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Short Communication

Phylogenetic relationships in the *Drosophila fasciola* species subgroup (Diptera, Drosophilidae) inferred from partial sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene

Erica C.C. Silva-Bernardi[1], Adriana C. Morales[2], Fabio M. Sene[1] and Maura H. Manfrin[2]

[1]*Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil.*
[2]*Departamento de Biologia, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil.*

Abstract

The *repleta* group is one of the largest and most complex species groups in the genus *Drosophila*, representing an important biological model for the elaboration of evolutionary and biogeographical hypotheses on the American Continent. It is divided into six subgroups, of which the *fasciola* subgroup is the only one with most of its species found in the humid forests of Central and South America. With the aim of understanding the origin and evolution of the *fasciola* subgroup, and consequently adding information about the *repleta* group, we analyzed partial sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene of nine *Drosophila* species (*D. carolinae*, *D. coroica*, *D. ellisoni*, *D. fascioloides*, *D. ivai*, *D. moju*, *D. onca*, *D. rosinae* and *D. senei*). The phylogenetic hypotheses obtained neither defined the relationships between the subgroups of the *repleta* group nor defined all the interspecific relationships in the *fasciola* subgroup. We found that the species *D. carolinae*, *D. coroica*, *D. onca*, *D. rosinae* and *D. senei* comprised a clade in which *D. carolinae*, *D. onca* and *D. senei* appeared together at a polytomy while *D. fascioloides* and *D. ellisoni* comprised another clade with a high bootstrap value.

Key words: fasciola subgroup, molecular phylogeny, mtDNA, systematics, *repleta* group.

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The *Drosophila repleta* species group, endemic to the American Continent, is one of the largest and most complex species groups in the genus *Drosophila*, because of which it represents an excellent biological model for speciation studies. This species group contains about one hundred described species (Vilela, 1983; Rafael and Arcos, 1989; Vilela and Bächli, 1990; Tidon-Sklorz and Sene, 1995a, 2001; Etges *et al.*, 2001; Bächli and Vilela, 2002) divided into the *D. fasciola*, *D. inca, D. hydei*, *D. mercatorum*, *D. mulleri* and *D. repleta* subgroups based on chromosome inversions and morphological characters (Wasserman, 1960, 1982, 1992; Vilela, 1983; Rafael and Arcos, 1989). The monophyly of the *repleta* group is defined on morphological (Throckmorton, 1975, 1982; Vilela, 1983), cytological (Wasserman, 1992) and molecular data (Tatarenkov and Ayala, 2001) but Durando *et al.* (2000) have suggested that the *repleta* group is not monophyletic, stating that '...this inference [of monophyly] may be the result of poor resolution at the base of the phylogenetic tree'.

The *repleta* group is widely distributed throughout the American Continent where it is mostly found in semiarid regions with open vegetation (Vilela, 1983; Vilela *et al.*, 1983; Tidon-Sklorz and Sene, 1995b). The *Drosophila* species in the *hydei*, *mercatorum* and *repleta* subgroups are mostly generalists, while those in the *inca* and *mulleri* subgroups use cacti as breeding sites (Pereira *et al.*, 1983; Rafael and Arcos, 1989).

The *fasciola* subgroup comprises 21 species (Vilela, 1983; Vilela and Bächli, 1990; Bächli and Vilela, 2002), of which 13 species (*D. carolinae*, *D. coroica*, *D. ellisoni*, *D. fascioloides*, *D. ivai*, *D. mapiriensis*, *D. moju*, *D. mojuoides*, *D. onca*, *D. papei*, *D. querubimae*, *D. rosinae* and *D. senei*) have been recorded in Brazil. Regarding the geographical distribution of the *fasciola* subgroup over the American Continent, five species (*D. ellisoni*, *D. fasciola*, *D. moju*, *D. mojuoides* and *D. pictura*) are found in South and Central America, ten species (*D. carolinae*, *D. coroica*, *D. fascioloides*, *D. ivai*, *D. mapiriensis*, *D. onca*, *D. papei*, *D. querubimae*, *D. rosinae* and *D. senei*) in South America,

Send correspondence to Erica Cristina de Carvalho Silva-Bernardi. Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900 Ribeirão Preto, SP, Brazil. E-mail: eccsilva@rge.fmrp.usp.br.

four species (*D. linearepleta*, *D. paraguttata, D. pictilis* and *D. prorepleta*) in Central America and two (*D. fulvalineata* and *D. hermionae*) in North America (Vilela, 1983; Vilela and Bächli, 1990; Bächli and Vilela, 2002).

