif of Galileo TPase was analyzed in this study, a similar A+T biased composition has also been reported for other mobile elements including retrotransposons and retroviruses from different hosts (Jia & Xue 2009; Moriyama et al. 1991; Zsiros et al. 1999; Turelli et al. 1997). As is pointed out by Lerat et al. (2002) some hypothesis proposed to explain the AT bias on TEs include a) mutational bias or natural selection acting on silent changes, b) influence of the site of insertion, since some TEs have shown preferences for specific DNA configuration, for instance low recombination regions, and c) inactivation of host genomes to limit TE invasion with processes like methylation or co-suppression. The underlying mechanism by which mobile elements have higher AT content still remains unknown.

Ancient cospeciation of Galileo and *Drosophila* species

In this study we tested the cospeciation hypothesis in Galileo and its host *Drosophila* species by comparing the phylogeny inferred for Galileo and the phylogeny inferred for host species. The results (Figures 14 and 15) are highly consistent with a long-term historical association of transposons and their hosts. This was corroborated with detection of the element in several populations of the *Sophophora* subgenus from Asia, where the ancestor of the subgenus had its origin *ca*. 40-56 Mya. The fact that Galileo TPases were amplified by PCR on samples of Old world and New world *Sophophora* species strongly suggests that the element is still active on these species. In addition ARR analysis of Galileo (Figure 8) also determined that the MRCA of *Sophophora* subgenus harbored Galileo in its genome. This is consistent with the hypothesis that TEs are ancient components of eukaryotic genomes (Kidwell 2002). It is notable that Galileo mirrored the phylogeny of its host, which is indicative of the cospeciation events between Galileo and its host. The results found here are not in agreement with genome wide-screenings that have

postulated a young origin of TEs families in *Drosophila*, in which the origin of TEs families is dated to be much more younger than for host species (Bowen & McDonald 2001; Bartolomé et al. 2009).

Horizontal transfer in the evolutionary dynamics of Galileo

Three kinds of evidence are generally used to infer HT of TEs: (i) discontinuous occurrence or patchy distribution, (ii) incongruence between host and TE phylogenies (iii) high sequence similarity between very distantly related species. All these three evidences have been found in this study. Alternative explanations for evidences (i) and (ii) such as ancestral polymorphism, inequality of substitutions rates in TE from different species are hard to dismiss conclusively (Loreto et al. 2008; Capy et al. 1998).

However a conclusive evidence of HT event can be inferred whenever the divergence among TE sequences is significantly lower than that observed for host genes under similar or higher selective constraints than those operating on the TEs themselves (Silva & Kidwell 2000). The cophylogenetic analysis of *Galileo-Drosophila* host species and the comparison of d_S values are consistent with punctual HT events during the long-term evolutionary history of Galileo.

The d_S values plotted for ten host species groups (Figure 13) shows that there are three cases in which Galileo divergence is significantly lower than the divergence of mitochondrial and nuclear genes in their host species. There are clearly many assumptions behind these estimates (e.g. G+C content or codon bias), and divergence rates can vary across lineages. Nevertheless, the d_S estimates found here are comparable with those obtained from Silva & Kidwell (2000) in the analysis of

divergence values from P element and three nuclear genes. In their study, the average value for d_S observed for the element was 5 to 10 times smaller than that for host genes. Here, the d_S value for Galileo is ca. 8 times smaller than the d_S value for the nuclear gene SinA and ca. 4 times smaller compared with the mitochondrial genes COI, COII and ND2.

Besides the evidences of HT found in the long-term evolutionary dynamics of Galileo; geographical, temporal and ecological overlapping between donor and recipient species must have happened so that HT events have been possible. Three events were detected in species from subgenera *Siphlodora* and *Drosophila* that intermingled in the *willistoni-saltans* clade belonging to the *Sophophora* subgenus. It has been estimated that these three subgenera split ca. 56 Mya (Russo et al. 2013). The taxa involved in HT events from different subgenera were: *Drosophila virilis*, *Drosophila americana* (*virilis* group); *Drosophila mediopictoides*, *Drosophila paramediostriata* (*tripunctata* group) and *D. subbadia* (*guarani* group).

According to the results obtained in the cophylogenetic analysis (Figures 14 and 15), *Drosophila equinoxialis* is the more likely donor species for HT events. Remarkably, the *willistoni* species group, particularly the *willistoni* subgroup (to which *D. equinoxialis* belong), has been proposed as a source of donor species in HT events of P elements within the genus *Drosophila* (Daniels et al. 1990).

Regarding the distribution and ecology of host species groups involved in HT events, the *virilis* lineage is one of the 30 species groups within the *virilis-repleta* radiation (Throckmorton 1975). Thirteen species are currently recognized in the virilis species group. *Drosophila virilis* and *D. americana* are closely related species (Spicer & Bell 2002; Powell 1997; Morales-Hojas et al. 2011). Southeastern Asia has been

postulated as the original geographical region for the *virilis* group (Throckmorton 1982). *Drosophila virilis* is one of the most ancestral lineages within the group (Caletka & McAllister 2004), originated in Asia and subsequently expanded to other regions of the world (Mirol et al. 2008). Nowadays, *D. virilis* is a widespread cosmopolitan species. *Drosophila americana* is widely distributed across Central and Eastern regions from North America (Fonseca et al. 2013). The time of divergence between *D. virilis* and D. *americana* has been estimated in ca. 4.1 Mya (Morales-Hojas et al. 2011).

The *tripunctata* species group encompasses *ca.* 78 species (Brake & Bächli 2008) and is considered one the most prolific forest dwelling groups of the Neotropical Region (Bächli et al. 2005; Vilela 1992). It was proposed that *tripunctata* and the *calloptera*, *guarani*, *pallidipennis* and *cardini* species groups diversified in the Neotropics during the so called *tripunctata* radiation (Throckmorton 1975; Da Lage et al. 2007). With the only exception of *D. tripunctata*, that is also found in North America (Jaenike 1987), this group is ubiquitous in tropical (Vilela 1992) and Andean forests (Acurio & Rafael 2009) of South America. *Drosophila mediopictoides* and *D. paramediostriata* are sibling species (Robe et al. 2010). The monophyly of the *tripunctata* radiation as a whole has been questioned because usually sister species groups such as *guarani*, appear intermingled in phylogenetic analyses (Remsen & O'Grady 2002; Robe et al. 2005; Hatadani et al. 2009; Robe et al. 2010).

The *guarani* species group encompasses around 12 species and is widely distributed in the Neotropical region (Ratcov & Vilela 2007; Vilela & Bächli 1990). *Drosophila subbadia* belongs to the *guarani* subgroup, one of the two subgroups recognized in the *guarani* group (King 1947; Robe et al. 2002). *Drosophila subbadia*

is a forest-dwelling species distributed in South America and Central Mexico (King 1947; Bächli 2013).

The *willistoni* species group encompasses *ca*. 23 species and *saltans* species group has *ca*. 21 species (Bächli 2013). According several authors (Throckmorton 1975; Russo et al. 2013; Powell 1997) , the tropical split of the subgenus *Sophophora* gave rise to the present Old World clade and the New World clade that include the *willistoni* and *saltans* groups. These two groups are endemic from Central and South America with a few lineages dispersed to Southern Mexico (Bächli 2013; Russo et al. 2013; Spassky et al. 1971).

The fact that the species from the *willistoni*, *saltans*, *tripunctata* and *guarani* species groups are endemic from South America suggests two possible places in the Neotropics where HT events could have happened: (i) Forested areas from South America, where the donor species from *willistoni* subgroup and recipient species from *tripunctata* and *guarani* species group are endemic and live in sympatry. (ii) Central America, the Northern limit distribution of the *willistoni* subgroup according to Spassky et al. (1971) and the Southern limit distribution of *D. americana*.

CONCLUSION

In this study the comprehensive search for the transposon Galileo has been performed in 113 species of Drosophilidae. The element was unequivocally detected in 51 *Drosophila* species using the most conserved region of its TPase. A total of 152 samples with a worldwide distribution in which Galileo was detected were cloned and sequenced to build a phylogenetic tree of the element. Simultaneously, the phylogeny of 174 from 31 species groups of Drosophilidae was inferred from partial coding sequences of genes COI, COII, ND2, SinA. The comparison of Galileo-*Drosophila*

host species phylogenies undercover the long-term historical association of this transposon with its host *Drosophila* species. This was corroborated with detection of the element in several populations of the *Sophophora* from Asia, where it is thought the ancestor of the subgenus has its origin *ca*. 40-56 Mya. The significant match found between host-and transposon phylogenies reveal cospeciation of Galileo in *Drosophila* and ancestral horizontal transfer events that involve the *willistoni*, *tripunctata*, *guarani* and *virilis* species groups.

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SUPPLEMENTARY MATERIAL

Table S1. Source and GenBank accession numbers for COI, COII, ND2 and SinA sequences of 174 taxa analyzed in this study.

	Genus	*Taxon*	Voucher ID	COI	Voucher ID	COII	Voucher ID	ND2	Voucher ID	SinA
1	*Drosophila*	*acanthoptera*	105622	EU493598	105622	EU493728	105622	EU494332	101823	EU341611
2	*Drosophila*	*adunca*	105818	EU493644	105818	EU493773	105818	EU493520	105818	O' Grady, P.
3	*Drosophila*	*affinis*	107540	EU493629	107540	EU493758	Unspecified	EF216219	107540	O' Grady, P.
4	*Drosophila*	*aldrichi*	119140	EU341603	Unspecified	JF736117	119140	EU341702	101824	EU341603
5	*Drosophila*	*ambigua*	107547	EU493630	107547	EU493889	107547	EU493513	107547	O' Grady, P.
6	*Drosophila*	*ambochila*	----	----	109433	EU493776	109433	EU493522	109433	O' Grady, P.
7	*Drosophila*	*americana*	Unspecified	DQ471597	Unspecified	AY646735	Unspecified	DQ471524	G9648	AY851033
8	*Drosophila*	*ananassae*	Unspecified	BK006336	Unspecified	BK006336	Unspecified	BK006336	7217	whole genome
9	*Drosophila*	*anceps*	Unspecified	JF736059	Unspecified	JF736093	Unspecified	JF736133	Unspecified	JF736324
10	*Drosophila*	*aracataca*	Unspecified	JF736077	Unspecified	JF736116	Unspecified	DQ471526	103962	O' Grady, P.
11	*Drosophila*	*arizonae*	106307	EU341676	106307	JF736122	106307	EU341707	106307	EU341620
12	*Drosophila*	*auraria*	109389	EU493624	109389	EU493753	109389	EU493511	109389	O' Grady, P.
13	*Drosophila*	*austrosaltans*	106314	EU493634	106314	EU493763	106314	EU493634	106314	O' Grady, P.
14	*Drosophila*	*baimaii*	109387	EU493625	109387	EU493754	----	----	109387	O' Grady, P.
15	*Drosophila*	*barbarae*	109391	EU493626	109391	EU493885	109391	EU493626	109391	O' Grady, P.
16	*Drosophila*	*bifasciata*	109382	EU493631	109382	EU493760	109382	EU493631	109382	O' Grady, P.
17	*Drosophila*	*bifurca*	Unspecified	JF736090	Unspecified	JF736130	Unspecified	JF736166	Unspecified	JF736378
18	*Drosophila*	*bipectinata*	Unspecified	AY757287	Unspecified	AY757275.1	----	----	42026	whole genome
19	*Drosophila*	*biseriata*	200201	HQ170757	200201	HQ170641	200201	HQ170868	unspecified	JQ413093
20	*Drosophila*	*borborema*	Unspecified	JF736081	Unspecified	JF736121	Unspecified	JF736157	Unspecified	JF736362
21	*Drosophila*	*bostrycha*	109445	EU493649	109445	EU493778	109445	EU493525	109445	O' Grady, P.

22	*Drosophila*	*buzzatii*	102049	DQ202051	102049	DQ202011	102049	DQ202091	102049	EU341621
23	*Drosophila*	*canalinea*	103953	EU493575	103953	EU493706	----	----	103953	JF736349
24	*Drosophila*	*canapalpa*	Unspecified	JF736086	Unspecified	JF736126	Unspecified	JF736162	Unspecified	JF736369
25	*Drosophila*	*capricorni*	108512	EU493637	108512	EU493766	108512	EU493637	108512	EU493518
26	*Drosophila*	*cardini*	103963	EU493576	103963	EU493707	----	----	103963	O' Grady, P.
27	*Drosophila*	*comatifemora*	106342	EU493650	106342	EU493779	106342	EU493526	106342	O' Grady, P.
28	*Drosophila*	*conformis*	105686	EU493652	105686	EU493781	105686	EU493528	105686	O' Grady, P.
29	*Drosophila*	*dunni*	103969	EU493577	103969	EU493708	103969	EU493470	103969	O' Grady, P.
30	*Drosophila*	*elegans*	Unspecified	AB032130	Unspecified	AF461307	----	----	30023	whole genome
31	*Drosophila*	*ellisoni*	105625	DQ202052	105625	DQ202012	105625	DQ202092	105625	JF736356
32	*Drosophila*	*emarginata*	107544	EU493635	107544	EU493764	107544	EU493517	----	----
33	*Drosophila*	*eohydei*	Unspecified	JF736083	Unspecified	JF736124	unspecified	JF736159	Unspecified	JF736366
34	*Drosophila*	*equinoxialis*	107548	EU493638	107548	EU493767	107548	EU493519	107548	O' Grady, P.
35	*Drosophila*	*erecta*	Unspecified	JQ679121	Unspecified	GQ244453	Unspecified	BK006335	Unspecified	XM001972967
36	*Drosophila*	*eremophila*	109208	DQ202053	109208	DQ202013	109208	DQ202093	Unspecified	JF736370
37	*Drosophila*	*euronotus*	----	----	15030	GU597484.1	----	----	----	----
38	*Drosophila*	*eurypeza*	109442	EU493653	109442	EU493782	109442	EU493529	----	----
39	*Drosophila*	*fraburu*	109373	EU493602	109373	EU493732	109373	EU493492	109373	O' Grady, P.
40	*Drosophila*	*fulvimacula*	Unspecified	JF736080	Unspecified	JF736120	Unspecified	JF736156	Unspecified	JF736361
41	*Drosophila*	*fulvimaculoides*	Unspecified	JF736060	Unspecified	JF736094	Unspecified	JF736134	Unspecified	JF736325
42	*Drosophila*	*fumipennis*	108511	EU493639	108511	EU493768	----	----	108511	O' Grady, P.
43	*Drosophila*	*funebris*	103952	EU493579	103952	EU493710	103952	EU493579	103952	O' Grady, P.
44	*Drosophila*	*gibberosa*	----	----	30029	EF468105	103960	EU493572	103960	O' Grady, P.
45	*Drosophila*	*grimshawi*	Unspecified	GU597459	Unspecified	GU597491	Unspecified	BK006341	Unspecified	O' Grady, P.
46	*Drosophila*	*guanche*	----	----	Unspecified	AF081354	Unspecified	EF216223	----	----
47	*Drosophila*	*guarani*	103966	EU493582	103966	EU493712	103966	EU493473	103966	O' Grady, P.
48	*Drosophila*	*guayllabambae*	Unspecified	JF736091	Unspecified	JF736131	Unspecified	JF736167	Unspecified	JF736380

49	*Drosophila*	*guttifera*	103968	EU493604	103968	EU493734	103968	EU493494	103968	O' Grady, P.
50	*Drosophila*	*haleakalae*	109330	EU493656	109330	EU493785	109330	EU493532	109330	AY348256
51	*Drosophila*	*hamatofila*	Unspecified	KC011819	Unspecified	KC011824	Unspecified	KC011834	Unspecified	KC011839
52	*Drosophila*	*huancavilcae*	Unspecified	KC011819	Unspecified	KC011824	Unspecified	KC011834	Unspecified	KC011839
53	*Drosophila*	*huaylasi*	Unspecified	KC011820	Unspecified	KC011825	Unspecified	KC011835	Unspecified	KC011840
54	*Drosophila*	*huichole*	109219	DQ202058	109219	DQ202018	109219	DQ202098	109219	JF736379
55	*Drosophila*	*hydei*	102059	DQ202060	102059	DQ202020	102059	DQ202100	102059	JF736328
56	*Drosophila*	*hystricosa*	109444	EU493659	109444	EU493916	109444	EU493534	504584	JQ413097
57	*Drosophila*	*immigrans*	103956	EU493586	103956	EU493716	103956	EU493477	103956	O' Grady, P.
58	*Drosophila*	*inca*	Unspecified	KC011821	Unspecified	KC011826	Unspecified	KC011836	Unspecified	KC011841
59	*Drosophila*	*iri*	109374	EU493601	109374	EU493731	109374	EU493491	109374	O' Grady, P.
60	*Drosophila*	*kambysellisi*	105683	EU493661	105683	EU493790	105683	EU493535	105683	O' Grady, P.
61	*Drosophila*	*kepulauana*	109407	EU493587	109407	EU493717	109407	EU493478	109407	O' Grady, P.
62	*Drosophila*	*kikkawai*	Unspecified	AF050746	OGS4	AY737608	OGS4	AY739953	unspecified	whole genome
63	*Drosophila*	*koepferae*	Unspecified	JF736061	Unspecified	JF736095	Unspecified	JF736135	Unspecified	whole genome
64	*Drosophila*	*kohkoa*	109399	EU493588	109399	EU493718	109399	EU493479	109399	O' Grady, P.
65	*Drosophila*	*lacertosa*	109370	EU493610	109370	EU493740	109370	EU493499	109370	O' Grady, P.
66	*Drosophila*	*leonis*	Unspecified	JF736062	Unspecified	JF736096	Unspecified	JF736136	Unspecified	JF736330
67	*Drosophila*	*littoralis*	Unspecified	NC011596	Unspecified	NC011596	Unspecified	NC011596	kemi96	EF635102
68	*Drosophila*	*longicornis*	Unspecified	DQ202061	Unspecified	DQ202021	Unspecified	DQ202101	Unspecified	JF736353
69	*Drosophila*	*machalilla*	E0035	KC011822	E0035	KC011827	E0035	KC011837	E0035	KC011842
70	*Drosophila*	*macroptera*	109393	EU493597	109393	EU493727	109393	EU493488	109393	O' Grady, P.
71	*Drosophila*	*mainlandi*	Unspecified	JX489217	102275	DQ202106	unspecified	AY739953	102275	EU341622
72	*Drosophila*	*malerkotliana*	105504	EU493627	105504	EU493756	105504	EU493512	105504	O' Grady, P.
73	*Drosophila*	*martensis*	Unspecified	JF736084	Unspecified	JF736125	unspecified	JF736160	Unspecified	JF736368
74	*Drosophila*	*mauritiana*	Unspecified	M57912	Unspecified	AF474081	unspecified	M57912	----	----
75	*Drosophila*	*mayaguana*	102279	DQ202067	102279	DQ202027	102279	DQ202107	102279	EU341623

76	*Drosophila*	*mediodiffusa*	109396	EU493616	109396	EU493745	109396	EU493505	109396	O' Grady, P.
77	*Drosophila*	*mediopictoides*	109395	EU493617	109395	EU493746	109395	EU493506	109395	O' Grady, P.
78	*Drosophila*	*mediostriata*	109394	EU493618	7269	AY847767	109394	EU493507	109394	O' Grady, P.
79	*Drosophila*	*melanica*	105499	EU493611	15030	EU390749	105499	EU493500	105499	O' Grady, P.
80	*Drosophila*	*melanogaster*	105503	EU493628	105503	EU493757	105503	EU493628	105503	O' Grady, P.
81	*Drosophila*	*melanoloma*	----	----	105708	EU493791	105708	EU493536	105708	O' Grady, P.
82	*Drosophila*	*mercatorum*	106304	EU493607	106304	EU493737	106304	EU493607	106304	JF736360
83	*Drosophila*	*meridiana*	Unspecified	JF736078	Unspecified	JF736118	Unspecified	JF736153	Unspecified	JF736357
84	*Drosophila*	*meridionalis*	109211	DQ202070	109211	DQ202030	109211	DQ202110	Unspecified	JF736372
85	*Drosophila*	*mettleri*	Unspecified	JF736063	Unspecified	JF736097	Unspecified	JF736137	Unspecified	JF736331
86	*Drosophila*	*microlabis*	----	----	Unspecified	EF216258	Unspecified	EF216231	----	----
87	*Drosophila*	*micromelanica*	109371	EU493612	109371	EU493741	109371	EU493501	109371	O' Grady, P.
88	*Drosophila*	*micromettleri*	Unspecified	JF736064	Unspecified	JF736098	Unspecified	JF736138	Unspecified	JF736332
89	*Drosophila*	*mimica*	205066	HQ170780	109331	EU493793	109331	EU493537	7270	AY348239
90	*Drosophila*	*miranda*	Unspecified	U51608	Unspecified	M95148	Unspecified	HQ110578	unspecified	whole genome
91	*Drosophila*	*mojavensis*	106302	EU493608	106302	EU493738	106302	EU493497	106302	EU341624
92	*Drosophila*	*moju*	Unspecified	JF736075	Unspecified	JF736112	Unspecified	JF736149	Unspecified	JF736347
93	*Drosophila*	*montana*	103959	EU493750	103959	EU493750	40370	DQ471461	40370	EF635103
94	*Drosophila*	*mulleri*	102305	EU341625	102305	DQ202032	102305	DQ202112	102305	EU341625
95	*Drosophila*	*multiciliata*	----	----	109439	EU493794	109439	EU493538	251469	AY348258
96	*Drosophila*	*nannoptera*	105440	EU493599	105440	EU493729	105440	EU493489	103845	JF736334
97	*Drosophila*	*nasuta*	103957	EU493589	103957	EU493719	103957	EU493589	NO	NO
98	*Drosophila*	*navojoa*	105433	EU493609	105433	EU493739	105433	EU493498	7232	EU341626
99	*Drosophila*	*nebulosa*	107549	EU493640	107549	EU532083	107549	EU493640	NO	NO
100	*Drosophila*	*neocordata*	107545	EU493636	107545	EU493765	30039	HQ110580	NO	NO
101	*Drosophila*	*neohypocausta*	109402	EU493590	109402	EU493720	109402	EU493481	NO	NO
102	*Drosophila*	*neorepleta*	102317	DQ202073	102317	DQ202033	102317	DQ202113	102317	JF736335

