Systematics and Evolution of the *Drosophila buzzatii* (Diptera: Drosophilidae) Cluster Using mtDNA

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Ann. Entomol. Soc. Am. 94(3): 333–346 (2001)

ABSTRACT The *Drosophila buzzatii* Patterson & Wheeler cluster (*repleta* group) includes seven species: *D. buzzatii* Patterson & Wheeler, *D. koepferae* Fontdevila et al. *D. serido* Vilela & Sene, *D. seriema* Tidon-Sklorz & Sene, *D. borborema* Vilela & Sene, *D.* sp. D and *D.* sp. B. These flies are widely distributed in South America outside the Amazon region. The systematics of this cluster has been based on chromosomal inversions and the aedeagus is used to identify species. These species use necrotic cactus tissues as breeding sites. The current hypothesis of differentiation and speciation of these species is related to expansion and retraction of cactus distribution in South America during Quaternary climatic cycles. We investigated the phylogenetic relationship among species of this cluster based on the mtDNA COI gene region and compared it with the relationship established using classical markers. The resulting phylogenetic hypothesis indicated that this cluster is a monophyletic group that can be divided into two sets of species: the one including *D. buzzatii* and *D. koepferae* and other with the remaining five species. The latter can also be divided into two clades. Although this branching pattern is similar to the one established by classical markers, some disagreement involving populations was observed that suggests secondary contact between populations of different species. The distribution pattern of COI haplotypes is partitioned geographically, which could be the result of limited gene flow between groups of species suggesting a longer history of differentiation than previously hypothesized for *D. buzzatii* cluster species.

KEY WORDS *Drosophila buzzatii* cluster, mtDNA COI, phylogeny, geographic variation.

TROPICAL AND SUBTROPICAL South America east of the Andes includes the Amazonian and Atlantic rainforests. Between these two humid plant formations, there is a corridor of xeric or xeromorphic vegetation, oriented along a NE-SW axis. This corridor of open formations includes the Caatinga and Chaco morphoclimatic domains, and shares a high diversity and density of cactus species with the Caribbean coasts of Colombia and Venezuela (Hueck 1972). Adjacent domains of forest also include cacti but as isolated populations. These cactus populations supposedly detached from the main body of cactus distribution as a result of repeated expansions and retractions of open vegetation during Quaternary glacial and interglacial periods, respectively. Thus, cactus populations may have become entrapped by the expansion of humid forest vegetation during the moister interglacial periods (Ab'Saber 1977, Bigarella and Andrade-Lima 1982, Sene et al. 1982). The history of cactus populations may have affected the diversification of associated species, such as cactophilic *Drosophila*, whose larvae feed exclusively on decaying cacti.

Systematics of *Drosophila buzzatii* Cluster

The *Drosophila buzzatti* cluster (*mulleri* subgroup, *Drosophila repleta* group) is composed of seven cactophilic species: *D. buzzatti* Patterson & Wheeler, *D. koepferae* Fontdevila, Pla, Hasson, Wasserman, Sanchez, Naveira and Ruiz, *D. serido* Vilela & Sene, *D. borborema* Vilela & Sene, *D. seriema* Tidon-Sklorz & Sene and two undescribed species *D.* sp. D and *D.* sp B, previously described as *D. serido* morphotypes D and B, respectively. This cluster is endemic to South America, and the presence of *D. buzzatti* species on other continents is due to human introduction of its host cactus (Barker et al. 1985). The *D. buzzatti* cluster, plus the *D. stalkeri* Wheeler and *D. martensis* Wasserman & Wilson clusters, belongs to the *D. buzzatti* complex (Ruiz and Wasserman 1993). Chromosomal inversion data suggest that the Caribbean and Floridian *D. stalkeri* cluster is the most primitive, the Venezuelan and Colombian *D. martensis* cluster is the most derived, and the *D. buzzatti* cluster is intermediate, occurring in almost all of South America (Ruiz and Wasserman 1993).