The species of the *fasciola* subgroup inhabit mostly humid forests but little is known about the substrates with which they are associated except for the following: *D. moju* is known to feed on fallen blossoms and drier fruits (Pipkin, 1965); *D. fasciola* feeds and breeds on the fallen flowers of *Aphelandra micans* (Acanthaceae), *Erythrina berteroana* (Fabaceae), *Heliconia latispatha* (Heliconiaceae), *Aroid* sp. (Araceae) and *Coffea arabica* fruits (Rubiaceae) (Pipkin, 1965; Pipkin *et al.*, 1966); *D. fulvalineata* was collected on fleshy fungi (Patterson and Wheeler, 1942); *D. onca* breeds in cladodes of decaying epiphytic cacti (*Rhipsalis* sp.) (Sene *et al.*, 1977); *D. rosinae* emerged from cladodes of decaying columnar cacti (*Cereus* sp.) (Tidon-Sklorz and Sene, 1995b). The last two examples suggest that species of the *fasciola* subgroup, even though inhabiting forests, may be associated with cacti. It has already been noted that *D. rosinae* associates with cacti of the genus *Cereus* and *Opuntia* (Vilela, 1983; Tidon-Sklorz and Sene, 1995b) and our work described in the present paper concerns *D. rosinae* specimens collected from dunes and rocky shores where *Cereus* and *Opuntia* were growing.

Most of the studies regarding the establishment of a phylogeny for the *fasciola* subgroup have been cytological (Wasserman, 1962, 1982, 1992; Kuhn *et al.*, 1995; Diniz and Sene, 2004). The existence of the 3c inversion in both the *mulleri* complex of the *mulleri* subgroup and in species of the *fasciola* subgroup supports the hypothesis of a common ancestry, with the *fasciola* subgroup stemming from the *mulleri* subgroup (Wasserman, 1982, 1992). However, Diniz and Sene (2004) recently showed that the fixed 3c inversion of the *mulleri* complex is not the same as the one observed in the *fasciola* subgroup, suggesting that the name of the inversion in the *mulleri* complex should be changed from 3c to 3u. This suggests that the ancestral chromosomal composition of the *fasciola* subgroup (Primitive VII) derived from the Primitive I, the ancestral sequence of the *repleta* group (Wasserman, 1960; Wasserman, 1992) (Figure 1). Furthermore, Diniz and Sene (2004) proposed two new species complexes within the *fasciola* subgroup, the *fascioloides* complex made up of *D. ellisoni* and *D. fascioloides* that share the 2d^3 inversion, two chromosomal centric fusions (2-4F and 3-5F) and karyotype similarity regarding the X chromosomes (Kuhn *et al.*, 1995) (Figure 1) and the *onca* complex, containing *D. carolinae* and *D. onca* which both share the 4f^2 inversion (Figure 1). Previously, chromosomal data had suggested two complexes, the *pictilis* complex (*D. pictilis* and *D. pictura*) and the *moju* complex (*D. moju*, *D. mojuoides* and *D. paraguttata*) (Wasserman, 1992) (Figure 1).

There are two hypotheses about the origin and diversification of the *repleta* species group. One hypothesis suggests that the ancestral populations of the *fasciola* subgroup were forest inhabitants that colonized the dry areas and led to the development of the species of the other *repleta* group subgroups (Throckmorton, 1975, 1982; Morais *et al.*, 1995; Tatarenkov and Ayala, 2001), while the other hypothesis considers that the *fasciola* subgroup is derived from species adapted to dry areas which later invaded forests (Wasserman, 1962, 1992) but this hypothesis was based on the sharing of the 3c inversion among species in the *fasciola* subgroup and the *mulleri* complex.