103	*Drosophila*	*nigella*	105820	EU493666	105820	EU493795	105820	EU493539	252916	AY348244
104	*Drosophila*	*nigra*	105821	EU493667	105821	EU493796	105821	EU493540	7272	AY348243
105	*Drosophila*	*nigricruria*	Unspecified	JF736067	Unspecified	JF736101	Unspecified	JF736141	Unspecified	JF736336
106	*Drosophila*	*nigrodumosa*	102319	EU341679	102319	JF736102	102319	EU341710	102319	EU341627
107	*Drosophila*	*nigrospiracula*	102321	DQ202074	102321	DQ202034	102321	DQ202114	102321	JF736337
108	*Drosophila*	*obscura*	Unspecified	GU220027	Unspecified	AF081356	Unspecified	EF216233	----	----
109	*Drosophila*	*ochracea*	----	----	109447	EU493797	109447	EU493668	----	----
110	*Drosophila*	*orena*	Unspecified	AY757281	Unspecified	AY757269	----	----	----	----
111	*Drosophila*	*ornatipennis*	103965	EU493573	103965	EU493704	103965	EU493467	103965	O' Grady, P.
112	*Drosophila*	*pachuca*	109212	DQ202078	109212	DQ202038	109212	DQ202118	109212	JF736373
113	*Drosophila*	*paramediostriata*	Unspecified	EF570013	Unspecified	AY162995	----	----	----	----
115	*Drosophila*	*paramelanica*	109372	EU493613	109372	EU493742	109372	EU493502	109372	O' Grady, P.
116	*Drosophila*	*paranaensis*	Unspecified	JF736088	Unspecified	JF736128	Unspecified	JF736164	Unspecified	JF736374
117	*Drosophila*	*pararubida*	109401	EU493591	109401	EU493721	109401	EU493482	109401	O' Grady, P.
118	*Drosophila*	*parisiena*	Unspecified	JF736068	Unspecified	JF736103	Unspecified	JF736142	Unspecified	JF736338
119	*Drosophila*	*paulistorum*	107546	EU493641	107546	EU493770	46793	HQ110581	107546	O' Grady, P.
120	*Drosophila*	*pectinitarsus*	----	----	109438	EU493798	109438	EU493542	109438	O' Grady, P.
121	*Drosophila*	*peninsularis*	Unspecified	JF736069	Unspecified	JF736104	Unspecified	JF736143	Unspecified	JF736339
122	*Drosophila*	*percnosoma*	200125	HQ170819	200125	HQ170715	200125	HQ170929	105685	O' Grady, P.
123	*Drosophila*	*persimilis*	MSH7	AF451101	Unspecified	M95143	Unspecified	EF216234	unspecified	O' Grady, P.
124	*Drosophila*	*polychaeta*	103958	EU493603	103958	EU493733	103958	EU493493	103958	O' Grady, P.
125	*Drosophila*	*pseudoananassae*	Unspecified	AY757280	Unspecified	AY757280	----	----	----	----
126	*Drosophila*	*pseudoobscura*	105505	EU493633	105505	EU493762	105505	EU493633	105505	O' Grady, P.
127	*Drosophila*	*pulaua*	109406	EU493592	109406	EU493722	109406	EU493483	109406	O' Grady, P.
128	*Drosophila*	*putrida*	103964	EU493615	103964	EU493744	103964	EU493504	103964	O' Grady, P.
129	*Drosophila*	*quinaria*	107542	EU493605	107542	EU493735	107542	EU493495	107542	O' Grady, P.
130	*Drosophila*	*repleta*	102340	EU341680	102340	JF736105	102340	EU341711	102340	EU341628

131	*Drosophila*	*rhopaloa*	unspecified	CONT8856	Unspecified	CONT23969	unspecified	CONT6279	Unspecified	whole genome
132	*Drosophila*	*richardsoni*	Unspecified	JF736070	Unspecified	JF736106	Unspecified	JF736144	Unspecified	JF736340
133	*Drosophila*	*ritae*	105431	DQ202082	105431	DQ202042	105431	DQ202122	105431	JF736354
134	*Drosophila*	*robusta*	103967	EU493614	Unspecified	GQ244457	103967	EU493503	103967	O' Grady, P.
135	*Drosophila*	*rubida*	109400	EU493593	109400	EU493723	109400	EU493484	109400	O' Grady, P.
136	*Drosophila*	*s.albostrigata*	109404	EU493595	109404	EU493725	109404	EU493486	109404	O' Grady, P.
137	*Drosophila*	*s.sulfurigaster*	109403	EU493596	109403	EU493726	109403	EU493487	109403	O' Grady, P.
138	*Drosophila*	*saltans*	Unspecified	AF045097	Unspecified	AF050741	Unspecified	HQ110585	----	----
139	*Drosophila*	*santomea*	Unspecified	JQ679120	156615	DQ382822	----	----	----	----
140	*Drosophila*	*sechelia*	Unspecified	M57908	Unspecified	GQ244459	Unspecified	M57908	GM25664	XM002030796
141	*Drosophila*	*serido*	Unspecified	JF736089	Unspecified	JF736129	Unspecified	JF736165	Unspecified	JF736376
142	*Drosophila*	*signata*	109405	EU493594	109405	EU493724	109405	EU493485	109405	O' Grady, P.
143	*Drosophila*	*simulans*	Unspecified	AF200844	Unspecified	AF200844	Unspecified	AF200844	105634	O' Grady, P.
144	*Drosophila*	*sonorae*	102346	DQ202084	102346	DQ202044	102346	DQ202124	102346	JF736341
145	*Drosophila*	*soonae*	109458	EU493672	109458	EU493801	109458	EU493544	----	----
146	*Drosophila*	*spenceri*	109217	DQ202087	109217	DQ202047	109217	DQ202127	109217	JF736377
147	*Drosophila*	*stalkeri*	102349	DQ202088	102349	DQ202048	102349	DQ202128	102349	JF736342
148	*Drosophila*	*starmeri*	Unspecified	JF736071	Unspecified	JF736107	Unspecified	JF736145	Unspecified	JF736343
149	*Drosophila*	*straubae*	Unspecified	JF736072	Unspecified	JF736108	Unspecified	JF736146	Unspecified	JF736344
150	*Drosophila*	*sturtevanti*	Unspecified	AY335205	14045	AF045082	Unspecified	HQ110595	----	----
151	*Drosophila*	*subbadia*	----	----	Unspecified	AY847772	----	----	----	----
152	*Drosophila*	*sucinea*	108510	EU493642	108510	EU532094	----	----	108510	O' Grady, P.
153	*Drosophila*	*tripunctata*	107541	EU493619	107541	EU493748	107541	EU493508	107541	O' Grady, P.
154	*Drosophila*	*tropicalis*	----	----	Unspecified	AF474103	----	----	----	----
155	*Drosophila*	*unipunctata*	109397	EU493620	109397	EU493749	109397	EU493509	109397	O' Grady, P.
156	*Drosophila*	*uniseta*	Unspecified	JF736074	Unspecified	JF736111	Unspecified	JF736148	Unspecified	JF736346
157	*Drosophila*	*venezolana*	106309	DQ202089	106309	DQ202049	106309	DQ202129	106309	JF736364

158	*Drosophila*	*virilis*	105500	EU493622	105500	EU493751	105500	EU493510	105500	JF736355
159	*Drosophila*	*wheeleri*	102367	EU341685	Unspecified	JF736110	102367	EU341705	102367	EU341616
160	*Drosophila*	*willistoni*	106322	EU493643	106322	EU493772	106322	EU493643	106322	O' Grady, P.
161	*Drosophila*	*yakuba*	Unspecified	NC001322	Unspecified	NC001322	Unspecified	NC001322	Unspecified	CM000159
162	*Drosophila*	*yangana*	Unspecified	KC011823	Unspecified	KC011828	Unspecified	KC011838	Unspecified	KC011843
163	*Drosophila*	*waddingtoni*	105687	HQ170825	105687	HQ170721	105687	HQ170935	105687	O' Grady, P.
164	*Scaptodrosophila*	*latifasciaformis*	105638	EU493684	105638	EU493813	105638	EU493553	105638	O' Grady, P.
165	*Scaptodrosophila*	*lebanonensis*	105639	EU493686	105639	EU493815	105639	EU493555	105639	O' Grady, P.
165	*Scaptodrosophila*	*pattersoni*	105497	EU493687	105497	EU493816	105497	EU493556	105497	O' Grady, P.
166	*Scaptomyza*	*hirtitibia*	109429	EU493658	109429	EU493915	109429	EU493533	109429	O' Grady, P.
167	*Scaptomyza*	*caliginosa*	105680	EU493676	105680	EU493805	----	----	105680	O' Grady, P.
168	*Scaptomyza*	*cyrtandrae*	109430	EU493678	109430	EU493807	109430	EU493548	109430	O' Grady, P.
169	*Scaptomyza*	*palmae*	106323	EU493550	106323	EU493809	106323	EU493680	106323	O' Grady, P.
170	*Zaprionus*	*badyi*	105640	EU493688	105640	EU493817	105640	EU493557	105640	O' Grady, P.
171	*Zaprionus*	*sepsoides*	105642	EU493690	105642	EU493819	105642	EU493559	105642	O' Grady, P.
172	*Zaprionus*	*tuberculatus*	105498	EU493691	105498	EU493820	105498	EU493560	105498	O' Grady, P.
173	*Zaprionus*	*indianus*	----	----	Unspecified	EF632396	----	----	----	----

Table S2. Source of 234 samples used in the search of Galileo. Numbers on brackets denote stocks from Drosophila Stock Center.

	Taxon	ID	Locatity	Country	Source
1	D. acanthoptera	G128	Huatulco	Mexico	Ruiz A.
2	D. aldrichi	G001	Zuata	Venezuela	Fontdevila A.
3	D. aldrichi	G032	Hatulco	Mexico	Etges W.
4	D. aldrichi	G036	Zapilote	Mexico	Etges W.
5	D. aldrichi	G099	Las Bocas	Mexico	Ruiz A.
6	D. aldrichi	G135	Punta Onah	Mexico	Ruiz A.
7	D. aldrichi	E011	Izhcayluma	Ecuador	Acurio A.
8	D. aldrichi	E022	San Jose	Ecuador	Acurio A.
9	D. americana	H5E34	Hurricane L.	USA	cracs.fc.up.pt
10	D. americana	W11E54	Wappapelo L.	USA	cracs.fc.up.pt
11	D. ananassae	BK006363	unknown	unknown	GenBank
12	D. ananassae	G048	unknown	unknown	[14024-0371.13]
13	D. ananassae	G071	Port-Louis	Mauritius	Cariou ML.
14	D. ananassae	G072	Tai 13-1610	unspecified	Cariou ML.
15	D. ananassae	G073	Borneo	Indonesia	Cariou ML.
16	D. ananassae	G074	Nago 181	Japan	Cariou ML.
17	D. ananassae	G075	Tahiti	France	Cariou ML.
18	D. ananassae	G076	Kirindy Forest	Madagascar	Cariou ML.
19	D. ananassae	G077	Kirindy Forest	Madagascar	Cariou ML.
20	D. ananassae	G078	Kirindy Forest	Madagascar	Cariou ML.
21	D. ananassae	G079	Monompana	Madagascar	Cariou ML.
22	D. ananassae	G080	Monompana	Madagascar	Cariou ML.
23	D. ananassae	G081	Monompana	Madagascar	Cariou ML.
24	D. ananassae	B129	unspecified	unspecified	Valente V.
25	D. anceps	G040	Michoacan	Mexico	[15081-1261.10]
26	D. aracataca	E037	Salango	Ecuador	Acurio A.
27	D. arizonae	G002	Tomatlan	Mexico	Heed C.

28	D. arizonae	G003	Punta Onah	Mexico	Etges W.
29	D. arizonae	G024	Punta Onah	Mexico	Etges W.
30	D. arizonae	G026	San Quintin	Mexico	Etges W.
31	D. arizonae	G028	Tomatlan	Mexico	Etges W.
32	D. arizonae	G029	Vaquerias	Mexico	Etges W.
33	D. arizonae	G095	Las Bocas	Mexico	Ruiz A.
34	D. arizonae	G096	El Choyudo	Mexico	Ruiz A.
35	D. arizonae	G098	Punta Onah	Mexico	Etges W.
36	D. austrosaltans	E024	San Antonio	Ecuador	Acurio A.
37	D. austrosaltans	E026	El Aromo	Ecuador	Acurio A.
38	D. bifurca	G122	El Tecolote	Mexico	Oliveira D.
39	D. bipectinata	KB463926	Chia	Taiwan	GenBank
40	D. bipectinata	G068	Katmandou	Nepal	Cariou ML.
41	D. bipectinata	G069	Myanmar	Myanmar	Cariou ML.
42	D. bipectinata	KB464248	Chia	Taiwan	GenBank
43	D. bipectinata	KB464408	Chia	Taiwan	GenBank
44	D. bipectinata	KB464390	Chia	Taiwan	GenBank
45	D. borborema	G041	Bahia	Brazil	[15081-1281.04]
46	D. buzzatii	B106	unspecified	unspecified	Valente V.
47	D. buzzatii	EU334685	unspecified	unspecified	GenBank
48	D. buzzatii	G012	Carboneras	Spain	Oliveira D.
49	D. buzzatii	G100	Guaritas	Brazil	Oliveira D.
50	D. buzzatii	G101	Trinkey	Australia	Oliveira D.
51	D. buzzatii	G102	Mazán	Argentina	Oliveira D.
52	D. buzzatii	G103	Wari	Peru	Oliveira D.
53	D. buzzatii	G104	Quilmes	Argentina	Oliveira D.
54	D. buzzatii	G105	Tichuco	Argentina	Oliveira D.
55	D. buzzatii	G106	Otamendi	Argentina	Oliveira D.
56	D. buzzatii	G107	Carboneras	Spain	Oliveira D.
57	D. buzzatii	G108	Carboneras	Spain	Oliveira D.
58	D. buzzatii	G109	Sardinia	Italy	Oliveira D.
59	D. buzzatii	G110	Carboneras	Spain	Oliveira D.

60	D. buzzatii	G111	Carboneras	Spain	Oliveira D.
61	D. capricorni	E003	Yangana	Ecuador	Acurio A.
62	D. capricorni	B130	Florianópolis	Brazil	Valente V.
63	D. cardini	E014	Islamar	Ecuador	Acurio A.
64	D. cardini	E016	Islamar	Ecuador	Acurio A.
65	D. cardini	B121	Itaqui	Brazil	Valente V.
66	D. desertorum	G119	Big Bend N.P.	USA	Oliveira D.
67	D. emarginata	E027	Mindo	Ecuador	Acurio A.
68	D. equinoxialis	B105	Mexico D.C.	Mexico	Valente V.
69	D. erecta	B125	unspecified	unspecified	Valente V.
70	D. erecta	G137	unspecified	unspecified	[14021-0224.01]
71	D. eremophila	G085	Las Bocas	Mexico	Ruiz A.
72	D. eremophila	G116	El Tecolote	Mexico	Oliveira D.
73	D. euronotus	S057	Tallahasee	USA	[15030-1131.01]
74	D. eurypeza	S055	Hawaii	USA	[15290-2581.00]
75	D. fulvimacula	G042	Veracruz	Mexico	Oliveira D.
76	D. fulvimacula	G118	los Tuxtlas B.S.	Mexico	Oliveira D.
77	D. funebris	B131	unspecified	unspecified	Valente V.
78	D. grimshawi	G138	Maui	USA	15287-2541.00]
79	D. guanche	G062	Mt Elgon	Kenya	Oliveira D.
80	D. guayllambae	E007	Islamar	Ecuador	Acurio A.
81	D. guayllambae	E018	Islamar	Ecuador	Acurio A.
82	D. guayllambae	E029	Guayllabamba	Ecuador	Acurio A.
83	D. hamatofila	G052	Superstition	USA	[15081-1301.07]
84	D. huancavilcae	E038	Manabi	Ecuador	Acurio A.
85	D. huaylasi	G004	Caraz	Peru	Oliveira D.
86	D. huaylasi	E040	Yangana	Ecuador	Acurio A.
87	D. huichole	G121	Zapotitlan	Mexico	Oliveira D.
88	D. hydei	G006	Pl. del Mercado	Cuba	Oliveira D.
89	D. hydei	G044	Sonora	Mexico	[15085-1641.67]
90	D. hydei	G092	Las Bocas	Mexico	Ruiz A.
91	D. hydei	G093	Punta Onah	Mexico	Ruiz A.

92	D. hydei	G094	El Choyudo	Mexico	Ruiz A..
93	D. hydei	B141	Florianópolis	Brazil	Valente V.
94	D. immigrans	E021	Izhcayluma	Ecuador	Acurio A.
95	D. inca	E012	Yangana	Ecuador	Acurio A.
96	D. inca	E017	Izhcayluma	Ecuador	Acurio A.
97	D. inca	E034	Yangana	Ecuador	Acurio A.
98	D. kikkawai	B144	unspecified	unspecified	Valente V.
99	D. koepferae	G005	Cébila	Argentina	Oliveira D.
100	D. leonis	G123	Ixtlan del Rio	Mexico	Oliveira D.
101	D. longicornis	G043	Tucson	USA	[15081-1311.20]
102	D. longicornis	E041	Guayllabamba	Ecuador	Acurio A.
103	D. machalilla	E035	San Jose	Ecuador	Acurio A.
104	D. mainlandi	G124	Catalina Is.	USA	Oliveira D.
105	D. malerkotliana	G065	unspecified	unspecified	Cariou ML.
106	D. malerkotliana	G066	318 A7	unspecified	Cariou ML.
107	D. malerkotliana	E009	Isla mar	Ecuador	Acurio A.
108	D. malerkotliana	E031	Mindo	Ecuador	Acurio A.
109	D. martensis	G007	Guaca	Venezuela	Oliveira D.
110	D. mauritiana	B100	unspecified	Mauritious	Valente V.
111	D. mayaguana	G011	Henderson P.	Jamaica	Oliveira D.
112	D. mediodiffusa	B123	Maricão	Puerto Rico	Valente V.
113	D. mediopictoides	B119	Boquete	Panama	Valente V.
114	D. mediopictoides	B145	Boquete	Panama	Valente V.
115	D. melanica	S050	Austin	USA	[15030-1141.03]
116	D. melanogaster	G112	Los Alamos	Mexico	Oliveira D.
117	D. melanogaster	G114	Las Bocas	Mexico	Oliveira D.
118	D. melanogaster	B113	Porto Alegre	Brazil	Valente V.
119	D. melanogaster	G139	unspecified	unspecified	[14021-0231.36]
120	D. mercatorum	G008	Comarada	Bolivia	Oliveira D.
121	D. mercatorum	G039	Tucson	USA	Oliveira D.
122	D. mercatorum	B111	Florianópolis	Brazil	Valente V.
123	D. mercatorum	G058	Palmira	Colombia	Oliveira D.

124	D. mercatorum	G059	Campo Grande	Brazil	Oliveira D.
125	D. meridiana	G056	Canal Zone	Panama	Oliveira D.
126	D. merina	G070	Reunion Island	France	Cariou ML.
127	D. mettleri	G038	Sonora	Mexico	Oliveira D.
128	D. mettleri	G127	El Choyudos	Mexico	Ruiz A.
129	D. microlabis	G061	unspecified	unspecified	Oliveira D.
130	D. micromelanica	S051	Smithville	USA	[15030-1151.01]
131	D. mimica	S056	Hawaii	USA	[15292-2561.08]
132	D. miranda	CM001516	Mt St. Helena	USA	GenBank
133	D. miranda	CM001519	Mt St. Helena	USA	GenBank
134	D. mojavensis	C	Catalina Island	USA	GenBank
135	D. mojavensis	D	Catalina Island	USA	GenBank
136	D. mojavensis	F	Catalina Island	USA	GenBank
137	D. mojavensis	X	Catalina Island	USA	GenBank
138	D. mojavensis	G009	Punta Onah	Mexico	Oliveira D.
139	D. mojavensis	G023	Catalina Island	USA	[15081-1352.22]
140	D. mojavensis	G031	Santiago	Mexico	Oliveira D.
141	D. mojavensis	G033	Punta Onah	Mexico	Oliveira D.
142	D. mojavensis	G034	Punta Onah	Mexico	Armella C.
143	D. mojavensis	G035	San Quintin	Mexico	Oliveira D.
144	D. mojavensis	G037	Providence	USA	Oliveira D.
145	D. mojavensis	G090	Punta Onah	Mexico	Ruiz A.
146	D. mojavensis	G091	El Choyudo	Mexico	Ruiz A.
147	D. mojavensis	G097	Las Bocas	Mexico	Ruiz A.
148	D. moju	E023	Yangana	Ecuador	Acurio A.
149	D. mulleri	G010	Panuco	Mexico	Richardson
150	D. nannoptera	G130	Joluxtla	Mexico	Oliveira D.
151	D. navojoa	G030	Chamela	Mexico	Oliveira D.
152	D. navojoa	G086	Las Bocas	Mexico	Ruiz A.
153	D. nebulosa	E008	Isla mar	Ecuador	Acurio A.
154	D. nebulosa	E010	San Jose	Ecuador	Acurio A.
155	D. nebulosa	B127	Porto Alegre	Brazil	Valente V.

156	D. neocardini	E025	Mindo	Ecuador	Acurio A.
157	D. neocardini	E036	San Antonio	Ecuador	Acurio A.
158	D. neocardini	B112	Ilha Campeche	Brazil	Valente V.
159	D. neoelliptica	B116	Joinville	Brazil	Valente V.
160	D. neorepleta	G057	Jalisco	Mexico	[15084-1601.07]
161	D. nigricruria	G126	Las Bocas	Mexico	Oliveira D.
162	D. nigricruria	E005	Yangana	Ecuador	Acurio A.
163	D. nigricruria	E020	Izhcayluma	Ecuador	Acurio A.
164	D. nigrospiracula	G084	Punta Onah	Mexico	Ruiz A.
165	D. obscura	G063	Canary Islands	Spain	Oliveira D.
166	D. orena	B139	unspecified	unspecified	Valente V.
167	D. pallidipennis	E015	San Antonio	Ecuador	Acurio A.
168	D. pallidipennis	B115	Joinville	Brazil	Valente V.
169	D. paramediostriata	B137	Porto Alegre	Brazil	Valente V.
170	D. paranaensis	G054	Chiapas	Mexico	[15082-1541.10]
171	D. paranaensis	E006	Ayampe	Ecuador	Acurio A.
172	D. paulistorum	B114	Ribeirão Preto	Brazil	Valente V.
173	D. peninsularis	G055	Bath	Jamaica	[15081-1401.05]
174	D. persimilis	G050	Mt St. Helena	USA	[14011-0111.49]
175	D. persimilis	BK0063	Mt St. Helena	USA	GenBank
176	D. polychaeta	S052	Hawaii	USA	[15100-1711.04]
177	D. polymorpha	B126	Florianópolis	Brazil	Valente V.
178	D. promeridiana	G014	Dagua	Colombia	Oliveira D.
179	D. promeridiana	E039	San Antonio	Ecuador	Acurio A.
180	D. pseudoananassae	G064	unspecified	unspecified	Cariou ML.
181	D. pseudoobscura	BK0063	Mesa Verde	USA	GenBank
182	D. pseudoobscura	G049	Mesa Verde	USA	[14011-0121.94]
183	D. pseudoobscura	G087	Punta Onah	Mexico	Oliveira D.
184	D. pseudoobscura	B138	Mesa Verde	USA	Valente V.
185	D. putrida	S054	Chadron	USA	[15150-2101.00]
186	D. repleta	G022	unspecified	unspecified	Oliveira D.
187	D. rhopaloa	KB450190	unspecified	Vietnam	GenBank

188	D. rhopaloa	KB452607	unspecified	Vietnam	GenBank
189	D. richardsoni	G045	Tortola Islands	U.K.	Oliveira D.
190	D. ritae	G047	Puebla	Mexico	Oliveira D.
191	D. robusta	G134	Chadron	USA	Oliveira D.
192	D. robusta	B107	unspecified	unspecified	Valente V.
193	D. saltans	B142	unspecified	unspecified	Valente V.
194	D. santomea	B103	unspecified	São Tomé & Príncipe	Valente V.
195	D. sechellia	G140	Cousin Island	Seychelles	[14021-0248.25]
196	D. simulans	G113	Los Alamos	Mexico	Oliveira D.
197	D. simulans	G115	Las Bocas	Mexico	Oliveira D.
198	D. simulans	E004	Isla mar	Ecuador	Acurio A.
199	D. simulans	B118	Solis	Uruguay	Valente V.
200	D. simulans	G141	unspecified	unspecified	[14021-0251.195]
201	D. spenceri	G089	Las Bocas	Mexico	Oliveira D.
202	D. spenceri	G120	Infiernillo	Mexico	Oliveira D.
203	D. stalkeri	G015	St. Petersburg	USA	Oliveira D.
204	D. starmeri	G016	Rio Hacha	Colombia	Oliveira D.
205	D. straubae	G017	Port Henderson	Jamaica	Oliveira D.
206	D. straubae	G046	Sigus Beach	Cuba	Oliveira D.
207	D. sturtevanti	E042	San Antonio	Ecuador	Acurio A.
208	D. sturtevanti	B101	Florianópolis	Brazil	Valente V.
209	D. subbadia	B140	El Naranjo	Mexico	Valente V.
210	D. sucinea	E033	Mindo	Ecuador	Acurio A.
211	D. sucinea	B102	Mexico DC	Mexico	Valente V.
212	D. teissieri	B124	unspecified	unspecified	Valente V.
213	D. tripunctata	E013	Madison	USA	Acurio A.
214	D. tropicalis	B108	unspecified	El Salvador	Valente V.
215	D. uniseta	G018	Salamanca	Colombia	Oliveira D.
216	D. venezolana	G020	Los Roques	Venezuela	Cerda
217	D. virilis	G019	unspecified	unspecified	[15010-1015.87]
218	D. virilis	B117	Bowling Green	USA	Valente V.
219	D. virilis	BK6359	unspecified	unspecified	GenBank

220	D. wassermani	G129	Infiernillo	Mexico	Heed W.
221	D. wheeleri	G021	Ejido	Mexico	Oliveira D.
222	D. wheeleri	G025	Punta Onah	Mexico	Oliveira D.
223	D. wheeleri	G027	Catalina Is.	USA	Oliveira D.
224	D. willistoni	G051	Guadaloupe Is.	France	[14030-0811.24]
225	D. willistoni	E028	Islamar	Ecuador	Acurio A.
226	D. willistoni	E032	Mindo	Ecuador	Acurio A.
227	D. willistoni	B109	unspecified	unspecified	Valente V.
228	D. willistoni	BK6360	Guadaloupe Is.	France	GenBank
229	D. yakuba	G143	unspecified	Ivory Coast	[14021-0261.01]
230	D. yangana	E030	Yangana	Ecuador	Acurio A.
231	S. latiefasciaeformis	B135	unspecified	unspecified	Valente V.
232	Z. indianus	E019	Yangana	Ecuador	Acurio A.
233	Z. indianus	E043	Izhcayluma	Ecuador	Acurio A.
234	Z. tuberculatus	B122	unspecified	unspecified	Valente V.