The systematics of the *D. buzzatti* cluster is based on observed chromosome inversions and the morphology of the aedeagi, the latter being a diagnostic species character (Vilela 1983, Tosi and Sene 1989, Silva and Sene 1991, Tidon-Sklorz and Sene 1995a). The relationship among the *D. buzzatii* cluster species has been under investigation, but the resulting data have

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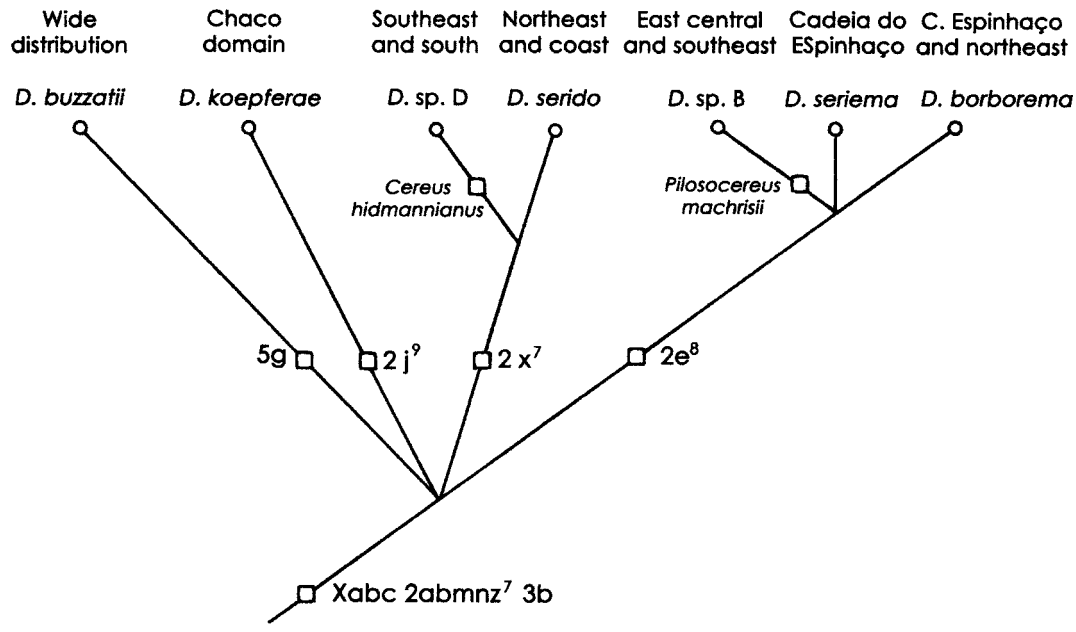

Fig. 1. Chromosomal phylogeny of the *Drosophila buzzatii* cluster using the fixed chromosomal inversions according to Ruiz and Wasserman (1993) and Ruiz et al. (1996). Xabc 2abmnz^7 3b is the standard chromosome inversion arrangement for the *D. buzzatii* cluster. 5g, 2j^9, 2x^7, 2e^8 are fixed chromosome inversions. *Cereus hildmannianus* and *Pilosocereus machrisii* are cactus genera known as breeding sites of species *D.* sp. D and *D.* sp. B, respectively.

not been systematically analyzed and have been compared only with the relationships based on chromosome inversions (Baimai et al. 1983, Barker et al. 1985). Interspecific and interpopulation hybridization tests have been performed and employed as indicators of phylogenetic relationships; however, these tests have little value in this group because no pattern of evolutionary relationship has been determined with different degrees of reproductive isolation, depending on the population being analyzed (Bizzo 1983, Moraes 1992, Marin et al. 1994, Madi-Ravazzi et al. 1997). The only consistent result is that *D. buzzatti* is more isolated from the other species in the cluster (Marin et al. 1994, Machado et al. 1999).

The monophyly of the *D. buzzatti* cluster has been defined on the basis of a complex arrangement of inversions (Ruiz and Wasserman 1993) (Fig. 1). However, phylogenetic relationships within the cluster are yet undefined; only four fixed inversions are useful to define relationships and for species identification (Fig. 1). Regarding the polymorphic inversions, each species presents its own pattern (Tosi and Sene 1989). *Drosophila buzzatii* is characterized by the fixed chromosomal inversion 5g. This species has a broad geographical distribution, occurring in almost the entire range of the cluster itself (Fig. 2). Populations of *D. buzzatti* are present at high density in the Chaco Domain and are associated with several cactus genera, mainly species of the genus *Opuntia* (Vilela et al. 1980, Hasson et al. 1992, Fanara et al. 1999). Outside the Chaco, there are many small isolated populations of *D. buzzatti*, also associated with several cactus genera, but the species is most common in *Opuntia ficus-indica* L. plantations (Pereira et al. 1983). These data, together with a high polymorphism of chromosomal inversions, suggest that *D. buzzatti* may have originated in the Chaco Domain and then dispersed to other areas of the American continent (Vilela et al. 1980, Barker et al. 1985, Figueiredo and Sene 1992).