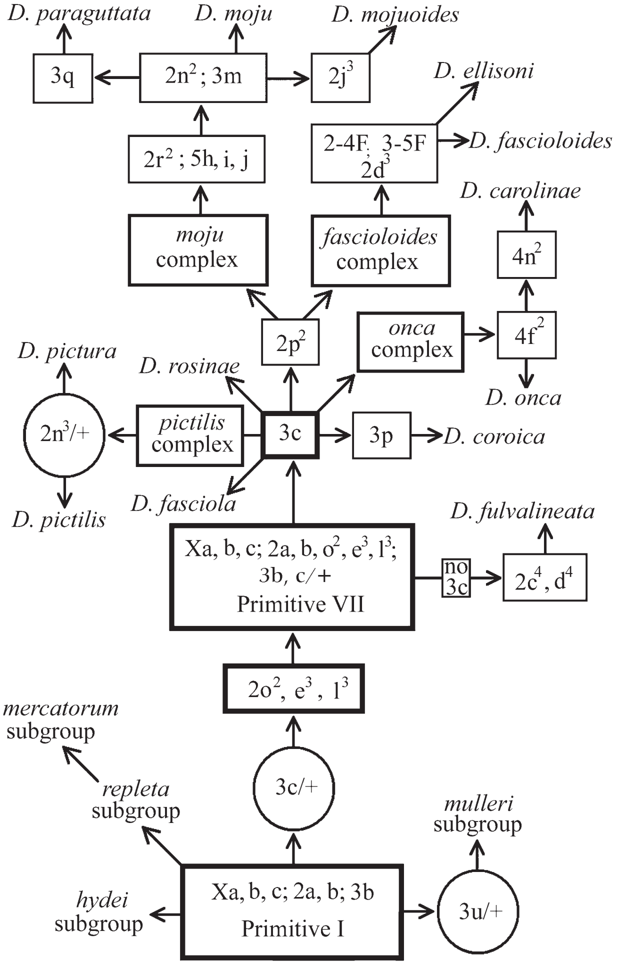

Figure 1 - Diagram illustrating the phylogenetic relationships among some species within the *fasciola* subgroup and between the subgroups in the *repleta* group (modified from Wasserman, 1992 and Diniz and Sene, 2004).

Many questions concerning the evolutionary relationships among the species in the *fasciola* subgroup remain obscure. To help resolve this we analyzed partial sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene of nine species of the *fasciola* subgroup (Table 1) with the aim of trying to understand the origin and evolution of the this subgroup and consequently adding to the current knowledge on the *repleta* group.

Table 1 - The *Drosophila* species of the *fasciola* subgroup used in this study, collection localities and type of vegetation.

Drosophila species grouped by vegetation type	Locality[1] (coordinates)
Atlantic rain forest	
D. carolinae	Ibiúna-SP (23°39' S, 47°13' W)
D. fascioloides	São Sebastião-SP (23°48' S, 45°23' W)
D. ellisoni	São Sebastião-SP (23°48' S, 45°43' W)
Mesophilic forest	
D. coroica	Cajuru-SP (21°16' S, 47°18' W)
D. ivai	Cajuru-SP (21°16' S, 47°18' W)
D. onca	Cajuru-SP (21°16' S, 47°18' W)
D. senei	Cajuru-SP (21°16' S, 47°18' W)
Caatinga[2]	
D. rosinae	Mucugê-BA (13°00' S, 41°22' W)
Unknown	
D. moju[3]	Panama (9° N, 80° W)

[1] SP = São Paulo state, Brazil; BA = Bahia state, Brazil.
[2] In Brazil this is an arid region of stunted vegetation and brushwood.
[3] Isoline provided by the Laboratório de *Drosophila*, Depto. de Genética, Instituto de Biociências, Universidade de São Paulo (IBUSP).

The species analyzed were collected using closed traps (Tidon-Sklorz and Sene, 1988) containing banana, orange and yeast. Flies from the *fasciola* subgroup were identified by morphological analysis of the *aedeagus* (male genitalia) (Vilela, 1983), males being directly identified and females indirectly by their male offspring.

We used *D. melanogaster* (NC_001709) and *D. yakuba* (NC_001322) as the outgroup in the phylogenetic analysis and *D. hydei* (*hydei* subgroup), *D. mercatorum* (*mercatorum* subgroup), *D. repleta* (*repleta* subgroup) and *D. serido* (*mulleri* subgroup) were used to provide information on the relationships between the *fasciola* subgroup and the other subgroups of the *repleta* group.

Genomic DNA was extracted from a single male of each species using the Wizard® Genomic DNA Purification kit (Promega) and the polymerase chain reaction (PCR) was used to amplify *COI* gene fragments using the primers TY-J-1460 - 5'TACAATTTATCGCCTAACTTC AGCC3' (Simon *et al.*, 1994) and 2191 - 5'TTGATTTTTT GGTCATCCAGAAGT3' (De Brito *et al.*, 1998). The following amplifications conditions were used: 94 °C for 1 min 30 s followed by 25 cycles of 40 s at 94 °C, 40 s at 46 °C and 2 min at 72 °C. The PCR products were purified with the ExoSAP-IT® kit (Amersham Biosciences Part of GE Healthcare) and sequenced directly. Sequencing reactions were prepared with the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and reaction products sequenced on an ABI Prism 377 Automated DNA Sequencer.