Table S3. Results obtained in the search of Galileo TE in 234 samples from 110 drosophilid species.

	Taxon	ID	detection (primers/contig)	sequence length (bp)	functional TPase	observations
1	*D. acanthoptera*	G128	no detected	NA	NA	NA
2	*D. aldrichi*	G001	PCR (1-3)	975	yes	325 aa
3	*D. aldrichi*	G032	PCR (5-6)	356	No	same as G036
4	*D. aldrichi*	G036	PCR (5-6) PCR (1-3)	356 953	No	same as G099
5	*D. aldrichi*	G099	PCR (5-6, 1-3)	356,974	No	deletion, mutation
6	*D. aldrichi*	G135	PCR (1-3)	974		mutation
7	*D. aldrichi*	E011	PCR (5-6, 1-3)	381,944	yes, no	127 aa, mutation
8	*D. aldrichi*	E022	PCR (5-6,1-3)	380,867	No	deletion
9	*D. americana*	H5E34	*in silico* (3485)	409	No	mutation
10	*D. americana*	W11E54	*in silico* (5443)	409	No	mutation
11	*D. ananassae*	BK006363	*in silico* (15556)*	531	yes	176 aa
12	*D. ananassae*	G048	PCR (7-8)	484	No	sequenced stock
13	*D. ananassae*	G071	PCR (7-8)	531	yes	176 aa
14	*D. ananassae*	G072	PCR (7-8)	387	yes	125 aa, intron
15	*D. ananassae*	G073	PCR (7-8)	387	yes	125 aa, intron
16	*D. ananassae*	G074	PCR (7-8)	531	yes	176 aa
17	*D. ananassae*	G075	PCR (7-8)	531	yes	176 aa
18	*D. ananassae*	G076	PCR (7-8)	531	yes	176 aa
19	*D. ananassae*	G077	PCR (7-8)	531	yes	176 aa
20	*D. ananassae*	G078	PCR (7-8)	531	yes	same as BK006363
21	*D. ananassae*	G079	PCR (7-8)	531	yes	176 aa
22	*D. ananassae*	G080	PCR (7-8)	531	yes	176 aa
23	*D. ananassae*	G081	PCR (7-8)	531	yes	same than G080
24	*D. ananassae*	B129	PCR (7-8)	531	yes	176 aa
25	*D. anceps*	G040	No detected	NA	NA	NA

26	*D. aracataca*	E037	No detected	NA	NA	NA
27	*D. arizonae*	G002	PCR (5-6, 1-3)	382, 975	no,yes	325 aa
28	*D. arizonae*	G003	PCR (5-6, 1-3)	380, 975	No	325 aa
29	*D. arizonae*	G024	PCR (5-6, 1-3)	381, 975	yes	127, 325 aa
30	*D. arizonae*	G026	PCR (5-6, 1-3)	381, 960	yes	same as G098,G095
31	*D. arizonae*	G028	PCR (5-6, 1-3)	380, 975	No	325 aa
32	*D. arizonae*	G029	PCR (5-6, 1-3)	381, 975	yes	same as G096,G098
33	*D. arizonae*	G095	PCR (5-6, 1-3)	381, 975	yes	same as G098
34	*D. arizonae*	G096	PCR (5-6, 1-3)	382, 975	no,yes	325 aa
35	*D. arizonae*	G098	PCR (5-6, 1-3)	381, 975	yes	127, 325 aa
36	*D. austrosaltans*	E024	PCR (11-12)	490	yes	same than E026
37	*D. austrosaltans*	E026	PCR (11-12)	490	yes	163 aa
38	*D. bifurca*	G122	no detected	NA	NA	NA
39	*D. bipectinata*	KB463926	*in silico* (459199271)	531	yes	176 aa
40	*D. bipectinata*	G068	No detected	NA	NA	NA
41	*D. bipectinata*	G069	No detected	NA	NA	NA
42	*D. bipectinata*	KB464248	*in silico* (459198949)	533	No	NA
43	*D. bipectinata*	KB464408	*in silico* (459198789)	531	No	NA
44	*D. bipectinata*	KB464390	*in silico* (459198807)	531	yes	176 aa
45	*D. borborema*	G041	PCR (5-6, 1-3)	333, 972	no	deletion, mutation
46	*D. buzzatii*	B106	PCR (5-6, 1-3)	975	No	mutation, (5-6) same as G100
47	*D. buzzatii*	EU334685	*in silico**	381	No	mutation
48	*D. buzzatii*	G012	PCR (5-6, 1-3)	381, 975	yes, no	127 aa, mutation
49	*D. buzzatii*	G100	PCR (5-6)	381	yes	127 aa
50	*D. buzzatii*	G101	PCR (5-6)	381	yes	127 aa
51	*D. buzzatii*	G102	PCR (5-6, 1-3)	381, 975	yes, no	127 aa, mutation
52	*D. buzzatii*	G103	PCR (5-6)	381	yes	127 aa
53	*D. buzzatii*	G104	PCR (5-6, 1-3)	381, 975	yes	127aa, 325 aa
54	*D. buzzatii*	G105	PCR (5-6)	381	yes	127 aa
55	*D. buzzatii*	G106	PCR (5-6, 1-3)	262, 975	no,yes	mutation,325 aa
56	*D. buzzatii*	G107	PCR (5-6, 1-3)	381, 975	yes, no	same as G108
57	*D. buzzatii*	G108	PCR (5-6, 1-3)	381, 975	yes, no	same as G012

58	*D. buzzatii*	G109	PCR (5-6, 1-3)	381, 975	no,yes	mutation,325 aa
59	*D. buzzatii*	G110	PCR (5-6, 1-3)	381, 975	no,yes	mutation,325 aa
60	*D. buzzatii*	G111	PCR (5-6, 1-3)	381, 975	yes, no	same as G012
61	*D. capricorni*	E003	PCR (11-12)	490	yes	same as B130
62	*D. capricorni*	B130	PCR (11-12)	490	yes	163 aa
63	*D. cardini*	E014	No detected	NA	NA	NA
64	*D. cardini*	E016	No detected	NA	NA	NA
65	*D. cardini*	B121	No detected	NA	NA	NA
66	*D. desertorum*	G119	No detected	NA	NA	NA
67	*D. emarginata*	E027	No detected	NA	NA	NA
68	*D. equinoxialis*	B105	PCR (11-12)	490	no	deletion
69	*D. erecta*	B125	No detected	NA	NA	NA
70	*D. erecta*	G137	No detected	NA	NA	sequenced stock
71	*D. eremophila*	G085	No detected	NA	NA	NA
72	*D. eremophila*	G116	No detected	NA	NA	NA
73	*D. euronotus*	S057	No detected	NA	NA	NA
74	*D. eurypeza*	S055	No detected	NA	NA	NA
75	*D. fulvimacula*	G042	PCR (5-6)	381	No	mutation
76	*D. fulvimacula*	G118	PCR (5-6)	381	No	same as G042
77	*D. funebris*	B131	No detected	NA	NA	NA
78	*D. grimshawi*	G138	No detected	NA	NA	sequenced stock
79	*D. guanche*	G062	no detected	NA	NA	NA
80	*D. guayllambae*	E007	no detected	NA	NA	NA
81	*D. guayllambae*	E018	no detected	NA	NA	NA
82	*D. guayllambae*	E029	no detected	NA	NA	NA
83	*D. hamatofila*	G052	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
84	*D. huancavilcae*	E038	no detected	NA	NA	NA
85	*D. huaylasi*	G004	PCR (5-6)	381	yes	same as E040
86	*D. huaylasi*	E040	PCR (5-6, 1-3)	381, 944	yes,no	127 aa, mutation
87	*D. huichole*	G121	no detected	NA	NA	NA
88	*D. hydei*	G006	No detected	NA	NA	NA
89	*D. hydei*	G044	No detected	NA	NA	NA

90	*D. hydei*	G092	No detected	NA	NA	NA
91	*D. hydei*	G093	No detected	NA	NA	NA
92	*D. hydei*	G094	No detected	NA	NA	NA
93	*D. hydei*	B141	No detected	NA	NA	NA
94	*D. immigrans*	E021	No detected	NA	NA	NA
95	*D. inca*	E012	No detected	NA	NA	NA
96	*D. inca*	E017	No detected	NA	NA	NA
97	*D. inca*	E034	No detected	NA	NA	NA
98	*D. kikkawai*	B144	PCR (7-8)	531	No	deletion
99	*D. koepferae*	G005	PCR (5-6)	381	yes	127 aa
100	*D. leonis*	G123	PCR (1-3)	975	yes	325 aa
101	*D. longicornis*	G043	PCR (5-6)	381	yes	127 aa
102	*D. longicornis*	E041	PCR (5-6)	381	yes	128 aa
103	*D. machalilla*	E035	No detected	NA	NA	NA
104	*D. mainlandi*	G124	PCR (5-6, 1-3)	378, 963	no	insertion, deletion
105	*D. malerkotliana*	G065	PCR (7-8)	522	yes	173 aa
106	*D. malerkotliana*	G066	PCR (7-8)	522	No	mutation
107	*D. malerkotliana*	E009	PCR (7-8)	531	No	same as E031
108	*D. malerkotliana*	E031	PCR (7-8)	531	No	mutation
109	*D. martensis*	G007	PCR (5-6, 1-3)	380	No	deletion
110	*D. mauritiana*	B100	No detected	NA	NA	NA
111	*D. mayaguana*	G011	PCR (1-3)	975	yes	325 aa
112	*D. mediodiffusa*	B123	No detected	NA	NA	NA
113	*D. mediopictoides*	B119	PCR (11-12)	490	yes	163 aa
114	*D. mediopictoides*	B145	PCR (11-12)	490	yes	163 aa
115	*D. melanica*	S050	No detected	NA	NA	NA
116	*D. melanogaster*	G112	No detected	NA	NA	NA
117	*D. melanogaster*	G114	No detected	NA	NA	NA
118	*D. melanogaster*	B113	No detected	NA	NA	NA
119	*D. melanogaster*	G139	No detected	NA	NA	sequenced stock
120	*D. mercatorum*	G008	PCR (5-6)	381	yes	127 aa
121	*D. mercatorum*	G039	PCR (5-6)	381	yes	127 aa

122	*D. mercatorum*	B111	PCR (5-6)	381	yes	same as G059
123	*D. mercatorum*	G058	PCR (5-6)	381	yes	same as G008, G058
124	*D. mercatorum*	G059	PCR (5-6)	381	yes	128 aa
125	*D. meridiana*	G056	PCR (5-6)	361	no	deletion
126	*D. merina*	G070	No detected	NA	NA	NA
127	*D. mettleri*	G038	No detected	NA	NA	NA
128	*D. mettleri*	G127	No detected	NA	NA	NA
129	*D. microlabis*	G061	No detected	NA	NA	NA
130	*D. micromelanica*	S051	No detected	NA	NA	NA
131	*D. mimica*	S056	No detected	NA	NA	NA
132	*D. miranda*	CM001516	*in silico* (480995225)	511	No	deletion
133	*D. miranda*	CM001519	*in silico* (480995219)	509	No	deletion
134	*D. mojavensis*	C	*in silico* (10758)*	381	yes	127 aa
135	*D. mojavensis*	D	*in silico* (9930)*	381	yes	127 aa
136	*D. mojavensis*	F	*in silico* (10369)*	381	yes	127 aa
137	*D. mojavensis*	X	*in silico* (10924)*	381	yes	128 aa
138	*D. mojavensis*	G009	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
139	*D. mojavensis*	G023	PCR (5-6, 1-3)	381, 975	yes	sequenced stock
140	*D. mojavensis*	G031	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
141	*D. mojavensis*	G033	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
142	*D. mojavensis*	G034	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
143	*D. mojavensis*	G035	PCR (5-6, 1-3)	381, 971	yes,no	127 aa
144	*D. mojavensis*	G037	PCR (5-6, 1-3)	381, 971	yes,no	127 aa
145	*D. mojavensis*	G090	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
146	*D. mojavensis*	G091	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
147	*D. mojavensis*	G097	PCR (5-6, 1-3)	381, 975	yes	127,325 aa
148	*D. moju*	E023	No detected	NA	NA	NA
149	*D. mulleri*	G010	PCR (5-6, 1-3)	381, 955	No	mutation, deletion
150	*D. nannoptera*	G130	No detected	NA	NA	NA
151	*D. navojoa*	G030	PCR (1-3)	975	yes	325 aa
152	*D. navojoa*	G086	PCR (1-3)	873	no	deletion
153	*D. nebulosa*	E008	PCR (11-12)	483	no	same as E010

154	*D. nebulosa*	E010	PCR (11-12)	483	no	deletion
155	*D. nebulosa*	B127	PCR (11-12)	485	no	deletion
156	*D. neocardini*	E025	No detected	NA	NA	NA
157	*D. neocardini*	E036	No detected	NA	NA	NA
158	*D. neocardini*	B112	No detected	NA	NA	NA
159	*D. neoelliptica*	B116	No detected	NA	NA	NA
160	*D. neorepleta*	G057	PCR (5-6)	381	yes	127 aa
161	*D. nigricruria*	G126	PCR (1-3)	948	yes	316 aa
162	*D. nigricruria*	E005	PCR (5-6)	381	yes	128 aa
163	*D. nigricruria*	E020	PCR (1-3)	833	no	deletion
164	*D. nigrospiracula*	G084	No detected	NA	NA	NA
165	*D. obscura*	G063	No detected	NA	NA	NA
166	*D. orena*	B139	No detected	NA	NA	NA
167	*D. pallidipennis*	E015	No detected	NA	NA	NA
168	*D. pallidipennis*	B115	No detected	NA	NA	NA
169	*D. paramediostriata*	B137	PCR (11-12)	490	yes	163 aa
170	*D. paranaensis*	G054	PCR 5-6	381	yes	127 aa
171	*D. paranaensis*	E006	PCR 5-6	381	yes	same as G054
172	*D. paulistorum*	B114	PCR (11-12)	476	no	deletion
173	*D. peninsularis*	G055	No detected	NA	NA	NA
174	*D. persimilis*	G050	no detected	NA	NA	sequenced stock
175	*D. persimilis*	BK0063	*in silico* (7729)*	502	yes	168 aa
176	*D. polychaeta*	S052	No detected	NA	NA	NA
177	*D. polymorpha*	B126	No detected	NA	NA	NA
178	*D. promeridiana*	G014	No detected	NA	NA	NA
179	*D. promeridiana*	E039	No detected	NA	NA	NA
180	*D. pseudoananassae*	G064	PCR (7-8)	537	no	deletion
181	*D. pseudoobscura*	BK0063	in silico (3151)*	511	no	mutation
182	*D. pseudoobscura*	G049	no detected	no	no	sequenced stock
183	*D. pseudoobscura*	G087	no detected	NA	NA	NA
184	*D. pseudoobscura*	B138	no detected	NA	NA	NA
185	*D. putrida*	S054	No detected	NA	NA	NA

186	*D. repleta*	G022	No detected	NA	NA	NA
187	*D. rhopaloa*	KB450190	*in silico* (452190070)	509	no	deletion
188	*D. rhopaloa*	KB452607	*in silico* (452187653)	488	no	deletion
189	*D. richardsoni*	G045	PCR (5-6)	381	yes	127 aa
190	*D. ritae*	G047	PCR (1-3)	870	no	deletion
191	*D. robusta*	G134	No detected	NA	NA	NA
192	*D. robusta*	B107	No detected	NA	NA	NA
193	*D. saltans*	B142	PCR (11-12)	472	no	deletion
194	*D. santomea*	B103	No detected	NA	NA	NA
195	*D. sechellia*	G140	No detected	NA	NA	sequenced stock
196	*D. simulans*	G113	No detected	NA	NA	NA
197	*D. simulans*	G115	No detected	NA	NA	NA
198	*D. simulans*	E004	No detected	NA	NA	NA
199	*D. simulans*	B118	No detected	NA	NA	NA
200	*D. simulans*	G141	No detected	NA	NA	sequenced stock
201	*D. spenceri*	G089	No detected	NA	NA	NA
202	*D. spenceri*	G120	No detected	NA	NA	NA
203	*D. stalkeri*	G015	PCR (5-6)	375	no	deletion
204	*D. starmeri*	G016	PCR (1-3)	975	yes	325 aa
205	*D. straubae*	G017	No detected	NA	NA	NA
206	*D. straubae*	G046	No detected	NA	NA	NA
207	*D. sturtevanti*	E042	PCR (11-12)	492	no	insertion
208	*D. sturtevanti*	B101	PCR (11-12)	472	no	deletion
209	*D. subbadia*	B140	PCR (11-12)	490	yes	163 aa
210	*D. sucinea*	E033	PCR (11-12)	488	no	same as B102
211	*D. sucinea*	B102	PCR (11-12)	488	no	deletion
212	*D. teissieri*	B124	No detected	NA	NA	NA
213	*D. tripunctata*	E013	No detected	NA	NA	NA
214	*D. tropicalis*	B108	PCR (11-12)	490	yes	163 aa
215	*D. uniseta*	G018	PCR (1-3)	967	no	mutation
216	*D. venezolana*	G020	PCR (1-3)	975	yes	325 aa
217	*D. virilis*	G019	no detected	NA	NA	sequenced stock

218	*D. virilis*	B117	no detected	NA	NA	NA
219	*D. virilis*	BK6359	*in silico* (16409)*	413	no	mutation
220	*D. wassermani*	G129	No detected	NA	NA	NA
221	*D. wheeleri*	G021	PCR (5-6, 1-3)	317, 974	yes, no	same as G025
222	*D. wheeleri*	G025	PCR (5-6, 1-3)	317, 974	yes,no	105 aa, mutation
223	*D. wheeleri*	G027	PCR (5-6, 1-3)	370, 975	yes	deletion, 325 aa
224	*D. willistoni*	G051	PCR (11-12)	490	yes	sequenced stock
225	*D. willistoni*	E028	PCR (11-12)	488	no	deletion
226	*D. willistoni*	E032	PCR (11-12)	490	yes	163 aa, same as BK006360
227	*D. willistoni*	B109	PCR (11-12)	490	yes	same as E032
228	*D. willistoni*	BK6360	*In silico* (10048)*	490	yes	163 aa
229	*D. yakuba*	G143	No detected	NA	NA	sequenced stock
230	*D. yangana*	E030	No detected	NA	NA	NA
231	*S. latiefasciaeformis*	B135	No detected	NA	NA	NA
232	*Z. indianus*	E019	No detected	NA	NA	NA
233	*Z. indianus*	E043	No detected	NA	NA	NA
234	*Z. tuberculatus*	B122	No detected	NA	NA	NA

Table S4. Significant hits retrieved from the *in silico* search of Galileo in the newly released *Drosophila* genomes. Searches were performed with a significance threshold of E-value = $\leq 10^{-3}$ for nucleotides.

Genome (Taxon ID)	Query	GenBank ID	Scaffold location	Identity	BLAST Score	E-value
1. D. bipectinata (42026)	Motif TPase GAL/ananassae	KB463974.1	7180000395832	376 / 395 (95.2%)	319	1 E-186
2. D. elegans (30023)	TIR GAL/ananassae	KB458613.1	7180000491255	337 / 373 (90.3%)	229	9 E-191
3. D. kikkawai (30033)	Motif TPase GAL/ananassae	KB459701.1	7180000302486	337 / 391 (86.2%)	175	1 E-99
4. D. rhopaloa (1041015)	Motif TPase GAL/ananassae	KB452318.1	7180000779902	380 / 396 (96%)	332	0
5. D. miranda (7229)	Motif TPase GAL/obscura	CM001517.2	chromosome XR	396 / 397 (99.7%)	393	0
6. D. americana (40366)	Motif TPase GAL/obscura	W11E_5443	ND	389/413 (94%)	316	0

Table S5. Estimates of d_S values for Galileo and 1360 element data set. Average values between 10 species groups of 51 *Drosophila* species. Values calculated on MEGA V4 based in the pairwise analysis of 152 sequences in a dataset of 426 positions, gaps and missing data were eliminated by pairwise deletion.Values above the diagonal show standard errors obtained by bootstrap procedure (500 replicates).