The populations of *D. koepferae*, which are endemic to the Chaco Domain (Fig. 2), are characterized by the fixed inversion 2j^9 (Fig. 1). Studies have shown that the emergence of individuals of this species happens mainly from columnar cacti (Fontdevila et al. 1988, Fanara et al. 1999).

Drosophila serido and *D.* sp. D share the fixed inversion 2x^7 but present distinct patterns of polymorphic inversions (Tosi and Sene 1989; Fig. 1). *Drosophila serido* is broadly distributed (Fig. 2), and polymorphic inversion data suggest the further splitting of its populations into two groups: northeastern Brazil populations and those of the Brazilian coast. The populations of *D.* sp. D are associated with the cactus *Cereus hildmannianus* K. Schum (Monteiro and Sene 1995), which occurs in gallery forests and mesophytic forests along the valleys of the Paraná-Paraguai River Basin (Fig. 2). *Drosophila serido* and *D.* sp. D are mostly allopatric, but hybrid populations have been observed on the coast of the Brazilian State of Rio Grande do Sul, which suggests secondary contact between the species (Cansian et al. 1996).

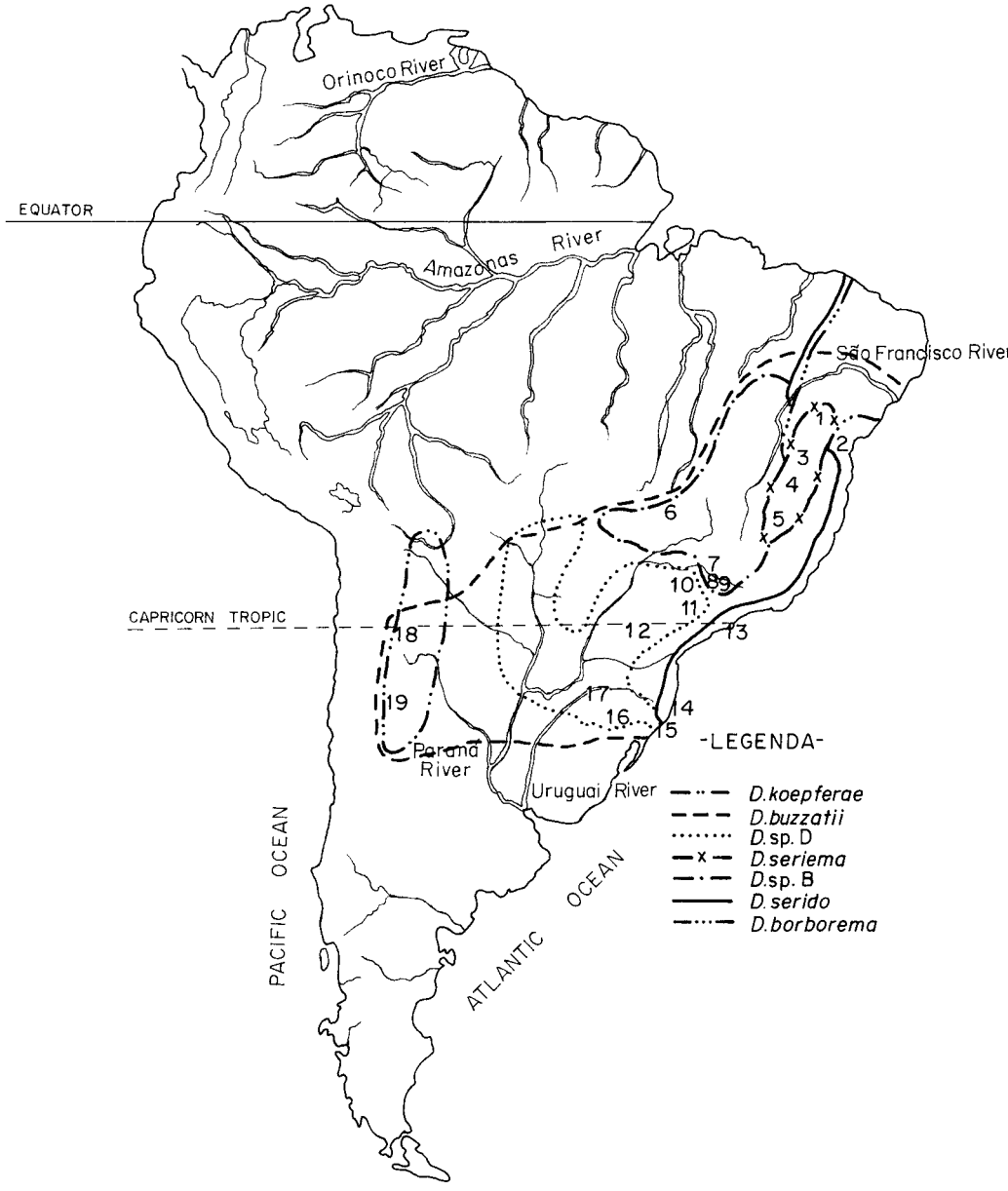

Fig. 2. Distribution in South America of the species of the *Drosophila buzzatii* cluster according to Silva and Sene (1991) and Vilela and Sene (1977). Numbers show the geographical location of sampled populations in the current study (See Table 1 for population identification).

The fixed inversion 2e[8] defines the clade including *D. borborema*, *D. seriema*, and *D.