Forward and reverse sequences of each taxa were compared, corrected and edited using the Chromas 2.3 program (http://www.technelysium.com.au/chromas_lite.html) and multiple sequence alignments were generated using the ClustalW 1.8 program (Thompson *et al.*, 1994). The presence of saturation in the data was checked using Dambe 4.2.13 program (Xia and Xie, 2001).

Phylogenetic reconstruction was performed in a maximum parsimony (MP) framework using the PAUP*4.0b10 program (Swofford, 2002) with the following settings: search type = heuristic; addition sequences = random; number of replicates = 100; and branch swapping = Tree Bisection Reconnection (TBR). Support at each node was assessed using bootstrap proportions (Felsenstein, 1985) with 1,000 replicates.

Reyes *et al.* (1999) and Bull *et al.* (2003) have suggested likely saturation problems for third mitochondrial codon positions and explored the use of a priori differential weighting of molecular characters. Bull *et al.* (2003) obtained the best resolution of the maximum parsimony analyses when third mtDNA codon positions were weighted by 0.5 and 0.1 with transitions excluded entirely. Based on this we used two weighting methods, one based on equal weighting of all informative characters and another omitting the transitions (zero weight) and assigning weights of 0.5 for the third codon positions with all other characters weighted equally.

Partial 624 nucleotide sequences from the *COI* gene were obtained from all the species studied except *D. repleta*. The first base of the sequences obtained corresponded to position 1,510 of the mitochondrial DNA of *D. yakuba* (NC_001322), the first codon from this base corresponding to the thirteenth amino acid of the *COI* gene. No gaps were found but there were 193 variable characters (including the outgroup species) and 137 parsimony informative characters. The third codon positions were the most variable, whereas the second codon positions were the most conserved in this *COI* gene region. There was an excess of T (38.9%) and A (28.9%) over G (16.7%) and C (15.5%). The saturation test (Figure 2) suggested likely saturation for transitions.

Phylogenetic analysis with equal weighting of all informative characters resulted in a single parsimony tree with consistency index (CI) of 0.450, retention index (RI) of 0.308 and a total length of 547 steps (Figure 3A). This parsimony tree contained a poorly resolved clade (*repleta* group). This polytomy can be explained by the reduced amount of phylogenetic information contained in the data due to the high level of saturation suggested for transitions (Figure 2) and/or for third codon positions. Multilocus sequence analysis or the inclusion of more *fasciola* subgroup taxa could provide enough information for a robust hypotheses. But inclusion of taxa could prove difficult since the strains might not be readily available.

On the basis of the likely saturation for transitions (Figure 2) and for third mtDNA codon positions (Allard and Carpenter, 1996; Reyes *et al.*, 1999; Bull *et al.*, 2003)

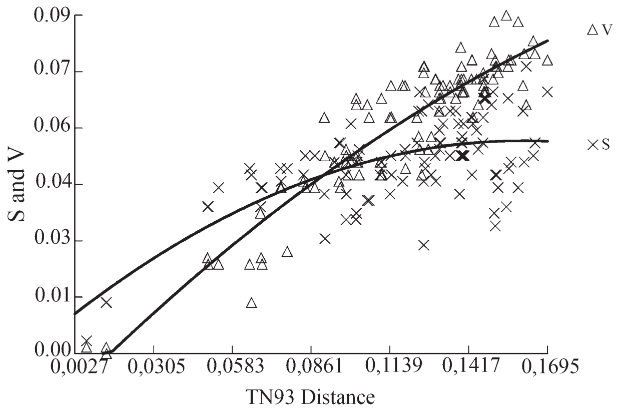

Figure 2 - Plot of the number of transitions (S) and transversions (V) *vs.* divergence among partial sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene. The curve (S) indicates the presence of saturation in the transitions.

we carried out a second maximum parsimony analysis with all transitions excluded (133 parsimony informative characters) and the third codons given a 0.5 weighting. This analysis resulted in a single parsimony tree with CI of 0.411, RI of 0.404 and a length of 137.5 steps (Figure 3B) with better resolution than the cladogram obtained with equal weighting of the characters (Figure 3A).