	1	2	3	4	5	6	7	8	9	10	11
[1] 1360		[0.120]	[0.097]	[0.127]	[0.159]	[0.108]	[0.112]	[0.112]	[0.106]	[0.108]	[0.134]
[2] repleta	0.577		[0.098]	[0.096]	[0.089]	[0.104]	[0.108]	[0.098]	[0.103]	[0.107]	[0.116]
[3] ananassae	0.448	0.448		[0.062]	[0.041]	[0.100]	[0.105]	[0.106]	[0.106]	[0.111]	[0.110]
[4] montium	0.509	0.448	0.188		[0.051]	[0.105]	[0.109]	[0.128]	[0.113]	[0.145]	[0.161]
[5] melanogaster	0.608	0.412	0.132	0.105		[0.123]	[0.109]	[0.114]	[0.117]	[0.113]	[0.125]
[6] obscura	0.514	0.499	0.412	0.414	0.479		[0.108]	[0.089]	[0.088]	[0.091]	[0.086]
[7] virilis	0.452	0.498	0.419	0.382	0.388	0.465		[0.076]	[0.073]	[0.080]	[0.094]
[8] saltans	0.561	0.501	0.484	0.529	0.494	0.408	0.315		[0.035]	[0.045]	[0.056]
[9] willistoni	0.520	0.501	0.459	0.459	0.505	0.405	0.301	0.123		[0.045]	[0.059]
[10] guarani	0.508	0.490	0.455	0.547	0.457	0.360	0.299	0.138	0.148		[0.065]
[11] tripunctata	0.587	0.523	0.455	0.618	0.491	0.369	0.338	0.176	0.194	0.181	

Table S6. Estimates of d_S values for COI data set. Average values between 28 species groups of Drosophilidae. Values calculated on MEGA V4 based in the pairwise analysis of 157 sequences in a dataset of 367 positions, gaps and missing data were eliminated by pairwise deletion. Values above the diagonal show standard errors obtained by bootstrap procedure (500 replicates).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28]
[1] Hawaiians		0.08	0.05	0.06	0.07	0.10	0.06	0.06	0.07	0.06	0.08	0.06	0.06	0.05	0.06	0.08	0.05	0.06	0.06	0.08	0.06	0.04	0.06	0.06	0.07	0.05	0.06	0.06
[2] Scaptodrosophila	0.63		0.08	0.07	0.06	0.11	0.09	0.07	0.11	0.09	0.11	0.08	0.07	0.08	0.07	0.07	0.08	0.07	0.07	0.10	0.10	0.06	0.09	0.08	0.08	0.06	0.09	0.07
[3] Scaptomyza	0.45	0.59		0.07	0.08	0.09	0.08	0.07	0.07	0.06	0.08	0.07	0.06	0.07	0.07	0.09	0.06	0.06	0.05	0.09	0.07	0.05	0.08	0.07	0.08	0.05	0.08	0.06
[4] Zaprionus	0.46	0.51	0.46		0.08	0.13	0.07	0.08	0.09	0.07	0.09	0.07	0.07	0.07	0.06	0.08	0.06	0.06	0.06	0.09	0.09	0.05	0.08	0.06	0.08	0.07	0.09	0.06
[5] ananassae	0.56	0.46	0.55	0.48		0.12	0.08	0.08	0.09	0.09	0.09	0.10	0.07	0.09	0.06	0.06	0.08	0.06	0.07	0.10	0.08	0.05	0.08	0.08	0.07	0.06	0.07	0.06
[6] annulimana	0.70	0.74	0.58	0.71	0.64		0.12	0.09	0.13	0.10	0.15	0.09	0.09	0.09	0.12	0.11	0.09	0.09	0.10	0.12	0.10	0.08	0.10	0.09	0.10	0.09	0.10	0.09
[7] atalaia	0.45	0.56	0.51	0.34	0.43	0.64		0.07	0.10	0.08	0.10	0.08	0.08	0.08	0.09	0.09	0.09	0.07	0.07	0.10	0.08	0.06	0.08	0.07	0.10	0.07	0.08	0.09
[8] calloptera	0.44	0.50	0.41	0.40	0.42	0.48	0.36		0.10	0.06	0.09	0.08	0.06	0.07	0.09	0.10	0.07	0.08	0.08	0.08	0.09	0.06	0.08	0.07	0.08	0.07	0.07	0.07
[9] canalinea	0.52	0.65	0.41	0.44	0.49	0.69	0.50	0.51		0.09	0.11	0.09	0.08	0.09	0.06	0.09	0.08	0.06	0.08	0.11	0.11	0.07	0.09	0.08	0.07	0.07	0.10	0.07
[10] cardini	0.48	0.60	0.44	0.40	0.54	0.55	0.44	0.31	0.50		0.09	0.08	0.06	0.07	0.07	0.09	0.06	0.07	0.07	0.09	0.07	0.06	0.08	0.07	0.07	0.06	0.07	0.08
[11] funebris	0.57	0.67	0.50	0.49	0.51	0.74	0.52	0.48	0.57	0.53		0.10	0.09	0.08	0.08	0.11	0.09	0.08	0.10	0.12	0.10	0.08	0.11	0.10	0.09	0.10	0.12	0.09
[12] guarani	0.46	0.53	0.45	0.37	0.53	0.50	0.39	0.37	0.45	0.44	0.55		0.07	0.06	0.08	0.09	0.07	0.06	0.06	0.07	0.08	0.05	0.09	0.06	0.09	0.07	0.08	0.07
[13] immigrans	0.59	0.60	0.52	0.47	0.49	0.61	0.53	0.41	0.54	0.43	0.63	0.50		0.08	0.07	0.08	0.06	0.07	0.06	0.08	0.09	0.05	0.08	0.07	0.07	0.05	0.07	0.07
[14] melanica	0.52	0.65	0.49	0.45	0.57	0.54	0.50	0.47	0.56	0.49	0.51	0.38	0.64		0.06	0.07	0.06	0.06	0.06	0.10	0.08	0.05	0.08	0.06	0.07	0.06	0.08	0.06
[15] melanogaster	0.60	0.54	0.56	0.41	0.43	0.76	0.57	0.56	0.38	0.51	0.52	0.50	0.58	0.51		0.05	0.06	0.06	0.07	0.09	0.08	0.05	0.08	0.06	0.06	0.06	0.08	0.06
[16] montium	0.65	0.51	0.62	0.51	0.38	0.67	0.53	0.58	0.51	0.53	0.65	0.52	0.59	0.54	0.44		0.07	0.06	0.07	0.09	0.11	0.06	0.10	0.09	0.07	0.07	0.09	0.06
[17] nanoptera	0.52	0.59	0.47	0.44	0.55	0.57	0.54	0.41	0.52	0.41	0.58	0.42	0.53	0.49	0.51	0.53		0.06	0.06	0.10	0.08	0.05	0.07	0.07	0.07	0.06	0.06	0.07
[18] obscura	0.58	0.58	0.49	0.46	0.50	0.62	0.48	0.52	0.44	0.53	0.61	0.46	0.58	0.51	0.49	0.51	0.53		0.05	0.09	0.07	0.05	0.08	0.06	0.06	0.06	0.08	0.05
[19] polychaeta	0.50	0.54	0.43	0.43	0.51	0.60	0.44	0.49	0.49	0.47	0.59	0.38	0.48	0.53	0.55	0.53	0.50	0.48		0.08	0.07	0.04	0.07	0.06	0.08	0.05	0.07	0.06
[20] putrida	0.54	0.62	0.53	0.44	0.52	0.57	0.44	0.39	0.51	0.43	0.58	0.34	0.53	0.55	0.55	0.48	0.55	0.54	0.44		0.08	0.07	0.10	0.08	0.09	0.08	0.10	0.10
[21] quinaria	0.43	0.59	0.43	0.44	0.45	0.53	0.36	0.40	0.50	0.37	0.49	0.40	0.55	0.45	0.51	0.60	0.50	0.46	0.44	0.36		0.07	0.08	0.07	0.09	0.07	0.08	0.09
[22] repleta	0.53	0.63	0.51	0.45	0.53	0.61	0.53	0.47	0.52	0.52	0.59	0.46	0.55	0.50	0.55	0.59	0.52	0.56	0.49	0.52	0.52		0.06	0.05	0.06	0.05	0.05	0.04
[23] robusta	0.48	0.62	0.55	0.46	0.49	0.57	0.41	0.42	0.51	0.48	0.61	0.46	0.58	0.51	0.59	0.62	0.53	0.61	0.49	0.51	0.44	0.51		0.07	0.08	0.07	0.07	0.07
[24] rubrifrons	0.45	0.55	0.45	0.31	0.48	0.45	0.35	0.38	0.39	0.40	0.54	0.27	0.48	0.41	0.43	0.58	0.43	0.46	0.38	0.37	0.34	0.48	0.36		0.08	0.06	0.08	0.07
[25] saltans	0.56	0.63	0.58	0.54	0.50	0.65	0.61	0.49	0.47	0.51	0.56	0.54	0.58	0.53	0.52	0.54	0.58	0.53	0.59	0.53	0.53	0.56	0.60	0.57		0.06	0.09	0.06
[26] tripunctata	0.67	0.62	0.57	0.58	0.56	0.69	0.59	0.55	0.55	0.57	0.74	0.54	0.59	0.61	0.59	0.58	0.57	0.61	0.56	0.58	0.57	0.60	0.65	0.55	0.65		0.06	0.05
[27] virilis	0.53	0.69	0.54	0.57	0.54	0.63	0.49	0.45	0.62	0.52	0.70	0.53	0.63	0.57	0.66	0.61	0.50	0.70	0.55	0.60	0.49	0.55	0.46	0.50	0.72	0.63		0.07
[28] willistoni	0.53	0.54	0.48	0.44	0.42	0.60	0.58	0.42	0.47	0.55	0.55	0.43	0.56	0.49	0.49	0.48	0.52	0.45	0.46	0.59	0.56	0.48	0.51	0.47	0.48	0.57	0.59	

Table S7. Estimates of d_S values for COII data set. Average values between 29 species groups of Drosophilidae. Distances calculated on MEGA V4 based in the pairwise analysis of 174 sequences in a dataset of 658 positions, gaps and missing data were eliminated by pairwise deletion. Values above the diagonal show standard errors obtained by bootstrap procedure (500 replicates).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29]
[1] Hawaiians		0.06	0.05	0.04	0.05	0.08	0.07	0.06	0.07	0.05	0.06	0.05	0.06	0.04	0.06	0.04	0.04	0.05	0.04	0.05	0.06	0.05	0.04	0.05	0.06	0.04	0.05	0.05	0.05
[2] Scaptodrosophila	0.69		0.07	0.06	0.06	0.11	0.09	0.08	0.07	0.06	0.10	0.08	0.07	0.05	0.07	0.06	0.07	0.06	0.05	0.07	0.09	0.06	0.05	0.07	0.08	0.06	0.06	0.06	0.05
[3] Scaptomyza	0.64	0.71		0.06	0.07	0.12	0.08	0.09	0.08	0.07	0.07	0.07	0.08	0.06	0.07	0.06	0.07	0.07	0.06	0.07	0.08	0.07	0.05	0.07	0.08	0.06	0.06	0.06	0.06
[4] Zaprionus	0.46	0.61	0.57		0.06	0.09	0.07	0.07	0.08	0.05	0.08	0.05	0.06	0.05	0.06	0.04	0.04	0.05	0.05	0.05	0.06	0.05	0.04	0.05	0.06	0.04	0.05	0.05	0.05
[5] ananassae	0.55	0.53	0.67	0.49		0.12	0.09	0.06	0.08	0.05	0.09	0.06	0.08	0.04	0.07	0.04	0.05	0.06	0.05	0.05	0.07	0.07	0.05	0.07	0.08	0.05	0.05	0.06	0.05
[6] annulimana	0.68	0.77	0.85	0.66	0.74		0.12	0.12	0.13	0.10	0.10	0.11	0.10	0.10	0.10	0.09	0.09	0.09	0.10	0.12	0.10	0.12	0.07	0.11	0.14	0.09	0.10	0.10	0.09
[7] atalaia	0.63	0.72	0.68	0.56	0.65	0.73		0.11	0.09	0.07	0.08	0.09	0.12	0.07	0.08	0.08	0.09	0.07	0.08	0.09	0.10	0.09	0.06	0.06	0.09	0.07	0.08	0.09	0.08
[8] calloptera	0.59	0.60	0.73	0.53	0.47	0.71	0.76		0.08	0.07	0.08	0.07	0.07	0.06	0.07	0.07	0.06	0.07	0.06	0.07	0.09	0.07	0.06	0.08	0.09	0.07	0.07	0.08	0.07
[9] canalinea	0.62	0.64	0.72	0.58	0.57	0.72	0.58	0.55		0.08	0.10	0.09	0.09	0.07	0.07	0.07	0.07	0.08	0.07	0.08	0.09	0.08	0.06	0.08	0.11	0.07	0.08	0.08	0.08
[10] cardini	0.50	0.60	0.67	0.45	0.48	0.67	0.55	0.58	0.58		0.07	0.05	0.07	0.05	0.06	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.05	0.07	0.06	0.05	0.05	0.07	0.05
[11] funebris	0.53	0.74	0.62	0.53	0.58	0.61	0.52	0.59	0.67	0.46		0.09	0.11	0.07	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.08	0.06	0.07	0.07	0.07	0.06	0.08	0.08
[12] guarani	0.53	0.62	0.66	0.42	0.41	0.71	0.63	0.45	0.60	0.43	0.53		0.07	0.05	0.07	0.06	0.05	0.06	0.05	0.05	0.06	0.07	0.05	0.06	0.07	0.05	0.04	0.07	0.06
[13] guttifera	0.56	0.57	0.65	0.41	0.56	0.64	0.78	0.45	0.58	0.54	0.66	0.52		0.05	0.07	0.06	0.06	0.07	0.06	0.07	0.07	0.06	0.06	0.08	0.09	0.07	0.06	0.06	0.06
[14] immigrans	0.57	0.59	0.64	0.49	0.45	0.76	0.60	0.54	0.59	0.51	0.59	0.49	0.45		0.05	0.04	0.05	0.05	0.05	0.05	0.06	0.05	0.04	0.05	0.06	0.04	0.05	0.05	0.05
[15] melanica	0.57	0.62	0.69	0.54	0.58	0.73	0.58	0.60	0.58	0.57	0.63	0.55	0.52	0.53		0.06	0.05	0.07	0.05	0.07	0.07	0.06	0.05	0.06	0.08	0.05	0.06	0.06	0.06
[16] melanogaster	0.56	0.59	0.68	0.44	0.41	0.70	0.65	0.59	0.57	0.58	0.71	0.51	0.52	0.52	0.57		0.04	0.06	0.04	0.05	0.07	0.05	0.04	0.05	0.07	0.05	0.05	0.05	0.05
[17] montium	0.56	0.65	0.70	0.44	0.40	0.70	0.72	0.49	0.53	0.57	0.70	0.46	0.48	0.53	0.51	0.44		0.06	0.04	0.05	0.06	0.05	0.04	0.06	0.08	0.05	0.05	0.06	0.05
[18] nannoptera	0.58	0.65	0.70	0.49	0.54	0.66	0.56	0.59	0.63	0.55	0.63	0.52	0.58	0.52	0.69	0.57	0.57		0.05	0.06	0.08	0.06	0.05	0.06	0.08	0.05	0.06	0.06	0.05
[19] obscura	0.60	0.62	0.70	0.50	0.47	0.83	0.65	0.57	0.58	0.57	0.63	0.52	0.60	0.57	0.58	0.49	0.49	0.59		0.06	0.06	0.05	0.04	0.05	0.08	0.04	0.05	0.05	0.05
[20] polychaeta	0.62	0.67	0.71	0.48	0.48	0.83	0.73	0.60	0.68	0.55	0.64	0.49	0.57	0.54	0.63	0.57	0.55	0.62	0.63		0.07	0.06	0.05	0.07	0.06	0.05	0.05	0.07	0.05
[21] putrida	0.57	0.70	0.68	0.46	0.49	0.67	0.69	0.62	0.65	0.46	0.48	0.44	0.46	0.55	0.52	0.55	0.50	0.63	0.57	0.58		0.07	0.06	0.08	0.09	0.07	0.06	0.07	0.07
[22] quinaria	0.47	0.50	0.60	0.42	0.50	0.73	0.60	0.43	0.58	0.44	0.53	0.47	0.38	0.49	0.50	0.48	0.45	0.48	0.49	0.53	0.43		0.05	0.07	0.08	0.06	0.05	0.06	0.06
[23] repleta	0.57	0.61	0.65	0.50	0.55	0.66	0.59	0.64	0.57	0.57	0.59	0.53	0.56	0.57	0.57	0.54	0.54	0.58	0.59	0.63	0.65	0.54		0.05	0.06	0.03	0.05	0.04	0.04
[24] robusta	0.58	0.64	0.70	0.49	0.58	0.77	0.48	0.63	0.60	0.60	0.53	0.51	0.62	0.55	0.53	0.53	0.58	0.53	0.57	0.69	0.66	0.52	0.58		0.07	0.05	0.06	0.07	0.06
[25] rubifrons	0.55	0.68	0.66	0.48	0.59	0.77	0.61	0.62	0.69	0.48	0.44	0.48	0.57	0.53	0.58	0.56	0.63	0.60	0.71	0.55	0.58	0.51	0.58	0.57		0.06	0.06	0.08	0.06
[26] saltans	0.51	0.57	0.65	0.40	0.41	0.68	0.53	0.55	0.56	0.49	0.55	0.45	0.54	0.49	0.51	0.45	0.48	0.46	0.49	0.53	0.55	0.45	0.49	0.49	0.44		0.05	0.06	0.04
[27] tripunctata	0.56	0.59	0.66	0.48	0.49	0.75	0.63	0.57	0.65	0.49	0.50	0.41	0.49	0.51	0.60	0.52	0.53	0.56	0.56	0.59	0.47	0.46	0.58	0.57	0.52	0.51		0.06	0.05
[28] virilis	0.57	0.61	0.61	0.48	0.53	0.69	0.66	0.59	0.57	0.56	0.60	0.56	0.50	0.53	0.57	0.51	0.52	0.56	0.61	0.65	0.55	0.52	0.54	0.60	0.62	0.52	0.60		0.05
[29] willistoni	0.55	0.59	0.65	0.45	0.45	0.68	0.64	0.56	0.59	0.50	0.60	0.50	0.46	0.52	0.57	0.48	0.52	0.53	0.55	0.58	0.58	0.50	0.52	0.58	0.51	0.38	0.57	0.52	

Table S8. Estimates of ds values for ND2 data set. Average values between 28 species groups of Drosophilidae. Distances calculated on MEGA V4 based in the pairwise analysis of 157 sequences in a dataset of 479 positions, gaps and missing data were eliminated by pairwise deletion. Values above the diagonal show standard errors obtained by bootstrap procedure (500 replicates).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28]
[1] Hawaiians		0.08	0.08	0.07	0.06	0.06	0.08	0.12	0.08	0.10	0.07	0.07	0.07	0.06	0.05	0.07	0.06	0.06	0.06	0.08	0.06	0.05	0.06	0.06	0.05	0.06	0.06	0.05
[2] Scaptodrosophila	0.68		0.11	0.10	0.09	0.08	0.12	0.11	0.10	0.12	0.09	0.10	0.09	0.08	0.10	0.10	0.09	0.07	0.09	0.10	0.10	0.08	0.08	0.11	0.09	0.08	0.11	0.11
[3] Scaptomyza	0.55	0.80		0.10	0.09	0.08	0.12	0.10	0.09	0.11	0.07	0.12	0.09	0.08	0.08	0.09	0.08	0.07	0.08	0.14	0.10	0.07	0.08	0.09	0.07	0.08	0.09	0.08
[4] Zaprionus	0.48	0.69	0.56		0.08	0.08	0.12	0.13	0.08	0.12	0.08	0.09	0.08	0.06	0.08	0.09	0.07	0.08	0.07	0.11	0.09	0.07	0.08	0.07	0.07	0.07	0.08	0.08
[5] ananassae	0.46	0.65	0.58	0.41		0.07	0.09	0.09	0.07	0.12	0.07	0.07	0.08	0.06	0.05	0.07	0.07	0.07	0.08	0.07	0.05	0.05	0.06	0.07	0.06	0.05	0.07	0.06
[6] annulimana	0.54	0.64	0.56	0.51	0.49		0.11	0.09	0.08	0.11	0.06	0.07	0.07	0.06	0.06	0.07	0.06	0.06	0.07	0.11	0.07	0.05	0.07	0.08	0.05	0.06	0.07	0.06
[7] atalaia	0.52	0.78	0.66	0.61	0.48	0.61		0.13	0.08	0.18	0.08	0.10	0.10	0.10	0.09	0.12	0.09	0.10	0.09	0.11	0.09	0.08	0.09	0.10	0.08	0.09	0.12	0.08
[8] calloptera	0.69	0.66	0.53	0.64	0.50	0.54	0.66		0.09	0.17	0.09	0.11	0.09	0.10	0.11	0.13	0.08	0.08	0.10	0.14	0.09	0.08	0.09	0.10	0.10	0.08	0.11	0.11
[9] cardini	0.52	0.63	0.50	0.41	0.36	0.48	0.41	0.43		0.18	0.07	0.08	0.09	0.07	0.06	0.10	0.06	0.06	0.07	0.09	0.08	0.06	0.06	0.10	0.08	0.07	0.09	0.08
[10] funebrs	0.64	0.75	0.62	0.63	0.67	0.68	0.79	0.74	0.85		0.13	0.18	0.09	0.11	0.10	0.10	0.10	0.10	0.12	0.17	0.10	0.10	0.10	0.13	0.11	0.11	0.10	0.12
[11] guarani	0.48	0.59	0.42	0.43	0.38	0.40	0.38	0.42	0.32	0.65		0.08	0.07	0.08	0.06	0.08	0.06	0.06	0.07	0.09	0.07	0.05	0.07	0.08	0.06	0.06	0.07	0.08
[12] guttifera	0.47	0.66	0.64	0.41	0.37	0.41	0.50	0.54	0.38	0.78	0.37		0.11	0.07	0.07	0.10	0.07	0.07	0.07	0.09	0.08	0.07	0.08	0.07	0.07	0.08	0.07	0.08
[13] immigrans	0.65	0.73	0.68	0.58	0.62	0.60	0.67	0.62	0.59	0.65	0.52	0.65		0.08	0.07	0.07	0.07	0.07	0.07	0.10	0.07	0.06	0.07	0.07	0.08	0.07	0.08	0.08
[14] melanica	0.45	0.58	0.51	0.37	0.39	0.45	0.56	0.52	0.39	0.59	0.44	0.38	0.62		0.07	0.08	0.06	0.06	0.07	0.09	0.07	0.05	0.06	0.08	0.06	0.05	0.07	0.08
[15] melanogaster	0.43	0.70	0.55	0.46	0.34	0.44	0.52	0.63	0.36	0.58	0.36	0.38	0.61	0.43		0.07	0.06	0.07	0.07	0.08	0.06	0.05	0.06	0.07	0.05	0.06	0.07	0.05
[16] montium	0.50	0.77	0.59	0.56	0.47	0.50	0.64	0.72	0.55	0.63	0.47	0.54	0.62	0.57	0.47		0.07	0.07	0.09	0.12	0.08	0.07	0.07	0.08	0.06	0.07	0.09	0.07
[17] nannoptera	0.50	0.68	0.56	0.40	0.44	0.47	0.53	0.49	0.38	0.59	0.37	0.37	0.55	0.42	0.41	0.54		0.05	0.07	0.10	0.07	0.05	0.06	0.08	0.05	0.06	0.07	0.06
[18] obscura	0.56	0.63	0.55	0.53	0.47	0.50	0.62	0.51	0.42	0.64	0.38	0.47	0.61	0.47	0.49	0.57	0.46		0.07	0.09	0.07	0.05	0.06	0.07	0.05	0.06	0.07	0.07
[19] polychaeta	0.58	0.74	0.57	0.45	0.56	0.57	0.62	0.57	0.45	0.73	0.47	0.48	0.65	0.55	0.57	0.67	0.52	0.61		0.12	0.08	0.05	0.07	0.07	0.06	0.06	0.08	0.07
[20] putrida	0.58	0.69	0.74	0.57	0.42	0.66	0.59	0.69	0.47	0.81	0.44	0.45	0.73	0.53	0.48	0.69	0.67	0.58	0.78		0.09	0.09	0.09	0.09	0.09	0.07	0.09	0.09
[21] quinaria	0.45	0.68	0.54	0.49	0.31	0.47	0.51	0.50	0.40	0.52	0.35	0.36	0.51	0.44	0.40	0.45	0.43	0.49	0.54	0.47		0.06	0.08	0.07	0.06	0.06	0.07	0.07
[22] repleta	0.50	0.71	0.54	0.47	0.44	0.50	0.61	0.55	0.47	0.66	0.42	0.51	0.61	0.44	0.45	0.56	0.47	0.55	0.53	0.61	0.44		0.05	0.06	0.05	0.05	0.07	0.05
[23] robusta	0.45	0.59	0.53	0.48	0.41	0.47	0.55	0.48	0.33	0.61	0.42	0.46	0.55	0.37	0.39	0.46	0.42	0.46	0.52	0.54	0.49	0.45		0.06	0.06	0.05	0.07	0.07
[24] rubrifrons	0.46	0.70	0.53	0.37	0.41	0.50	0.54	0.54	0.45	0.67	0.40	0.33	0.55	0.43	0.42	0.47	0.47	0.48	0.49	0.50	0.40	0.48	0.33		0.07	0.06	0.08	0.07
[25] saltans	0.40	0.67	0.45	0.45	0.39	0.46	0.48	0.58	0.43	0.61	0.36	0.39	0.62	0.42	0.33	0.44	0.38	0.46	0.51	0.50	0.39	0.45	0.42	0.45		0.06	0.06	0.04
[26] tripunctata	0.53	0.64	0.58	0.45	0.37	0.51	0.57	0.52	0.43	0.69	0.40	0.46	0.64	0.45	0.45	0.56	0.53	0.50	0.55	0.47	0.40	0.50	0.44	0.43	0.49		0.06	0.06
[27] virilis	0.52	0.82	0.59	0.52	0.49	0.54	0.71	0.61	0.52	0.60	0.44	0.42	0.67	0.52	0.53	0.58	0.53	0.55	0.63	0.54	0.46	0.57	0.54	0.51	0.45	0.52		0.08
[28] willistoni	0.44	0.77	0.50	0.49	0.41	0.50	0.48	0.58	0.41	0.66	0.45	0.45	0.66	0.51	0.34	0.47	0.46	0.54	0.56	0.51	0.44	0.43	0.43	0.42	0.31	0.51	0.54	