The cladograms obtained support monophyly of the *repleta* group (Figure 3) when tested with *D. melanogaster* and *D. yakuba* (not a strong test), which is in agreement with other studies in the literature (Throckmorton, 1975, 1982; Wasserman, 1992; Tatarenkov and Ayala, 2001). On the other hand, Durando *et al.* (2000) suggest that the *repleta* group is not monophyletic, although these authors cautioned that this inference '...may be the result of poor resolution at the base of the phylogenetic tree'.

The *repleta* and *mercatorum* subgroups appeared as sister taxa only in the maximum parsimony analysis with equal character weighting, but support for this clade was low with a bootstrap value of only 58% (Figure 3A). This relationship has previously been suggested by morphological (Throckmorton, 1975), cytological (Wasserman, 1992) (Figure 1) and molecular data (Durando *et al.*, 2000; Tatarenkov and Ayala, 2001). The relationships among the other subgroups in the *repleta* group were not resolved by our analysis, supporting previous work by Throckmorton (1975), Wasserman (1992) and Durando *et al.* (2000).

The phylogeny with equal character weighting (Figure 3A) did not suggest monophyly of the *fasciola* subgroup. We used the nonparametric Templeton test (Templeton, 1983) function of the PAUP* program to test if this departure from the conventional notion of the *fasciola* subgroup relationships was significantly supported by the *COI* gene data. This analysis showed significant differences (p = 0.0001) between the constrained topology monophyly of the *fasciola* subgroup (522 steps) and the unconstrained topology (547 steps) (Figure 3A), with the constrained topology explaining the data significantly better than the unconstrained topology. Therefore, the Templeton test showed that the data do not reject monophyly of the *fasciola* subgroup. The phylogeny obtained using differential weightings (Figure 3B) suggested monophyly of the *fasciola* subgroup, but again, support for this clade was low with a 53% bootstrap and the Templeton test indicated no significant differences (p = 0.6844) between the topology of the Figure 3B (137.5 steps) and the constrained topology