Table S9. Estimates of ds values for SinA data set. Average values between 29 species groups of Drosophilidae. Distances calculated on MEGA V4 based in the pairwise analysis of 154 sequences in a dataset of 132 positions, gaps and missing data were eliminated by pairwise deletion. Values above the diagonal show standard errors obtained by bootstrap procedure (500 replicates).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29]
[1] Hawaiian		0.10	0.03	0.08	0.13	0.05	0.06	0.07	0.06	0.08	0.05	0.07	0.05	0.06	0.06	0.14	0.11	0.06	0.08	0.05	0.07	0.05	0.05	0.06	0.05	0.15	0.07	0.06	0.13
[2] Scaptodrosophila	0.71		0.13	0.15	0.13	0.10	0.13	0.11	0.12	0.11	0.11	0.09	0.11	0.09	0.11	0.15	0.11	0.13	0.09	0.12	0.11	0.11	0.08	0.10	0.11	0.15	0.09	0.09	0.10
[3] Scaptomyza	0.15	0.82		0.09	0.12	0.05	0.06	0.08	0.07	0.09	0.06	0.09	0.06	0.08	0.06	0.13	0.11	0.07	0.09	0.05	0.07	0.06	0.05	0.06	0.07	0.18	0.08	0.06	0.15
[4] Zaprionus	0.45	0.95	0.53		0.16	0.09	0.08	0.10	0.09	0.08	0.08	0.09	0.07	0.09	0.09	0.15	0.12	0.09	0.11	0.09	0.09	0.07	0.08	0.11	0.08	0.16	0.08	0.09	0.13
[5] ananassae	0.79	0.85	0.75	0.94		0.15	0.15	0.13	0.16	0.20	0.14	0.12	0.14	0.13	0.12	0.07	0.07	0.16	0.10	0.14	0.16	0.16	0.13	0.12	0.19	0.30	0.13	0.13	0.22
[6] annulimana	0.29	0.72	0.32	0.58	0.86		0.05	0.07	0.06	0.08	0.06	0.09	0.06	0.08	0.05	0.14	0.12	0.05	0.10	0.06	0.07	0.05	0.04	0.06	0.07	0.20	0.07	0.04	0.16
[7] atalaia	0.26	0.80	0.29	0.45	0.85	0.24		0.09	0.07	0.09	0.07	0.10	0.06	0.08	0.06	0.17	0.15	0.04	0.09	0.06	0.08	0.07	0.05	0.07	0.06	0.17	0.08	0.06	0.15
[8] callopera	0.39	0.70	0.46	0.53	0.83	0.44	0.50		0.08	0.06	0.08	0.09	0.08	0.09	0.08	0.16	0.11	0.10	0.12	0.07	0.08	0.06	0.07	0.09	0.07	0.19	0.05	0.08	0.17
[9] canalinea	0.34	0.79	0.39	0.54	0.88	0.29	0.32	0.48		0.08	0.06	0.10	0.07	0.08	0.07	0.19	0.15	0.07	0.10	0.08	0.08	0.06	0.04	0.07	0.07	0.14	0.08	0.07	0.14
[10] cardini	0.47	0.80	0.54	0.48	1.04	0.52	0.48	0.32	0.51		0.07	0.08	0.08	0.08	0.10	0.23	0.17	0.09	0.14	0.08	0.09	0.06	0.08	0.11	0.06	0.19	0.06	0.10	0.15
[11] funebris	0.24	0.79	0.34	0.43	0.79	0.32	0.35	0.41	0.31	0.41		0.08	0.06	0.08	0.06	0.15	0.11	0.07	0.09	0.06	0.05	0.04	0.05	0.07	0.04	0.23	0.06	0.06	0.17
[12] guarani	0.39	0.62	0.47	0.54	0.75	0.47	0.54	0.50	0.51	0.51	0.44		0.08	0.05	0.08	0.12	0.10	0.11	0.08	0.08	0.10	0.07	0.07	0.08	0.08	0.18	0.07	0.09	0.14
[13] guttifera	0.25	0.76	0.30	0.41	0.83	0.37	0.31	0.46	0.35	0.43	0.26	0.41		0.07	0.06	0.13	0.11	0.07	0.08	0.06	0.07	0.05	0.05	0.07	0.06	0.17	0.06	0.06	0.13
[14] immigrans	0.45	0.71	0.54	0.62	0.87	0.51	0.52	0.55	0.50	0.58	0.50	0.36	0.47		0.07	0.12	0.09	0.08	0.09	0.08	0.09	0.07	0.06	0.07	0.07	0.18	0.06	0.06	0.13
[15] melanica	0.30	0.74	0.34	0.56	0.73	0.32	0.34	0.47	0.38	0.58	0.36	0.42	0.36	0.49		0.13	0.10	0.07	0.10	0.06	0.08	0.06	0.04	0.03	0.07	0.20	0.08	0.05	0.14
[16] melanogaster	0.80	0.91	0.77	0.86	0.44	0.82	0.91	0.88	0.97	1.07	0.84	0.69	0.78	0.75	0.76		0.04	0.16	0.08	0.14	0.17	0.15	0.15	0.12	0.20	0.40	0.12	0.11	0.25
[17] montium	0.71	0.81	0.70	0.75	0.48	0.74	0.84	0.68	0.87	0.95	0.70	0.61	0.69	0.68	0.65	0.28		0.14	0.07	0.11	0.15	0.12	0.11	0.11	0.15	0.37	0.09	0.10	0.20
[18] nannoptera	0.34	0.83	0.38	0.50	0.89	0.31	0.20	0.60	0.38	0.54	0.41	0.61	0.38	0.57	0.43	0.89	0.84		0.09	0.08	0.08	0.07	0.06	0.08	0.07	0.16	0.08	0.06	0.17
[19] obscura	0.49	0.64	0.53	0.68	0.63	0.56	0.48	0.71	0.59	0.76	0.54	0.46	0.49	0.56	0.57	0.47	0.48	0.51		0.09	0.09	0.09	0.08	0.09	0.09	0.20	0.08	0.09	0.15
[20] polychaeta	0.26	0.81	0.29	0.53	0.85	0.34	0.35	0.36	0.43	0.46	0.33	0.43	0.32	0.52	0.35	0.81	0.68	0.45	0.55		0.07	0.05	0.06	0.07	0.06	0.15	0.06	0.06	0.12
[21] putrida	0.36	0.82	0.43	0.49	0.92	0.40	0.40	0.48	0.43	0.49	0.23	0.60	0.35	0.57	0.45	0.99	0.90	0.47	0.59	0.40		0.05	0.07	0.09	0.05	0.23	0.08	0.07	0.18
[22] quinaria	0.23	0.75	0.34	0.39	0.86	0.29	0.30	0.34	0.31	0.33	0.13	0.40	0.24	0.47	0.34	0.83	0.70	0.41	0.52	0.28	0.20		0.05	0.07	0.04	0.20	0.06	0.06	0.16
[23] repleta	0.30	0.66	0.36	0.52	0.82	0.32	0.31	0.48	0.23	0.55	0.33	0.44	0.34	0.44	0.32	0.91	0.78	0.40	0.55	0.38	0.46	0.33		0.05	0.06	0.17	0.07	0.05	0.13
[24] robusta	0.33	0.69	0.37	0.65	0.75	0.35	0.40	0.53	0.43	0.66	0.42	0.43	0.42	0.48	0.12	0.73	0.68	0.50	0.56	0.40	0.53	0.42	0.35		0.08	0.24	0.08	0.05	0.19
[25] rubifrons	0.27	0.80	0.38	0.45	0.94	0.39	0.32	0.37	0.35	0.35	0.16	0.48	0.31	0.49	0.38	1.02	0.87	0.38	0.52	0.35	0.19	0.14	0.36	0.45		0.20	0.06	0.06	0.16
[26] saltans	0.82	0.94	0.92	0.90	1.28	1.07	0.93	0.98	0.79	0.98	1.03	0.97	0.91	1.02	1.01	1.70	1.45	0.90	1.05	0.86	1.07	0.97	0.92	1.14	0.98		0.19	0.21	0.04
[27] tripunctata	0.50	0.73	0.57	0.52	0.83	0.53	0.50	0.32	0.51	0.40	0.40	0.51	0.42	0.52	0.54	0.77	0.66	0.59	0.57	0.43	0.50	0.38	0.52	0.58	0.37	1.04		0.07	0.15
[28] virilis	0.31	0.64	0.36	0.56	0.84	0.26	0.29	0.48	0.35	0.61	0.35	0.52	0.32	0.44	0.25	0.67	0.65	0.32	0.52	0.35	0.38	0.34	0.32	0.27	0.36	1.09	0.54		0.16
[29] willistoni	0.76	0.72	0.84	0.78	1.14	0.93	0.84	0.95	0.84	0.91	0.91	0.87	0.80	0.93	0.84	1.23	1.07	0.94	0.91	0.79	0.94	0.86	0.82	1.01	0.90	0.22	0.93	0.96	

V. CONCLUSIONS

V. CONCLUSIONS

In this study, a cophylogenetic analysis of a DNA transposon and its host has been performed. The long-term evolutionary dynamic of Galileo is studied in the context of the phylogeny of 174 species of Drosophilidae. In order to obtain a robust host species phylogeny, the evolutionary relationships at different taxonomic levels were revised with the addition of new taxa and using different phylogenetic methodologies. The following conclusions can be drawn from this work:

1. In a taxonomic inventory of Drosophilidae a new species of cactophilic spotted-thorax diptera was discovered and has been formally described as *Drosophila machalilla*.
2. Based in a cladistics analysis of 52 morphological traits of males, females and inmatures stages of the new species and representative taxa of four species groups, the new species group *atalaia* is erected.

3. The molecular phylogenetic analysis uncovered that the *nannoptera* species group is closely related to D. *machalilla*. The dating analysis estimates that these lineages diverged around 16.9 Mya.

4. For the first time the *inca* subgroup has been included in a phylogenetic and biogeographical analysis. The *inca* clade, endemic from Ecuador and Peru is the first diverging lineage within the *repleta* radiation. This support the hypothesis of a South American origin of this lineage.

5. The results obtained in the biogeographical analysis of 51 taxa of the *repleta*, *nannoptera* and *atalaia* species groups are the bases to propose that

diversification of the *repleta* radiation is associated with the uplift of the Central Andes.

6. A robust phylogenetic tree of 174 taxa of *Drosophila*, *Scaptodrosophila*, *Hawaiian Drosophila* and *Zaprionus* have been inferred using three mitochondrial (COI, COII, ND2, ND2) and one nuclear (SinA) genes.

7. Galileo transposon was detected in 51 species from ten species groups of *Sophophora*, *Drosophila* and *Siphlodora* subgenera.

8. Galileo was detected in samples of *Drosophila* from seven zoo-geographical regions: Nearctic, Neotropic, Paleartic, Orient, Australian, Madagascar and Africa.

9. The results obtained support the hypothesis of an ancient cospeciation of Galileo in *Drosophila* host species. The element was detected in several populations of the *Sophophora* subgenus from Asia, where it is thought the ancestor of this subgenus has its origin ca. 40-56 Mya.

10. The significant match found between host-and transposon phylogenies reveal cospeciation of Galileo in *Drosophila* and ancestral horizontal transfer events that involve the *willistoni*, *tripunctata*, *guarani* and *virilis* species groups.

11. The partial matching between Galileo-host phylogenies reflect a history of synchronous evolution and cospeciation combined with a few horizontal transfer events.

12. The fact that Galileo and its host species recover highly congruent trees inplies that these entities diversified over the same period of time, therefore codiversification on transposon and host species has been linked through ecological and geographical associations.

VI. APPENDICES

Appendix 1.

LABORATORY PROTOCOLS

Cetyl-Trimethyl-Ammonium-Bromide (CTAB) DNA extraction protocol.

Protocol used for DNA extraction on the project: Evolutionary Dynamic of Galileo TE in the genus Drosophila. Adapted from the original protocol Doyle & Doyle, 1987. Prepare CTAB buffer at 60ºC.

2. Put 1 to 3 frozen flies into a 1.5 Eppendorf tube and grind in liquid nitrogen with pestles, keeping tissue frozen the entire time. Use a new pestle for every sample. Soak pestles in bleach water for at least ½ hour before rinsing and autoclaving.

3. Add 500 uL of CTAB buffer and mix the tubes. Make sure the tissue is in solution and not in a clump at the bottom of the tube. Incubate at 55°C for at least one hour, mixing once after 30 minutes. They can stay in the water bath for a few hours if necessary.

4. Add add 500 uL of chloroform and mix by gently shaking tubes. Change gloves immediately if you spill chloroform on them. Be careful not to drip chloroform onto the tubes, it has a low viscosity and drips out of the tip

5. Centrifuge for 7 minutes at 16000 rcf.

6. Transfer the aqueous phase (top layer) into the new labeled tube. Be careful to avoid transferring any chloroform.

7. Estimate the volume of the aqueous phase and add the same volume of cold isopropanol. Mix by inverting tubes 20-30 times.Incubate on ice for 30-40 minutes.

8. Centrifuge for 3 minutes at 16000 rcf.

9. Discard supernatant into isopropanol chemical waste jar. Be careful not to dislodge pellet.

10. Add 700 uL 70% EtOH, invert tubes 5-10 times.

11. Centrifuge for 1 minute at 16000 rcf.

12. Discard supernatant; be careful not to dislodge pellet.

13. Use small pieces of Whatman paper to dry the walls of the tube and the use desiccator of propylene (Kartell) for 15 minutes or until pellet looks dry.

Hydrate pellets with 20 uL of water. Allow to resuspend overnight at room temperature. Store the DNA in the refrigerator the next day.

CTAB Buffer (Sigma)

100 ml 1 M Tris HCl pH 8.0

280 ml 5 M NaCl

40 ml of 0.5 M EDTA

20 g of CTAB (cetyltrimethyl ammonium bromide)

Bring total volume to 1 L with ddH_2O.

TE Buffer

10 ml 1 M Tris HCl pH 8.0

2 ml 0.5 M EDTA

Bring total volume to 1 L with ddH_2O.

1 M Tris HCl pH 8.0

121.1 g Tris

Dissolve in about 700 ml of H_2O.

Bring pH down to 8.0 by adding concentrated HCl (you'll need about 50 ml).

Bring total volume to 1 L with ddH_2O.

0.5 M EDTA

186.12 g EDTA

Add about 700 ml H^2O

16-18 g of NaOH pellets

Adjust pH to 8.0 by with a few more pellets, EDTA won't dissolve until the pH is near 8.0

Bring total volume to 1 L with ddH_2O.

5 M NaCl

292.2 g of NaCl

700 ml H_2O

Dissolve (don't add NaCl all at once, it will never go into solution) and bring to 1 L.

Agarose gel procedure

Procedure for agarose gel used on the project: Evolutionary Dynamic of Galileo TE in the genus *Drosophila*, modified from protocol to running agarose gels by St. Olaf College.

1. Assemble the gel casting tray and comb. The comb should not touch the bottom of the tray.

2. Add agarose to 100 mL of TAE (Tris-Acetate-Edta) Buffer, 1X. Using a microwave, melt the agarose solution.

3. When the agarose solution has cooled to about 50°C, pour solution directly into the casting tray, ensuring that no bubbles get into the gel.

4. Allow the gel to cool. It will solidify and become slightly opaque within 20 to 30 minutes. Remove black end pieces.

5. Submerge the gel by adding approximately 1L of TAE 1X running buffer to cover the gel by about a half a centimeter.

6. Carefully remove the comb by lifting it gently at one end, tilting the comb as it comes out. Ensure that the wells are submerged and filled with buffer.

7. Prepare the DNA samples for loading using gel loading buffer 6X (0.25% bromophenol blue and 0.25% xylene cyanol plus 30% glycerol).

8. Once all the samples are loaded place the cover on the gel apparatus. Connect the leads so that the red (positive) lead is at the end of the gel to which the DNA will migrate and the black (negative) lead is at the end of the gel containing the wells.

9. Run at a constant voltage of 50-70 volts. When the blue tracking dye (which runs in these gels along with a DNA fragment of about 200-400 bp) has migrated about 75% of the distance to the end of the gel (usually within 60-90 minutes), turn off the power supply and disconnect the power leads.

10. Transfer the gel into a plastic dish and add enough Ethidium Bromide staining solution to cover the gel. Set in a dark drawer for 30 minutes. Visualize the DNA with UV light. Dispose of the gel into the trash. Rinse the light box and tray with water and dry it with paper towels.

PCR clean-up protocol

Protocol used for PCR clean- up of Galileo fragments performed previous the Cloning protocol and the sample sequencing. Modified from NucleoSpin PCR Clean-up Manual.

1. Adjust DNA binding condition

For very small sample volumes < 30 µL adjust the volume of the reaction mixture to 50–100 µL with water. Mix 1 volume of sample with 2 volumes of Buffer NTI (*e.g.*, mix 100 µL PCR reaction and 200 µL Buffer NTI). Note: For removal of small fragments like primer dimers dilutions of Buffer NTI can be used instead of 100 % Buffer NTI.

2. Bind DNA

Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 µL sample.

Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.

Load remaining sample if necessary and repeat the centrifugation step.

3. Wash silica membrane

Add 700 µL Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.

4. Dry silica membrane

Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

5. Elute DNA

Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube. Add 15–30 µL Buffer NE and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x g.

Cloning protocol.

Protocol used for Cloning of Galileo TE sequences. Adapted from the StrataClone PCR Cloning Manual.

Ligating the insert

Mixture by combining the following components. Add the components in the order given below and mix gently by repeated pipetting.

3 µl StrataClone Cloning Buffer

2 µl of PCR product (5–50 ng, typically a 1:10 dilution of a robust PCR reaction)

1 µl StrataClone Vector Mix amp/kan. Incubate at room temperature for 5 minutes, then place the reaction on ice.

Transforming the competent cells

Add 1 µl of the cloning reaction mixture to a tube of thawed StrataClone SoloPack competent cells. Mix gently (do not mix by repeated pipetting). Incubate the transformation mixture on ice for 20 minutes. Then the transformation mixture must be exposed to heat-shock at 42°C for 45 seconds. Incubate the transformation mixture on ice for 2 minutes. Add 250 µl of LB medium (pre-warmed to 42°C). Allow the cells to recover at 37°C with agitation for at least 1 hour. Plate 5 µl and 100 µl of the transformation mixture on LB–ampicillin plates that have been spread with 40 µl of 2% X-gal.Incubate the plates overnight at 37°C.

Analyzing the Transformants: Pick white or light blue colonies for plasmid DNA analysis. Do not pick dark blue colonies. Positive clones may be identified by PCR analysis of plasmid DNA using the T3/T7 primer pair.

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved

Cool to 55°C, Add 10 ml of 10-mg/ml filter-sterilized Ampicillin

Pour into petri dishes

(~25 ml/100-mm plate)

2% X-Gal (per 10 ml)

0.2 g of 5-bromo-4-chloro-3-inodlyl-β-Dgalactopyranoside

(X-Gal)

10 ml of dimethylformamide (DMF)

Store at –20°C, spread 40 µl per LB-agar plate.

Appendix 2.

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Radiation of the *Drosophila nannoptera* species group in Mexico

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Keywords:

asymmetric male genitalia;
molecular phylogeny;
multilocus analysis;
nannoptera species group;
reproductive characters;
species divergence estimates.

Abstract

The *Drosophila nannoptera* species group, a taxon of Mexican cactophilic flies, is an excellent model system to study the influence of abiotic and biotic factors on speciation, the genetic causes of ecological specialization and the evolution of unusual reproductive characters. However, the phylogenetic relationships in the nannoptera species group and its position within the virilis-repleta phylogeny have not been thoroughly investigated. Using a multilocus data set of gene coding regions of eight nuclear and three mitochondrial genes, we found that the four described nannoptera group species diverged rapidly, with very short internodes between divergence events. Phylogenetic analysis of repleta group lineages revealed that *D. inca* and *D. canalinea* are sister to all other repleta group species, whereas the annulimana species *D. aracataca* and *D. pseudotalamancana* are sister to the nannoptera and bromeliae species groups. Our divergence time estimates suggest that the nannoptera species group radiated following important geological events in Central America. Our results indicate that a single evolutionary transition to asymmetric genitalia and to unusual sperm storage may have occurred during evolution of the nannoptera group.

Introduction

Species of the genus *Drosophila*, because of their well-defined phylogenetic relationships and diverse ecologies and life histories, provide an attractive group of model organisms for the study of evolution (Markow & O'Grady, 2007). A few taxa in Drosophilidae have evolved the ability to feed and breed in necrotic cactus, predominantly in the repleta and nannoptera species groups (Markow & O'Grady, 2007). The nannoptera species group consists of only four described species: *Drosophila nannoptera* (Wheeler, 1949), *D. acanthoptera* (Wheeler, 1949), *D. wassermani* (Pitnick & Heed, 1994) and *D. pachea* (Patterson & Wheeler, 1942). Even though all species of the nannoptera group live on columnar cacti, they exhibit diverse degrees of ecological specialization. Whereas *D. nannoptera* can live on a variety of host plants of the genera *Stenocereus*, *Pachycereus*, *Escontria* and *Myrtillocactus* (Heed, 1982), *D. acanthoptera* and *D. wassermani* are restricted to species in the genus *Stenocereus* (Heed, 1982). An even more tight ecological specialization links *D. pachea* to a single host plant, the senita cactus *Lophocereus schottii* (Engelmann, 1852), which is toxic to the other three species of the nannoptera group (Heed & Kircher, 1965; Etges *et al.*, 1999) and which provides a particular sterol (lathosterol) absolutely required for *D. pachea* survival (Heed & Kircher, 1965; Lang *et al.*, 2012).