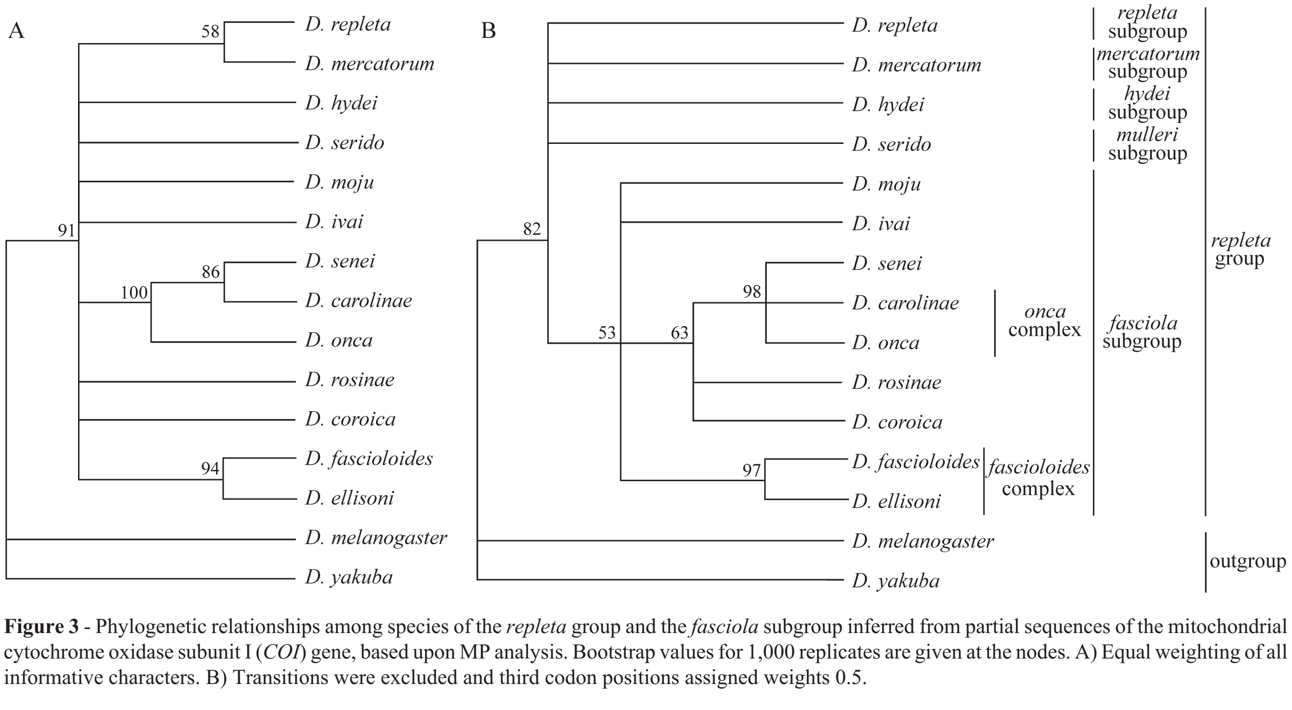

Figure 3 - Phylogenetic relationships among species of the *repleta* group and the *fasciola* subgroup inferred from partial sequences of the mitochondrial cytochrome oxidase subunit I (*COI*) gene, based upon MP analysis. Bootstrap values for 1,000 replicates are given at the nodes. A) Equal weighting of all informative characters. B) Transitions were excluded and third codon positions assigned weights 0.5.

of monophyly of the *fasciola* subgroup (136.5 steps). Thus, our *COI* gene data neither rejected nor supported the concept of monophyly of the *fasciola* species subgroup.

The phylogenies did not define all the interspecific relationships in the *fasciola* subgroup. In both of the hypotheses described above *D. ellisoni* and *D. fascioloides* appear as sister species (Figure 3), which is in agreement with the cytogenetic data produced by Diniz and Sene (2004) (Figure 1). In the differentially weighted phylogeny (Figure 3B) the species *D. carolinae*, *D. coroica*, *D. onca*, *D. rosinae* and *D. senei* formed a single clade with a 63% bootstrap in which *D. carolinae*, *D. onca* and *D. senei* formed a nested polytomy with a 98% bootstrap. However, the equally weighted cladogram (Figure 3A) indicated that *D. onca, D. carolinae* and *D. senei* comprised one clade in which *D. carolinae* and *D. senei* appeared as sister species, contradicting the morphological data suggesting that *D. carolinae* and *D. onca* are sister taxa (Vilela, 1983) but which shows no relationship between *D. carolinae* and *D. senei*. The relationships between the other species were not defined in the equally weighted cladogram. Our results are in agreement with theories on the *fascioloides* and *onca* complexes proposed by Diniz and Sene (2004). It is possible that *D. senei* also belongs to the *onca* complex, but additional cytological data are necessary to confirm this hypothesis.

Because the relationships between the subgroups were not defined our results show no evidence that the ancestral populations of the *repleta* group inhabited forests (Morais *et al.*, 1995; Throckmorton, 1975, 1982; Tatarenkov and Ayala; 2001) or arid areas (Wasserman, 1962, 1992).

However, the hypothesis that the *fasciola* subgroup represents the oldest lineage of the *repleta* group is supported by data produced by Throckmorton (1982) suggesting that the species groups of the *virilis*-*repleta* radiation, closely related to the *repleta* group (*castanea*, *canalinea*, *dreyfusi* and *mesophragmatica* groups), occur in forests and that, morphologically, the *fasciola* subgroup is the least derived *repleta* group subgroup. Furthermore, Morais *et al.* (1995) conducted ecological studies of *Drosophila* yeast communities associated to species of the *fasciola* subgroup and produced data suggesting that this subgroup represents the lineage from which the South American species of the *repleta* group evolved. More recently, Tatarenkov and Ayala (2001) used the nuclear genes *Ddc* and *amd* to construct a molecular phylogeny of the *virilis*-*repleta* radiation which strongly indicates that the *fasciola* subgroup is the earliest offshoot in the *repleta* group. It thus seems that, as with the *fasciola* subgroup, the ancestral *repleta* group probably inhabited forests.

While supporting monophyly of the *repleta* group, our data clearly does not resolve the phylogenetic relationships in the *fasciola* subgroup, possibly due to saturation in relation to the mitochondrial gene used to construct the phylogeny (Allard and Carpenter, 1996; Reyes *et al*., 1999; Durando *et al.*, 2000; Bull *et al.*, 2003). Research using nuclear genes could help elucidate the phylogenetic relationships in the *fasciola* subgroup and between this and the other subgroups of the *repleta* group.

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Internet Resources

Chromas Software, http://www.technelysium.com.au/chromas_lite.html.

Associate Editor: Louis Bernard Klaczko