The geographic distribution of *D. pachea* coincides with the distribution of senita cactus, which is restricted to the Sonoran desert in north-west mainland Mexico and to the Baja California peninsula (Fig. 1; Lindsay, 1963; Hastings *et al.*, 1972). The Gulf of California and the Sierra Madre Occidental mountain range on the mainland separate the distribution of *D. pachea* from *D. wassermani* (Fig. 1b). The other nannoptera species are found in an overlapping region in southern Mexico (Heed, 1982; Markow & O'Grady, 2005). *Drosophila nannoptera* generally localizes in highlands, whereas *D. wassermani* is primarily found in lowlands (Heed,

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1982). Specimens of *D. acanthoptera* were also reported from Venezuela (Hunter, 1970), but this single sampling record, based on morphological characterization, remains dubious.

The nannoptera species group inhabits a zone of important geological history. About 15 million years ago (Ma), seismic activity along a volcanic arc formed the Isthmus of Panama that connected Central and South America (Montes *et al.*, 2012a,b). The formation of the isthmus had a huge biological and climatic impact and provided a means for terrestrial fauna to move between the two continents (Webb, 1976; Leigh *et al.*, 2014). More recently, about 6–3 Ma, the Baja California peninsula formed as a result of a series of complex geological events that caused the successive separation of landmasses from mainland Mexico (Lizarralde *et al.*, 2007; Umhoefer, 2011). Increased desertification of North and South America in the past 10 Ma due to a global climate change and an uplift period of the Andes during the late Miocene-Pliocene (Gregory-Wodzicki, 2000; Capitanio *et al.*, 2011) was accompanied by radiations of major succulent plant lineages (Arakaki *et al.*, 2011). Whether these geological events might have influenced speciation within the nannoptera species group is unknown.

In addition to the ability to utilize cactus tissue as a resource, some unusual reproductive characters have evolved in the nannoptera species group. For instance, sperm gigantism was observed in *D. pachea* and *D. nannoptera*, but not in the other two members of the group, *D. acanthoptera* and *D. wassermani* (Pitnick *et al.*, 1995). Two additional very curious reproductive characters, genital asymmetry (Vilela & Baechli, 1990; Pitnick & Heed, 1994; Lang & Orgogozo, 2012) and site of sperm storage in females (Pitnick *et al.*, 1999), have also been reported in a few species of the nannoptera group. Whereas *D. nannoptera*, like most other Drosophilidae, has fully symmetric genitalia (Vilela & Baechli, 1990; Huber *et al.*, 2007), the other three species of the nannoptera group possess diverse genital organs with conspicuous left-right asymmetric morphologies. *Drosophila pachea* displays an epandrial lobe size asymmetry (Pitnick & Heed, 1994; Lang & Orgogozo, 2012),

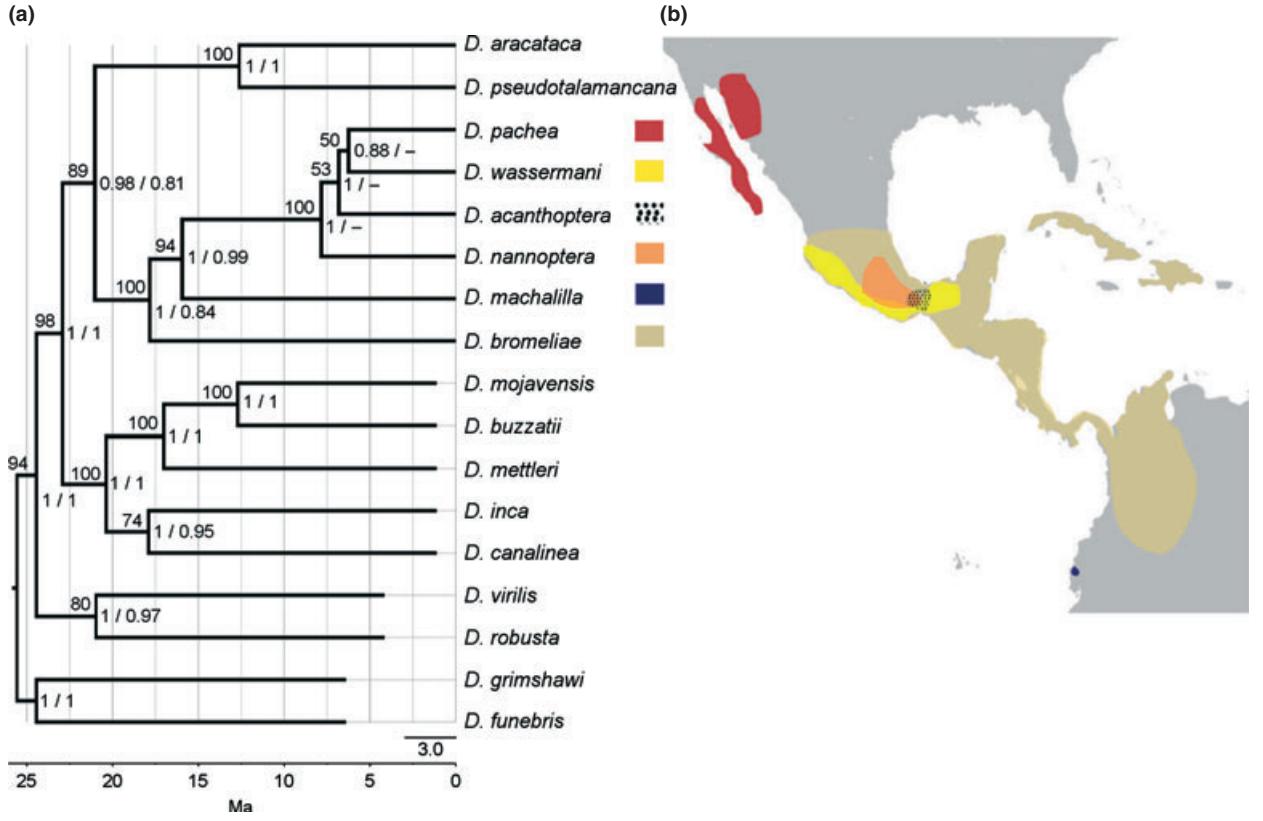

Fig. 1 Phylogenetic relationships of the nannoptera group and related species. (a) Phylogenetic tree generated in BEAST based on the concatenated data set with nine partitions. Bootstrap support from maximum likelihood (PhyML) analysis is presented on the left side of each node. Bayesian posterior probabilities are presented on the right side of each node for the BEAST analysis/and *BEAST analysis, respectively. The time scale was calculated according to estimates B in Table 1. (b) Distributions of the species of the nannoptera group and of *Drosophila machalilla* and *D. bromeliae*, reproduced from Heed (1982) and Markow & O'Grady (2005).

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D. acanthoptera possesses an asymmetric phallus (Vilela & Baechli, 1990), and *D. wassermani* has a left–right concave–convex-shaped cercus (Pitnick & Heed, 1994; Fig. 2). Furthermore, *D. nannoptera*, like most other *Drosophila* species, uses two types of organs for post-copulatory storage of sperm in females: the paired spermathecae and the single seminal receptacle. However, *D. acanthoptera*, *D. wassermani* and *D. pachea* use only the spermathecae (Pitnick *et al.*, 1999). Therefore, with regard to the evolution of asymmetric genitalia and unusual sperm storage, the most parsimonious scenario would be that *D. nannoptera* is an out group relative to the other three species of the nannoptera group.

For the reasons mentioned previously, the nannoptera group thus represents an interesting model system to tackle a variety of important questions in evolutionary biology, such as the influence of abiotic and biotic factors on speciation, the genetic causes of ecological specialization and the evolution of reproductive characters. To address these questions and to trace back the evolution of different characters across the nannoptera species group, a reliable phylogeny of the four species and related taxa is required. Whereas relationships within the repleta group have been characterized to a great extent (Van der Linde *et al.*, 2010; Oliveira *et al.*, 2012), previous phylogenetic studies of the nannoptera group have led to equivocal and conflicting results (Pitnick *et al.*, 1995, 1997, 1999; Van der Linde *et al.*, 2010; Oliveira *et al.*, 2012; Yassin, 2013). Some of these analyses were based on relatively few genetic loci (Pitnick *et al.*, 1995, 1997, 1999) and others that included more loci either lacked appropriate out groups (Oliveira *et al.*, 2012) or did not examine all members of the nannoptera species group (Van der Linde *et al.*, 2010; Yassin, 2013). In a morphological analysis based on internal reproductive organ morphology, Heed (1982) proposed a phylogeny of the nannoptera group, with *D. wassermani* and *D. pachea* forming two sister species, which are in turn sister to *D. acanthoptera*, and with *D. nannoptera* being out group relative to the other

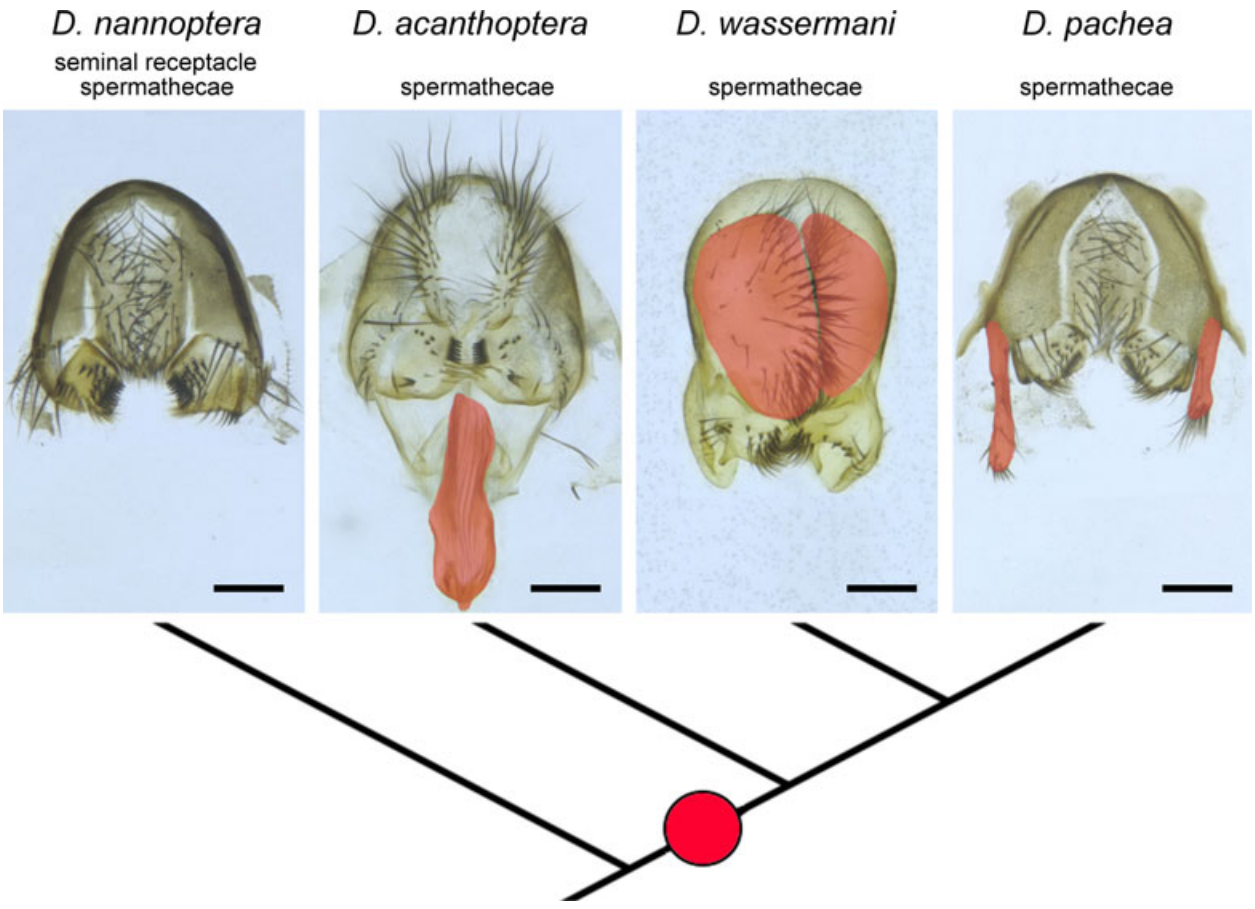

Fig. 2 Hypothetical character evolution of asymmetric male genitalia in the nannoptera species group. The red dot indicates the putative origin of both left–right asymmetric male genitalia and spermathecae-restricted sperm storage. Images below each species names illustrate male external genitalia of each species. Asymmetric parts were artificially coloured in red. Sperm storage organs are indicated below each species name. The scale bar is 100 μm.

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three species. According to this topology, the evolution of asymmetric genitalia and the evolutionary change in sperm storage would have occurred only once, whereas sperm gigantism would have evolved twice within the nannoptera group.

The closest relatives of the nannoptera group are thought to be the bromeliae species group (Van der Linde *et al.*, 2010; Yassin, 2013) and a newly described species, *D. machalilla* (Acurio *et al.*, 2013; A. Acurio, K. Goodman, D.C. Oliveira, V. Rafael & A. Ruiz, unpublished) that was proposed to belong to a new species group, the atalaia group (Acurio *et al.*, 2013). Whereas the nannoptera and bromeliae species groups are part of the virilis-repleta group radiation (Throckmorton, 1975), the particular branching order of lineages leading to the nannoptera group has never been fully resolved (Van der Linde *et al.*, 2010; Oliveira *et al.*, 2012; Yassin, 2013).

Here, we address the phylogenetic relationships of the four species of the nannoptera group and related taxa. Using a multilocus data set of gene coding regions of eight nuclear and three mitochondrial genes, we found that the four described nannoptera group species diverged rapidly. We discuss the nannoptera group radiation with respect to important geological events in Central America. Furthermore, our results allow us to propose a scenario for the evolution of reproductive traits in the nannoptera group.

Materials and methods

Taxon sampling

In addition to the four described species of the nannoptera group, we sequenced two species, *D. bromeliae* and *D. machalilla,* that are hypothesized to be close relatives of the nannoptera group based on morphology and previous phylogenetic analyses (Van der Linde *et al.*, 2010; Oliveira *et al.*, 2012; Yassin, 2013; A. Acurio, K. Goodman, D.C. Oliveira, V. Rafael & A. Ruiz, unpublished). We also included the annulimana group species *D. aracataca* and *D. pseudotalamancana,* and *D. canalinea* of the canalinea group to represent sister lineages of the repleta group. From the repleta group, we included four representative species, *D. inca* (inca subgroup), *D. mettleri* (mulleri subgroup), *D. buzzatii* (mulleri subgroup, buzzatii species complex) and *D. mojavensis* (mulleri subgroup, mojavensis species complex). *Drosophila virilis* and *D. robusta* were chosen as distant lineages of the repleta-virilis radiation (Van der Linde *et al.*, 2010; Yassin, 2013) and *D. funebris* and *D. grimshawi* were used to root the phylogeny. The latter two species belong to different species radiations of the *Drosophila* subgenus (Throckmorton, 1975; Van der Linde *et al.*, 2010; Yassin, 2013). Flies were obtained from the *Drosophila* Species Stock Center (Table S1), except for *D. machalilla and D. inca* (both collected by A. Acurio) and *D. buzzatii* (provided by Jean David).

DNA sequencing

Genomic DNA was obtained in a single extraction per species including 2–5 adults using the DNeasy blood and tissue kit (QIAGEN, Hilden, Germany). Partial genomic regions of eight nuclear genes (*amy, amyrel, boss, fkh, marf, sinA, snf, wee*) and three mitochondrial genes (*ND2*, *COI*, *COII*) were amplified by PCR with gene-specific or degenerate primers (Liu & Beckenbach, 1992; Bonacum *et al.*, 2001; Wang *et al.*, 2006; Da Lage *et al.*, 2007; Table S2). Degenerate oligonucleotides optionally contained T7 or SP6 universal primer sequences at their 5′ end (Table S2), following Bonacum *et al.* (2001). For PCR amplifications, we used 0,4 μM oligonucleotides, 1 u GoTaq® DNA Polymerase (Promega, Fitchburg, WI, USA) per 35 μL reaction volume, 2 mM $MgCl_2$ and 200 μM dNTP, and reactions were carried out using standard thermocycle conditions. PCR products were purified and Sanger-sequenced with gene-specific primers or with T7, SP6 universal primers at Cogenics (www.cogenics.com, Beckman Coulter, Brea, California, USA). Sequence data (GenBank accession numbers KF632591-KF632711; Table S3) were examined and aligned with Geneious 6.1.3 (Biomatters, Auckland, New Zealand). Additional sequence data were retrieved from GenBank (Table S3). We generated a data set that contained all the selected homologous genomic regions of all species. DNA sequences were aligned using MAFFT (Katoh *et al.* 2002). Nuclear loci were tested for recombination with the pairwise homoplasy index (Φ_w) statistics using Phi-Pack (Bruen *et al.*, 2006). No evidence of recombination was detected (Table S4). A total of 121 polymorphic sites were detected within single sequences based on the presence of double peaks in sequencing chromatograms. Thirty-eight of these polymorphic sites were found in the nannoptera species group sequences. All polymorphic sites were excluded from the analysis. Furthermore, noncoding DNA sequences were removed from the data set, as well as a short region of the *wee* locus (sequences homologous to positions 267–306 in *D. pachea wee*, accession number KF632622), which was difficult to align and that did not contain any parsimony informative sites in the nannoptera species group. The extremities of each locus-specific alignment were also trimmed to be in codon frame. Alignments were re-aligned with the Geneious translation alignment program and either used separately or concatenated in the following order: amy-amyrel-boss-fkh-marf-sinA-snf-wee-ND2-COI-COII. The number of informative sites was calculated using MEGA4 (Tamura *et al.*, 2007).

Phylogenetic analysis

Phylogenetic analysis was performed using both maximum likelihood and Bayesian approaches. For all analyses, models of nucleotide substitution were selected using the Akaike Information Criterion as cal-

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culated in jModelTest 2.1.3 (Posada, 2008; Darriba *et al.*, 2012). Maximum likelihood inference was carried out on the concatenated data set in PhyML (Guindon & Gascuel, 2003) using a GTR+I+G model of nucleotide substitution. Node support was determined by performing 100 bootstrap replicates.

Two types of Bayesian analyses were carried out in BEAST v1.7.5 (Drummond *et al.*, 2012). The first analysis was carried out on the concatenated data set using nine partitions with individual and unlinked models of nucleotide substitution (Table S5). The partitions corresponded to the eight nuclear loci (*amy*, *amyrel*, *boss*, *fkh*, *marf*, *sinA*, *snf* and *wee*) plus a ninth partition for mitochondrial sequences (*ND2*, *COI*, *COII*). Mitochondrial genes were combined into one partition because they are located in the same order and orientation in the mitochondrial genome, and largely evolve as a single unit with little to no recombination (Ballard, 2000). Clock models were linked, and a common strict clock rate was assumed for all partitions using the Yule birth process tree prior. We also estimated a species tree using *BEAST (Heled & Drummond, 2010). For the *BEAST analysis, tree and clock models were unlinked for each partition and a relaxed exponential clock model was assumed.

For species divergence time estimates, we set priors for most recent common ancestors (MRCA) using estimates from Obbard *et al.* (2012) for the splits *D. grimshawi* – *D. virilis*: 13 ± 2.5 Ma and *D. mojavensis* – *D. virilis*: 10 ± 2.5 Ma (estimates A). Alternatively, calibration dates for the divergence of *D. grimshawi* – *D. virilis*: 42.9 Ma ± 8.7 (Tamura *et al.*, 2004), *D. mojavensis* – *D. virilis*: 26 ± 6 Ma (Russo *et al.*, 1995; Spicer & Bell, 2002; Oliveira *et al.*, 2012), *D. mojavensis* – *D. buzzatii*: 11.3 ± 2 Ma and *D. mojavensis* – *D. mettleri*: 16.3 ± 2 Ma (Oliveira *et al.*, 2012) were used (estimates B). Priors were assumed to follow a normal distribution with the mean and a standard deviation according to the literature estimates. Markov-Chain Monte-Carlo (MCMC) runs were performed with a chain length of 10^8 generations and were recorded every 1000 generations. Estimates were computed with Tracer version 1.5 (Rambaut & Drummond, 2009), and MCMC output analysis was carried out using TreeAnnotator (Drummond *et al.*, 2012). The first 2000 sampled trees were discarded as the burn-in. Phylogenies were visualized and annotated with Figtree version 1.4 (Rambaut & Drummond, 2012).

Results

Phylogenetic analysis

To analyse the phylogenetic relationships of the nannoptera group, we gathered DNA sequences of partial coding gene regions of eight nuclear genes (*amy*, *amyrel*, *boss*, *fkh*, *marf*, *sinA*, *snf*, *wee*) and three mitochondrial genes (*ND2*, *COI*, *COII*) from 17 species. The entire data set comprised 6810 aligned positions, including 4208 constant positions, 695 single variable positions and 1907 parsimony informative (28%) positions (Table S3).

Phylogenetic analysis of the concatenated, nine-partition data set was performed using a maximum likelihood approach in PhyML (Guindon & Gascuel, 2003) and by Bayesian inference in BEAST (Drummond *et al.*, 2012). Phylogenetic relationships inferred from these analyses resulted in identical tree topologies, but with varying node support values (Fig. 1). The resulting phylogeny supports Acurio *et al.*'s findings (A. Acurio, K. Goodman, D.C. Oliveira, V. Rafael & A. Ruiz, unpublished) that the nannoptera species group is a sister clade of the atalaia species group, with *D. machalilla* being more closely related to the nannoptera clade than to *D. bromeliae* (bromeliae group). In our phylogeny, *D. inca* and *D. canalinea* form a lineage sister to the repleta group. Furthermore, both the Bayesian and the maximum likelihood phylogeny provided, for the first time, strong support for the monophyly of annulimana species, *D. aracataca* and *D. pseudotalamancana,* which we found to be more closely related to the nannoptera group than to the repleta group. Phylogenetic relationships within the nannoptera group were relatively well-resolved in the Bayesian analysis, but not in the maximum likelihood analysis. Our results from the Bayesian analysis were congruent with the phylogenetic relationships previously suggested based on morphological data (Heed, 1982) (Fig. 1a).

Analysis of concatenated multilocus data has recently been criticized as it poorly integrates locus-specific phylogenetic signals and can lead to false phylogenetic inferences with high statistical support (Song *et al.*, 2012). Therefore, we also analysed our data set with *BEAST (Heled & Drummond, 2010), an extension of the BEAST package that incorporates coalescence models to estimate a species tree from multiple gene-specific phylogenies. The topology of the species tree inferred in *BEAST was similar to the phylogeny obtained with the concatenated data set (Fig. 1a, Fig. S1), except that relationships within the nannoptera group differed, with *D. pachea* being sister to the clade containing *D. acanthoptera* and the sister species pair *D. nannoptera* and *D. wassermani* (Fig. S1). However, the posterior probability for the corresponding nodes were low, suggesting that our data set might not contain enough information for species tree estimation using *BEAST. Within the nannoptera group, we observed only 120 parsimony informative sites across all genes in the data set (Table S3). The mean length of DNA sequence per nuclear locus was 477 ± 178 bp (SD), which, on average, included only 5 ± 3 (SD) parsimony informative sites among the nannoptera species group (Table S3). We wondered whether the number of informative sites per locus was too low for a

coalescent multilocus phylogenetic approach to produce a well-resolved species tree. To estimate the number of loci that would be necessary to reliably establish nannoptera group relationships using *BEAST, we produced partial data sets containing 2, 4, 6 or 8 loci and inferred the phylogeny of each data set. The average node support was calculated and the number of necessary loci was approximated by a logarithmic regression (Fig. S2). Whereas we are aware that the number of parsimony informative sites per locus is low and that the data sets are partially redundant, this analysis showed that for the entire phylogeny, the average posterior probability was 0.90 when six and eight loci were used. Support for the nodes within the nannoptera group also increased with the number of loci, but at a much lower rate. We estimated that approximately 60 loci would be required to obtain a posterior probability of 0.90 for the internal nodes within the nannoptera group.

Divergence time estimates

We estimated the divergence times of the nannoptera group radiation and the splits of *D. machalilla* and *D. bromelia* from the branch leading to the nannoptera species group. There is conflicting information about species divergence times in *Drosophila*. Most estimates are based on the phylogeny of Hawaiian Drosophilidae where species divergence times can be approximated based on the ages of the islands they inhabit (Price & Clague, 2002). Recently, Obbard *et al.* (2012) proposed a refinement of this approach to take lineage-specific variation of mutation rates into account (Obbard *et al.*, 2012). This new approach suggested a younger age for the virilis-repleta radiation, of about 10 Ma compared to the previous estimates of 20 Ma (see Material and methods). We computed species divergence times either based on Obbard *et al.* (2012) (dates A) or based on previous species divergence estimates (dates B) (Table 1).

As the calibration estimates in A were about half the ages in B, divergence time estimates of dates A were expectedly also half the age compared to estimates B.

Table 1 Divergence time estimates.

	Divergence time estimates*	
Dated nodes	*A*	*B*
D. mojavensis – D. buzzatii	5.5 Ma (3.5–7.5)	12.2 Ma (10.0–14.5)
D. mojavensis – D. mettleri	7.5 Ma (4.8–10.1)	16.7 Ma (13.7–19.6)
D. pachea – D. wassermani	3.0 Ma (1.9–4.1)	6.7 Ma (5.3–8.1)
D. pachea – D. nannoptera	3.7 Ma (2.4–5.0)	8.3 Ma (6.7–10.0)
D. pachea – D. machalilla	7.5 Ma (4.9–10.2)	16.9 Ma (13.7–20.1)
D. pachea – D. bromeliae	8.4 Ma (5.4–11.3)	17.9 Ma (15.8–20.0)
D. virilis – D. mojavensis	10.6 Ma (7.0–14.3)	18.9 Ma (15.3–22.4)

*Estimates are the posterior means with 95% highest posterior density intervals.

We estimated that the nannoptera group lineage diverged about 3.7 Ma (dates A) or 8.3 Ma (dates B). Furthermore, the most recent split of *D. pachea* and *D. wassermani* was estimated to have occurred shortly thereafter, about 3.0 Ma (dates A) or 6.7 Ma (dates B). The *D. machalilla* lineage separated from the nannoptera group about 7.5 Ma (dates A) or 16.9 Ma (dates B) and the bromeliae group separated from the nannoptera group lineage about 8.4 Ma (dates A) or 17.9 Ma (dates B). Based on the conflicting calibration, these dates do not precisely estimate speciation events, but they put the nannoptera group radiation into an approximate time frame.

Discussion

Origin of the nannoptera group

The four species of the nannoptera group are endemic to distinct regions of Mexico (Fig. 1b). Our phylogenetic analysis uncovered three closely related out groups to the nannoptera group: *D. machalilla*, the *bromeliae* species group (comprising five species including *D. bromeliae*) and the members of the annulimana group. Members of these species groups are primarily found in South America but also in Central America (Fig. 1b) (Sturtevant, 1916; Duda, 1927; Pavan & da Cunha, 1947; Do Val & Marques, 1996; Da Silva *et al.*, 2004; Markow & O'Grady, 2005; Acurio *et al.*, 2013). These species distributions thus suggest that the ancestor of the four nannoptera group species may have originated from South America. Interestingly, our dating analysis estimates that the nannoptera group diverged from *D. machalilla* around 16.9 Ma (B) – 7.5 Ma (A). This time period corresponds to the closure of the Isthmus of Panama, about 15–9 Ma (Montes *et al.*, 2012b), suggesting that the ancestor of the nannoptera group may have migrated over the newly formed isthmus from South America. Most species were found to migrate across the isthmus much later, at about 3–2 Ma (Leigh *et al.*, 2014). However, exceptions are known such as the extinct carnivora *Cyonasua* and ground sloths, which migrated about 9 Ma from north to south and south to north, respectively (Webb, 1976). Furthermore, recent data suggest that the isthmus was already passable for stingless bees at late Eocene and early Miocene times (20–15 Ma), which migrated from South to Central America (Roubik & de Camargo, 2012). The isthmus might have faced multiple events of temporary land bridge formations and disconnections, allowing a few species to cross continents before a permanent land bridge formed about 4–3 Ma (Webb, 1976; Roubik & de Camargo, 2012; Stone, 2013).

Drosophila species have been extensively sampled in Mexico and Central America (Patterson & Stone, 1952), but multiple areas known as biodiversity hotspots in South America are still unexplored. An origin of the

nannoptera group in South America would suggest that yet undescribed close out group species of the nannoptera group might be present in these geographic areas.

Radiation of the nannoptera species group

Within the nannoptera species group, we observed only 120 parsimony informative sites in our entire data set (Table S6), whereas 850 nucleotide changes were lineage-specific. The distribution of mutations indicates that the four species of the nannoptera group diverged within a relatively short time period. Phylogenetic inference can be particularly difficult in these cases especially if node support remains low with increasing data. One interpretation of the current data is that the ancestral lineage diverged nearly simultaneously into the four described extant lineages (Walsh *et al.*, 1999; Humphries & Winker, 2010). Such scenario might be expected from species with a large geographic range where peripatric speciation can occur in different regions. One well-studied example is the *D. simulans* species complex, where *D. mauritiana* and *D. sechellia* diverged independently on islands that were geographically separated from the cosmopolitan species *D. simulans* (Garrigan *et al.*, 2012).

Alternatively, very short internode distances could result from a rapid succession of divergence events that could be inferred with increasing amounts of data (soft polytomy). Our current sequence data are insufficient to distinguish between a soft and a hard polytomy in the nannoptera species group. The rapid and ongoing decrease in high-throughput sequencing costs now makes it more practical to sequence and to compare whole genomes for future studies aiming at a better resolution of the nannoptera group phylogeny.

Phylogenetic relationships and evolution of reproductive traits within the nannoptera group

Under the hypothesis of a soft polytomy in the nannoptera species group, the phylogeny of the nannoptera group that we inferred using the concatenated data (Fig. 1a) appears to propose the most plausible scenario, despite a low node support in one of our analyses (PhyML maximum likelihood analysis). Indeed, several lines of evidence corroborate this topology. First, compared to the previous molecular phylogenetic analysis of Oliveira *et al.* (2012), which hypothesized different relationships for the nannoptera group, our phylogeny is based on a higher number of loci and on an increased number of relevant out group species close to the nannoptera group.

Second, our inferred topology recapitulates the species relationships presented by Heed (1982) based on internal reproductive organ anatomy and by Pitnick *et al.* (1999) independently based on cytochrome oxidase data. Third, it is congruent with chromosome inversions. Comparisons of polytene chromosome banding patterns revealed that *D. nannoptera* and *D. wassermani* have a homosequential 'ancestral-like' chromosomal organization, whereas *D. acanthoptera* and *D. pachea* are derived with three and one inversion, respectively (Ward & Heed, 1970). A fourth, polymorphic, inversion is also found in *D. pachea* and is not detected in the other species of the nannoptera group (Etges *et al.*, 1999).

Fourth, our inferred topology is consistent with a parsimonious scenario of the evolution of the unusual reproductive characters within the nannoptera group. Genital asymmetry is found in *D. acanthoptera* (Vilela & Baechli, 1990), *D. wassermani* (Pitnick & Heed, 1994) and *D. pachea* (Pitnick & Heed, 1994; Lang & Orgogozo, 2012) (Fig. 2), whereas *D. nannoptera* (Vilela & Baechli, 1990), as well as the species *D. bromeliae*, *D. speciosa* and *D. aguape* of the bromeliae group and *D. machalilla* (atalaia group) have symmetric genitalia (Do Val & Marques, 1996; Da Silva *et al.*, 2004; Acurio *et al.*, 2013). Therefore, a single evolutionary transition to asymmetric genitalia might have occurred in the nannoptera group. Even though the asymmetry involves different male genitalia organs in each species, a common genetic and developmental process may underlie these distinct morphological asymmetries. We currently are trying to unravel the genetic factors that determine the asymmetric development of male genitalia in the three nannoptera species. In particular, we are testing whether genitalia clockwise rotation (Feuerborn, 1922; Suzanne *et al.*, 2010) during pupal development could be the signal that triggers differential growth between the left and right parts of various organs in distinct species. Furthermore, our inferred topology is consistent with a single evolutionary change in sperm storage in the nannoptera group. After copulation, females of *D. acanthoptera*, *D. wassermani* and *D. pachea* exceptionally use only the spermathecae to store the sperm and not the seminal receptacle as is typical for Drosophilidae, including *D. nannoptera* (Pitnick *et al.*, 1999). Future efforts are required to examine how copulation position might affect sperm transfer in the nannoptera species group and to determine whether asymmetric male genitalia and unusual sperm storage are functionally linked. Finally, according to our inferred phylogeny, sperm gigantism would have evolved twice independently in the *nannoptera* group, which is consistent with other reported instances of rapid evolution of sperm size in *Drosophila* (Pitnick *et al.*, 1995).

Evolutionary history of the nannoptera species group

Species divergence estimates for the virilis-repleta radiation vary greatly, from 30–20 Ma (Russo *et al.*, 1995; Spicer & Bell, 2002; Tamura *et al.*, 2004; Oliveira *et al.*, 2012) to 10 Ma (Obbard *et al.*, 2012) when adjusting

for lineage-specific mutation rates. We performed two separate estimations (Table 1) to account for two incompatible calibrations of species divergence estimates. The two sets of estimates (A and B) for the radiation of the nannoptera group approximate the lower and upper bounds of the geological time approximation of sea floor spreading of the southern Gulf of California. Formation of the Baja California peninsula started approximately 12 Ma due to changes in continental plate tectonics (Umhoefer, 2011). The peninsula itself formed along an almost north-south-directed rift, now partially covered by the Gulf of California. Landmasses separated from the continent as a result of complex geological events about 6–2.5 Ma and successively formed the peninsula (Lizarralde *et al.*, 2007; Umhoefer, 2011). Thus, the divergence of *D. wassermani* and *D. pachea* might have been influenced by the formation of the Baja California peninsula and by the separation of these landmasses from the continent (Heed, 1982). Whether senita cacti were already present in the forming Baja peninsula and whether *D. pachea* or its predecessors were already feeding on senita cactus when landmasses disconnected from the continent is unknown. A phylogenetic analysis of the senita cactus and its closely related species, together with estimations of divergence times, would be helpful to try to infer the evolutionary history of the close ecological relationship between *D. pachea* and its host cactus. The distribution area of *D. wassermani* is limited to the north by the Trans-Mexican Volcanic Belt and by the Sierra Madre Occidental mountain range (Fig. S3), which originated about 17–7 and 38–25 Ma, respectively (Ferrari *et al.*, 1999). A plausible scenario is that these mountains formed an obstacle for the ancestor of *D. wassermani* and *D. pachea*, which colonized further northern regions through the coastal lowlands of north-west Mexico (Heed, 1982). As this region successively re-arranged into the Baja California peninsula, the Gulf of California created a natural barrier and could have led to the isolation of *D. pachea* in the north and *D. wassermani* in the south (Fig. S3).

Drosophila machalilla, the most closely related outgroup of the nannoptera group, is a recently described species that was collected in traps containing *Opuntia* cactus, and the columnar cactus *Armatocereus cartwrightianus* (Britton & Rose, 1920) was proposed to be their native host plant (Acurio *et al.*, 2013). As all nannoptera group species also feed on columnar cacti, the MRCA of *D. machalilla* and the nannoptera species group was likely to be already cactophilic. Our results suggest that the major radiation of succulent plants, which occurred in the past 10 million years in North and South America (Arakaki *et al.*, 2011), could have then contributed to shifts in cactus hosts and to speciation in the nannoptera group.

In summary, our results indicate that the four species of the nannoptera group originated within a short time period. Our approximations of species divergence times suggest that the emergence of the southern Gulf of California might have been involved in the split between *D. pachea* and *D. wassermani*. The branching order of basal repleta lineages reveals that the annulimana species *D. aracataca* and *D. pseudotalamancana* are the most closely related taxa to the nannoptera and bromeliae species groups. Our phylogenetic analysis suggests that evolution of asymmetric genital and unusual sperm storage have evolved only once within the nannoptera group, and that the ancestor of the nannoptera group was already feeding on columnar cacti.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1 Species resources.
Table S2 Oligonucleotides used in this study.
Table S3 GenBank accession numbers of the data set.
Table S4 Partition specific models of nucleotide evolution and parameters.
Table S5 Test of recombination.
Table S6 Parsimony informative sites.
Figure S1 *BEAST analysis and gene-specific tree topologies.
Figure S2 Approximation of the number of loci required for a *BEAST multilocus analysis of the nannoptera group with reliable node support.
Figure S3 Hypothetical speciation scenario of *Drosophila wassermani* and *D. pachea*.

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Appendix 3.

Nueva especie cactofílica de Drosophila descubierta en Ecuador

02/2014 - **Biología.** **Ha sido descubierta en Ecuador una nueva especie de mosca endémica con manchas en el tórax, *Drosophila machalilla*, cuyo nombre específico hace referencia a una cultura prehispánica que habitó la región. Esta especie habita en cactus columnares y tiene una alta tolerancia a alcaloides tóxicos para otras especies. La futura secuenciación de su genoma permitiría buscar los genes implicados en la depuración de alcaloides tóxicos, así como la evolución de caracteres sexuales en dípteros.**

La nueva especie D. machalilla y el cactus columnar Armatocereus cartwrightianus, en donde ha sido colectada.

Un equipo conformado por investigadores de la UAB y la PUCE de Ecuador han descrito una nueva especie de mosca con manchas en el tórax que pertenece al género *Drosophila*, el organismo modelo más utilizado en investigación biológica, particularmente en Genética y Biomedicina.

El nombre de la nueva especie, *D. machalilla*, hace referencia a una cultura prehispánica (850-1400 d. C.) que habitó la región en la que fue descubierta y es endémica. El estudio publicado en la revista *Annals of Entomological Society of America*, fue realizado en el Departamento de Genética y Microbiología de la UAB e incluye una descripción morfológica completa de la especie en diferentes fases de su desarrollo, lo que ha servido para su clasificación.

Los especímenes tipo de *D. machalilla* se encuentran depositados en el Museo de Historia Natural de Nueva York. El análisis de su DNA ha determinado que las especies filogenéticamente más cercanas pertenecen al grupo *nannoptera*, moscas conocidas por habitar en cactus columnares y que tienen una alta tolerancia a alcaloides que son tóxicos para otras especies. Utilizando el reloj molecular se ha estimado que *D. machalilla* divergió del grupo *nannoptera* hace 7-17 millones de años.

El hallazgo de *D. machalilla* en Sudamérica abre interrogantes sobre cómo pudo haberse producido la separación de estos linajes, debido a que las especies más cercanas del grupo *nannoptera* habían sido registradas únicamente en zonas desérticas de Norteamérica. Según otro artículo publicado en *Journal of Evolutionay Biology*, realizado por investigadores de Francia, Estados Unidos y la UAB, el tiempo de divergencia estimado entre estas especies coincide con el período de formación del istmo de Panamá, sugiriendo que el ancestro del grupo *nannoptera* pudo haber migrado desde Sudamérica cuando se formó el itsmo.

Un futuro proyecto de investigación planea secuenciar el genoma de *Drosophila machalilla* y utilizar esta información en la búsqueda de genes implicados en la depuración de alcaloides tóxicos y la evolución de caracteres sexuales en dípteros.

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Acurio, A.; Rafael, V.; Céspedes, D.; Ruiz, A. Description of a new spotted-thorax Drosophila (Diptera, Drosophilidae) species and its evolutionary relationships inferred by a cladistic analysis of morphological traits. Annals of Entomological Society of America 106(6):695-705. 2013.
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Appendix 4.

Description of a new spotted-thorax *Drosophila* (Diptera, Drosophilidae) species and its evolutionary relationships inferred by a cladistic analysis of morphological traits.

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1. Background

In a taxonomic inventory of Drosophilidae in Ecuador (South America), we discovered a new species of cactophilic, spotted-thorax *Drosophila*. Analyzing its morphology, we found similarities with flies of two Neotropical spotted-thorax species groups of *Drosophila*, namely *repleta* and *peruensis*. Flies or DNA sequence data are unavailable for the latter species group, hindering a molecular approach (Fig 1).

Here we describe *Drosophila machalilla* sp. nov., and place it in the phylogeny of the genus *Drosophila* by performing a cladistic analysis using 52 morphological characters.

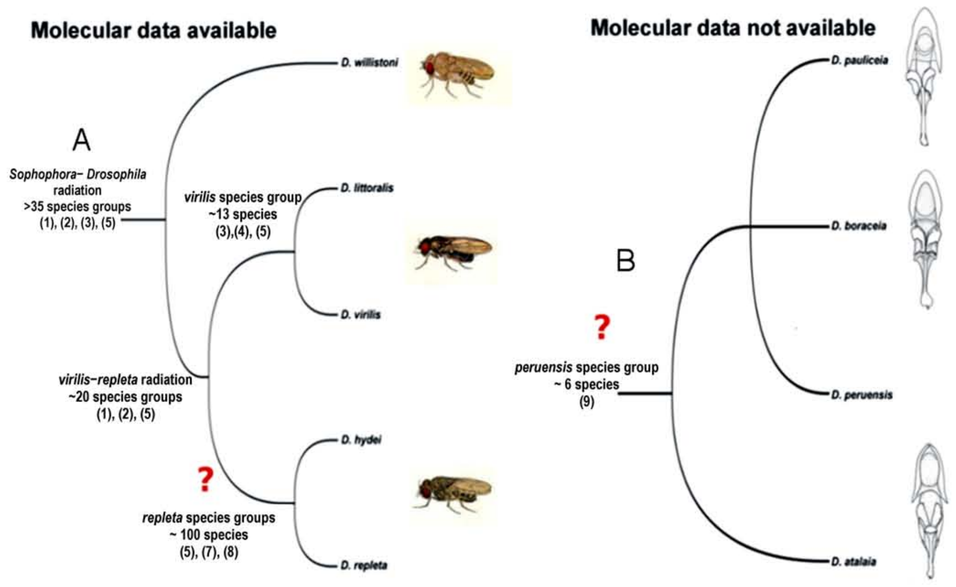
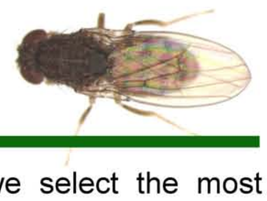
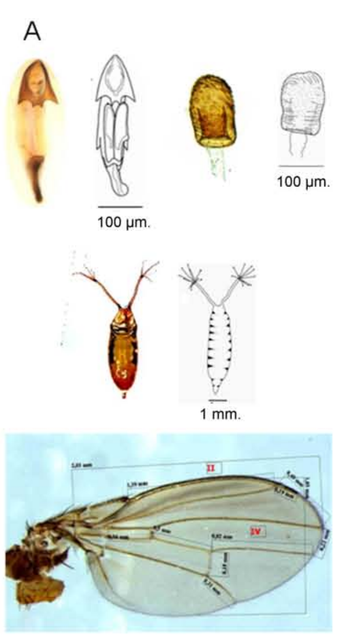


Figure 1. Evolutionary landscape of the species possible related to *D. machalilla* sp. nov. Numbers on parenthesis show phylogenetic studies supporting each evolutionary hypothesis. A: *Sophophora-Drosophila* radiation hypothesis, B: *peruensis* species group hypothesis.



2. Data and Methods

From our analysis (Fig 2A) and the literature, we select the most informative characters because (i) they correlate well with taxonomic grouping and (ii) they were independent. Our dataset contain 27 discrete and 25 continuous traits (Fig 2B). A Maximum Parsimony cladistic analysis was performed with TNT software (10). Continuous characters were analyzed as such and it optimized as additive, discrete characters were considered as unordered (11). To evaluate concordance between datasets two measures of group support were calculated, Jackknifing (P = 0.36) and Symmetric Resampling (P = 0.33) with 500 replications. Measures of raw frequency groups were calculated for both, the strict consensus tree obtained by discrete data set and the optimal tree obtained by the complete data set. Similarity on trees was estimated using SPR distances implemented on TNT.



B

Continuous	Discrete
0. top to bottom width ratio: [1.19-1.5]	25. dorsal branches on arista
1. frontal index: [0.78-1]	26. ventral branches on arista
2. check index: [3.5-12]	27. spot at base of setae
3. vibrissal index: [0.52-0.89]	28. rows of acrostichal setulae
4. eye index: [1.16-1.4]	29. prescutellars
5. or1/or3 ratio: [0.71-1]	30. shade at crossvein R-M
6. or2/or1 ratio: [0.5-0.7]	31. shade at crossvein dM-Cu
7. scut index: [0.72-1.08]	32. dark costal lappet
8. thorax lenght male: [0.73-1.55]	33. dark costal section apex
9. thorax lenght female: [0.51-1.41]	34. dark ring on tibia
10. sterno index: [0.48-0.87]	35. microtrichoses on surstylus
11. mid katepisternal: [29.5-54]	36. subapical aedeagal spurs
12. dc index: [0.58-0.77]	37. sclerotized median aedeagal teeth
13. h index: [0.61-1.6]	38. lower setae on hypandrium
14. wings lenght male: [1.52-3]	39. upper setae on hypandrium
15. wings lenght female: [1.9-3.07]	40. prensisetae on decasternum
16. lenght to width: [1.92-2.25]	41. outer setae on decasternum
17. Ac index: [1.9-3.09]	42. inner setae on decasternum
18. hb index: [0.38-0.61]	43. dorsal arch on hypandrium
19. C index: [1.69-3.79]	44. ventral rod on hypandrium
20. 4C index: [0.65-1.46]	45. marginal seta on hypandrium
21. 4v index: [1.47-2.22]	46. marginal ovisensilla
22. M index: [0.43-0.77]	47. discal ovisensilla
23. Prox.x index: [0.4-0.8]	48. lower m-d ovisensilla on oviscapt
24. 5X index: [1-2.14]	49. subterminal ovisensilla
	50. filaments on eggs
	51. traqueal branches on pupae

Figure 2. Morphological characters used on: (A) the taxonomical description of Drosophila machalilla sp. nov (B) The cladistic analysis, continuous characters with variation ranges on brackets.

3. Results

The implicit enumeration analysis of the 27 discrete characters alone, yielded two most parsimonious trees with a total adjusted homoplasy of 0.56 and 51 steps of length (Fig. 3 A, B), the strict consensus cladogram of which is shown in Fig. 3 C. *Drosophila machalilla* sp. nov., is a sister taxon of *D. atalaia*, and together conform a separate clade from *peruensis* and *repleta*. The *atalaia* clade is recovered using both discrete alone and complete dataset and supported by two synapomorphies (Fig 2B). The phylogenetic signal recovered with the discrete data alone, is good enough to recover the evolutionary relationships of species groups sampled. The addition of 25 continuous characters to the data matrix and an implicit enumeration search under the same parameters, it yielded the optimal tree of Fig. 3 D with a length of 129 steps. Autapomorphic features of *D. machalilla* sp. nov., are the sterno index, wing indices 4V and 5X.

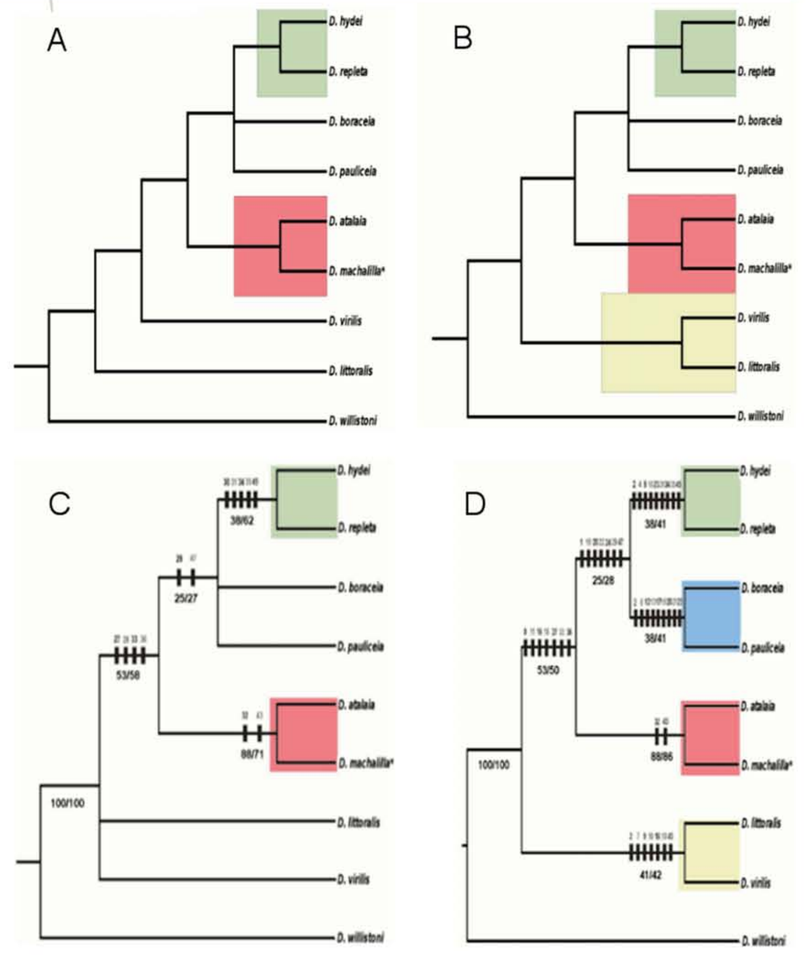


Figure 3. Cladograms obtained. In C and D synapomorphies (black rectangles) are mapped on trees, the numbers above rectangles refers to character numbers, the numbers beneath branching indicate group support Jackknifing (P=36)/Symmetric Resampling (P=33). Colors denote Drosophila clades: repleta clade (green), peruensis clade (blue), atalaia clade (red) virilis clade (yellow).

4. Study Implications

The results of this study are congruent with a molecular phylogenetic analysis using 5 molecular markers from *D. machalilla* sp. nov. and 53 taxa representative of *repleta*, *virilis* and *nannoptera* species groups (Acurio et al. in preparation).

Drosophila atalaia, previously classified as a member of the *peruensis* species group (9) and *Drosophila machalilla* sp. nov., are now grouped in the new *atalaia* species group on the basis of male and female genitalia, monophyly on a cladistic analysis (Figs.3,4), preference of substrate and habitat ecology.

Neotropical species of *Drosophila* with dark spots on mesonotum and a substrate preference for Cactaceae plants have been historically used as characters to identify species of the repleta group. In the light of our results, we recommend caution in the utilization of these traits for identification at lower taxonomical levels.

Currently it is unclear if the virilis-repleta radiation can be defined as monophyletic (7,12,13) in this context, high quality systematic research including both alpha-taxonomy and phylogenetically supported hypotheses becomes critical to better resolve the evolutionary relationships of a prime model system as *Drosophila*.

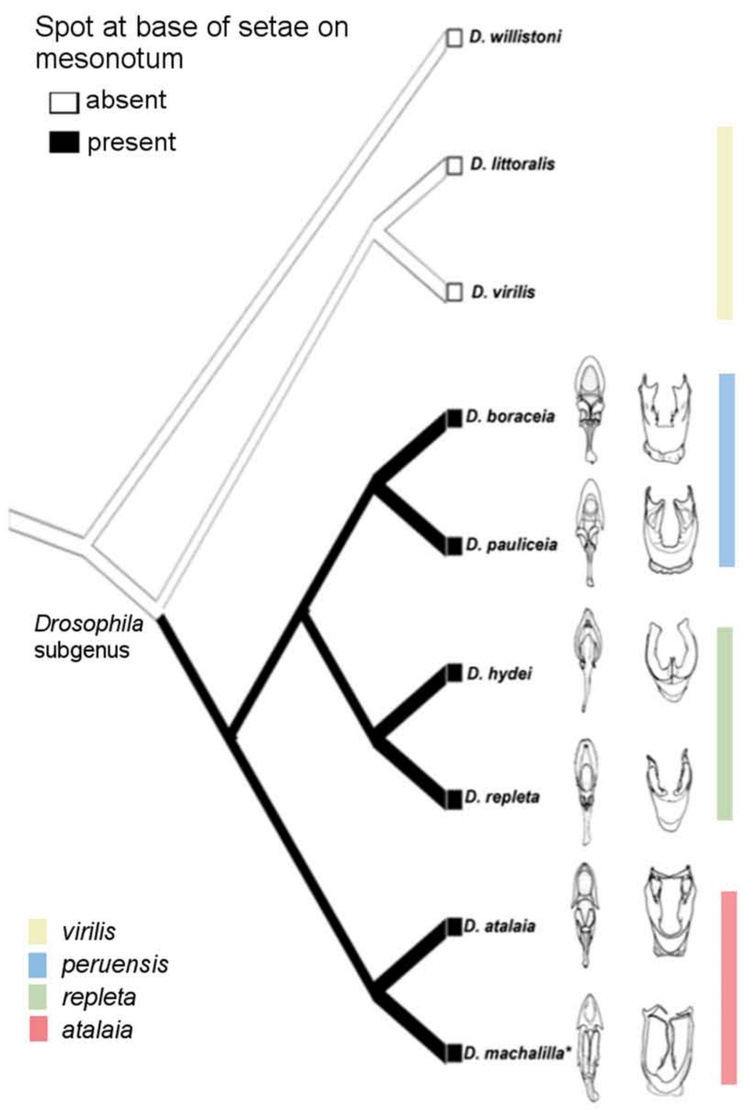


Figure 4. Phylogenetic tree of Drosophila relationships based in the cladistic analysis of 52 morphological traits with spotted- thorax character mapped onto it. Draws show the aedeagus and hypandrium structures of male genitalia

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Acknowledgments

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WHS XXXII Bostock 2013 100th Anniversary of the Birth of Willi Hennig

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Appendix 5.

Monophyly and placement of the *Drosophila inca* species subgroup corroborate the early South American diversification of the *Drosophila repleta* lineage

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Background

The *Drosophila repleta* species group is one of the largest in the subgenus *Drosophila*. This group has been used as a model system for genetic, ecological and evolutionary studies[1]. The *inca* subgroup[2] is the less well-known of six species subgroups of the *repleta* group. It was defined in 1989, to include three cactophilic species endemics to Ecuador (South America): *D. inca*, *D. huancavilcae* and *D. yangana*. The inclusion of these three species in the same subgroup was based on shared morphological traits, accordingly the evolutionary relationships of the *inca* subgroup in the repleta lineage is unclear. Here we include for the first time the *inca* subgroup in a molecular phylogenetic study in order to determinate its evolutionary relationships within the *Drosophila repleta* lineage.

Methods

Collections of *Drosophila* adults were carried out in xerophytic habitats of North Coast, Central and South of Ecuador (Acurio *et al. in preparation*). DNA was extracted from isofemale strains, amplified by PCR with specific primers and sequenced. Our dataset includes sequences of two mitochondrial (COI, COII) and two nuclear genes (Marf, SinA) generated by our collections and sequences of selected representatives from others five *repleta* species subgroups (*mulleri, fasciola*, *hydei*, *mercatorum, repleta*) drawn from a previous study[3]. *D. virilis* from the *virilis* group was used as outgroup. Two different phylogenetic approaches were used:
(1) Bayesian Inference: sequences were aligned with Clustal W, and analysed with BEAST[4] setting two partitions for mitochondrial and nuclear genes.
(2) Maximum Likelihood: sequences were aligned and a phylogenetic tree was simultaneously estimated using SATé[5].

Results

Both ML and partitioned Bayesian analyses produce single tress with the same well-supported topology (Figures 1 and 2). Both phylogenetic trees recover a monophyletic *inca* subgroup. On the *inca* clade, *D. inca* is most closely related to *D. huancavilcae* than *D. yangana,* which is the most ancestral from the three species. From the six species subgroups on the *Drosophila repleta* species group, the *inca* subgroup shows the most basal phylogenetic position.

Discussion

The Mexican Trans-Volcanic Region has been considered the center of diversification of the *repleta* group [6,7] because many years of collection efforts focused on this area. However, nowadays the diversity of *D. repleta* group species and other members of the *virilis-repleta* radiation[7] has become apparent in South America. A recent phylogenetic study[3] suggests a South American origin for the *repleta* lineage associated with their cactus host. The basal position in the phylogeny (Figures 1 and 2) of the three *inca* subgroup species that are seemingly endemics to Ecuador corroborates the hypothesis of the early South American diversification of the *Drosophila repleta* lineage.

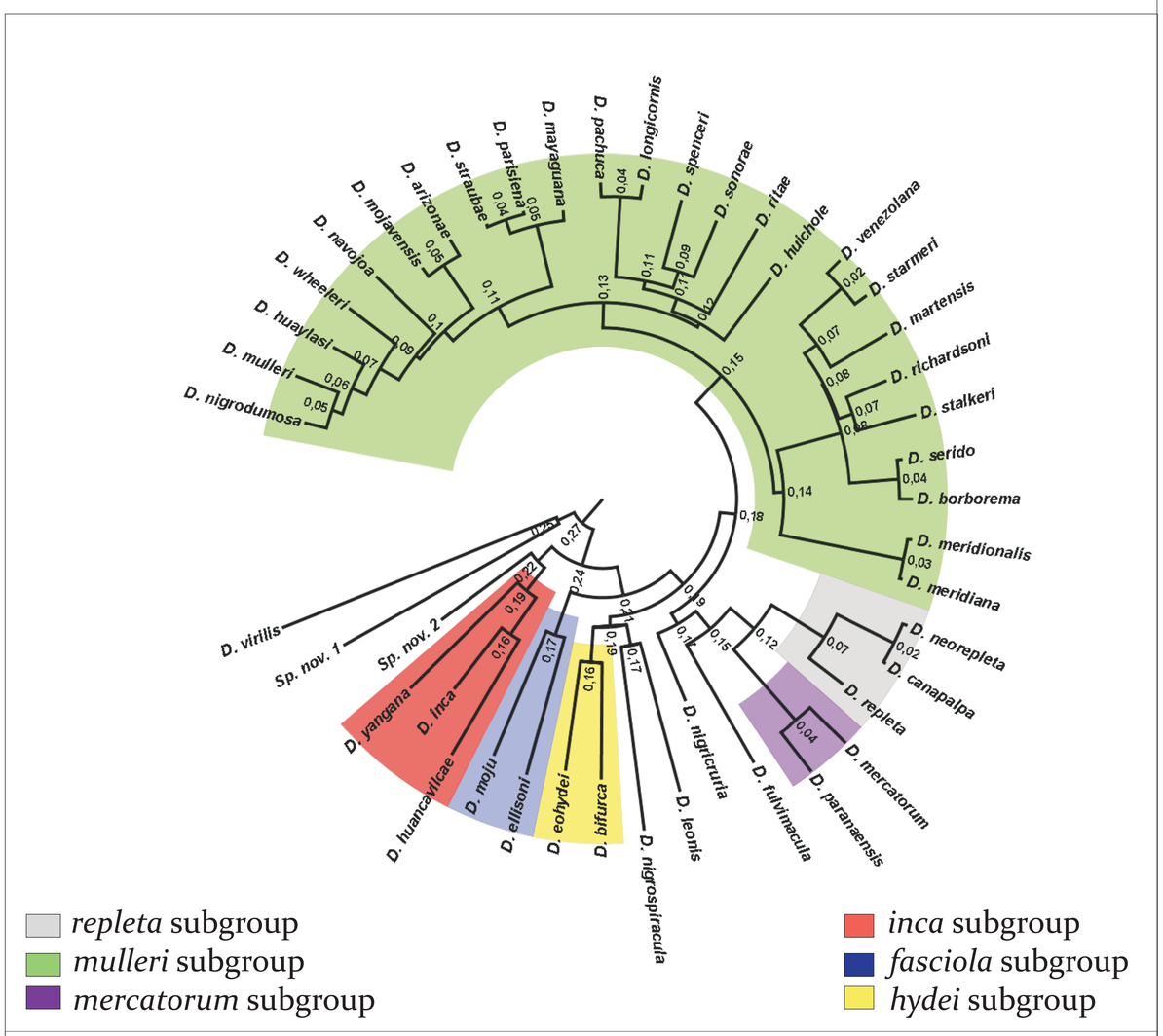

Figure 1. Molecular Phylogenetic tree obtained by Maximum Likehood with SATé setting MAFFT as aligner, RAXML as tree estimator and GTR GAMMA substitution model. Numbers denote support on each clade.

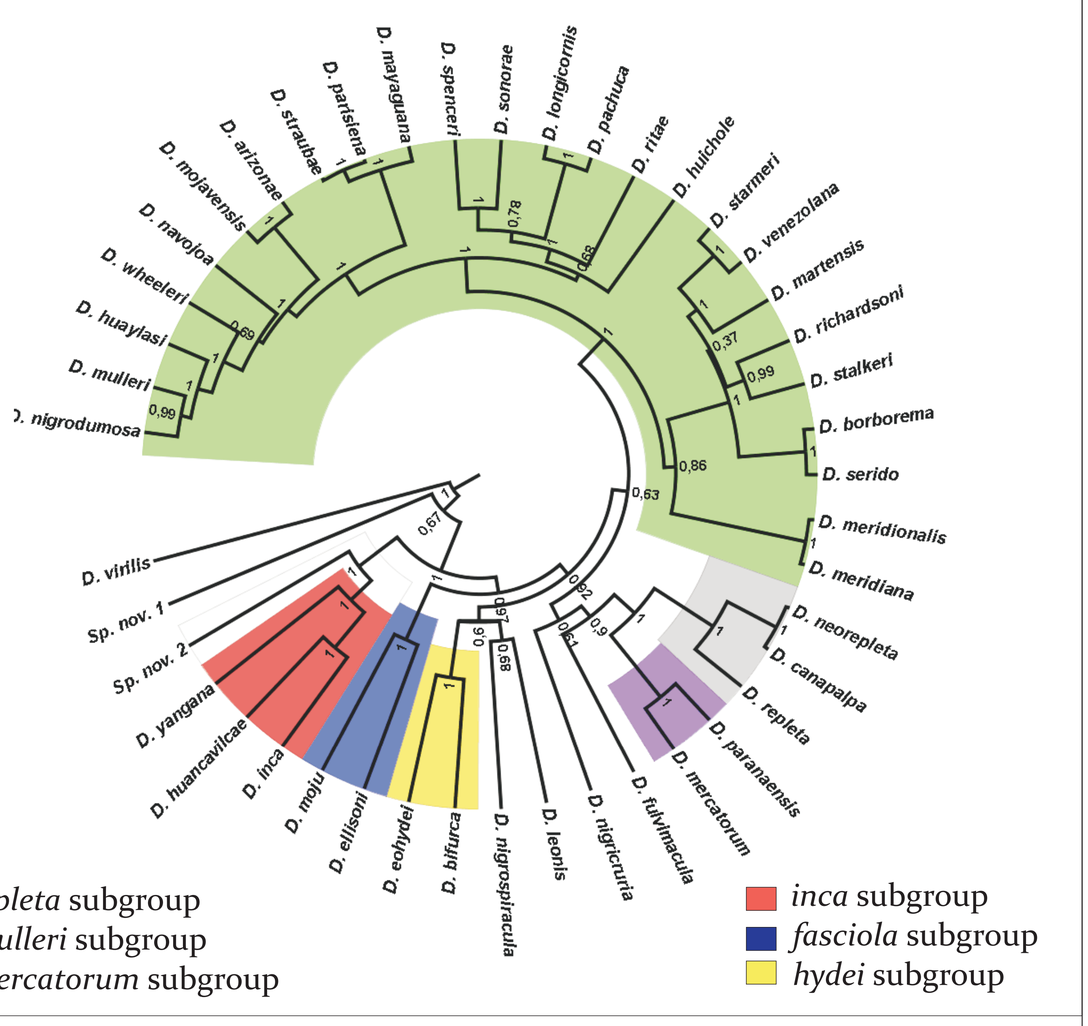

Figure 2. Molecular Phylogenetic tree obtained by Bayesian Inference with BEAST setting mitochondrial and nuclear partitions. Numbers denote BI posterior probability.

Acknowledgments

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Appendix 6.

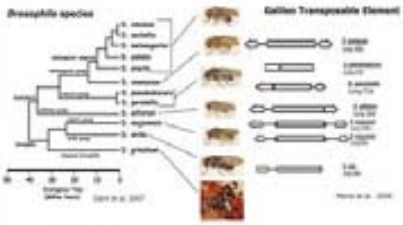
Dynamics and evolution of the transposable element *Galileo* in the genus *Drosophila*

Acurio A*, O' Grady P^, Oliveira CSG*, Etges W+, Cariou ML^^, Rafael Vº, Valente VLS~ & Ruiz A*

Aims

Galileo is a DNA transposon responsible for the generation of three chromosomal inversions in natural populations of *Drosophila buzzatii* (1,2,3).

Previously (4), in silico searches uncovered copies of Galileo in six of the twelve sequenced *Drosophila* genomes. A subfamily classification based on its TIR sequences resulted in five well supported groups: C,D, E, F and X (5).



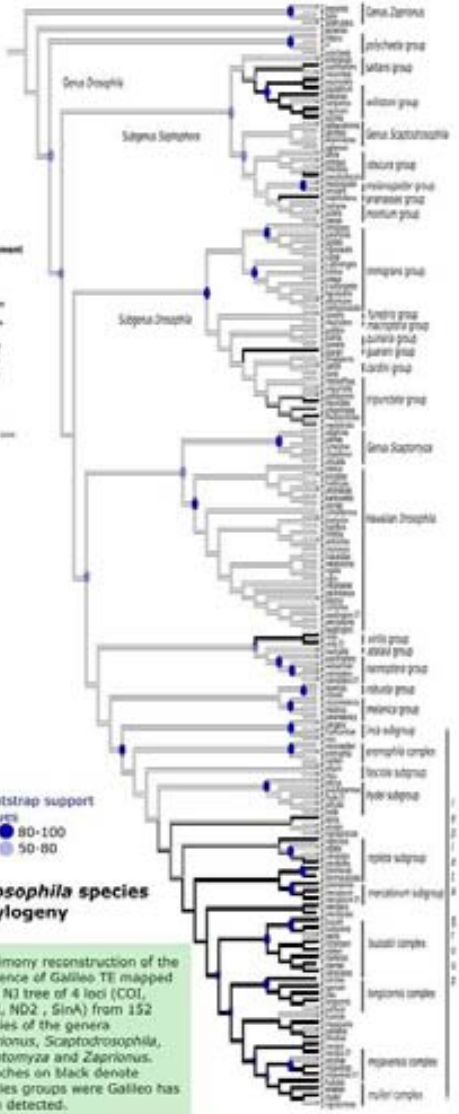
Material and Methods

We obtained a representative sample of genus *Drosophila*, gathered from field collections, fly donations, and *Drosophila* stock centers around the world. Using the information from the six *Drosophila* genomes sequenced, primers were designed to amplify by PCR the most conserved fragment of Galileo transposase region.

Results so far...

Galileo was inequivocall detected in 165 samples of 51 species from nine species groups: *repleta*, *willistoni*, *virilis*, *saltans*, *ananassae*, *montium*, *tripunctata*, *obscura* and *guarani*. Our results are consistent with an ancient origin of Galileo in the Drosophila genus and subsequent vertical transmission with some events of stochastic loss in some lineages. Galileo has a broad Neotropical distribution but was also found in samples from Europe and Africa.

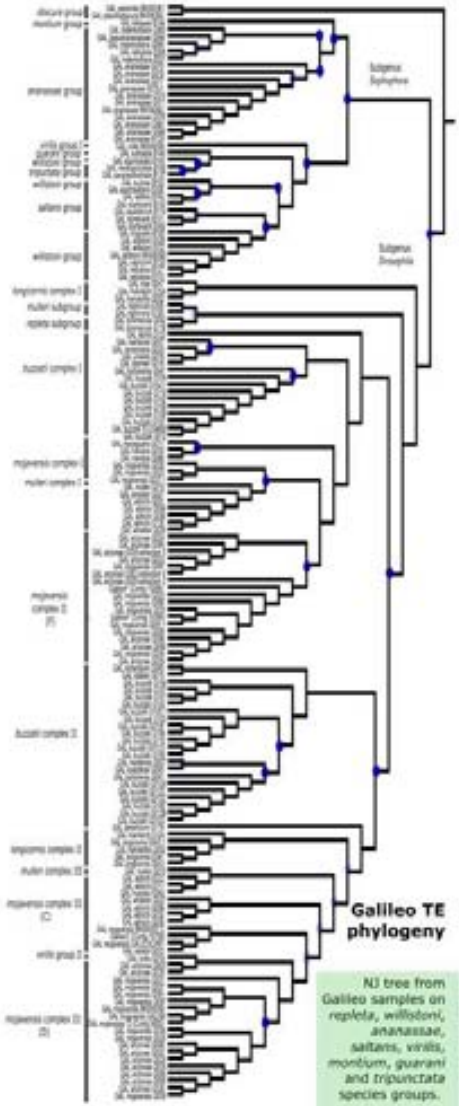
Galileo TE world distribution labeled on red dots.



Bootstrap support values
80-100
50-80

Drosophila species phylogeny

Parsimony reconstruction of the presence of Galileo TE mapped on a NJ tree of 4 loci (COI, COII, ND2 , SinA) from 152 species of the genera *Zaprionus*, *Scaptodrosophila*, *Scaptomyza* and *Zaprionus*. Branches on black denote species groups were Galileo has been detected.



Galileo TE phylogeny

NJ tree from Galileo samples on *repleta*, *willistoni*, *ananassae*, *saltans*, *virilis*, *montium*, *guarani* and *tripunctata* species groups.

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