* sp. B (Fig. 1). *Drosophila borborema* occurs in the Caatinga Domain and in rocky fields in the middle of the Espinhaço mountain range (Vilela et al. 1983, Tidon-Sklorz and Sene 1995a; Fig. 2). *Drosophila seriema* is endemic to the Espinhaço mountain range, its populations being isolated in the range in rocky fields above 1,000 m (Tidon-Sklorz and Sene 1995b) (Fig. 2). *Drosophila* sp. B is more broadly distributed along and to the west of the Espinhaço range (Fig. 2). Peripheral populations of *D.* sp. B in the southern portion of its distribution are associated with *Pilosocereus machrisis* Y. Dawson, a cactus species occurring in isolated rock outcrops (Monteiro and Sene 1995).

Tidon-Sklorz and Sene (1995a) defined the *D. serido* superspecies, within the *D. buzzatii* cluster, based on similarity of aedeagus morphology. This superspecies taxon includes all the *D. buzzatii* cluster species with the exception of the *D. buzzatti* species.

Populations of the *D. buzzatii* cluster species have been studied using metaphase chromosomes and es-

Table 1. Geographic localities of isofemale lines analyzed from species of *Drosophila buzzatii* cluster

Species	Geographic localities and map no.[a]	Isofemale lines code numbers	Coordinate
D. serido	Guaratuba,SP-BR (13)	H49F1M	23° 50′ S, 45° 53′ W
	Milagres,BA-BR (2)	1431.3	12° 51′ S, 39° 53′ W
D. sp. B	Analândia,SP-BR (9)	H32.1	22° 08′ S, 47° 39′ W
	Analândia,SP-BR (9)	H32M	22° 08′ S, 47° 39′ W
	Analândia,SP-BR (9)	H32.9	22° 08′ S, 47° 39′ W
	Furnas,MG-BR (7)	H24S3	20° 37′ S, 46° 15′ W
	Cristalina,GO-BR (6)	H35	16° 43′ S, 47° 40′ W
	Altinópolis,SP-BR (8)	H6G6	21° 05′ S, 47° 55′ W
D. sp. D	Capão da Canoa,RS-BR (14)	H42R2M	23° 47′ S, 50° 10′ W
	Sertãozinho,SP-BR (10)	H34G8	21° 10′ S, 48° 05′ W
	Cianorte,PR-BR (12)	D93S6M	23° 37′ S, 52° 31′ W
	Guaritas,RS-BR (16)	H44R2M	30° 45′ S, 43° 20′ W
	Arroio Teixeira,RS-BR (15)	H41S1	23° 46′ S, 50° 10′ W
	Arroio Teixeira,RS-BR (15)	H41S2	23° 46′ S, 50° 10′ W
	Campinas,SP-BR (11)	H48F1M	22° 58′ S, 46° 47′ W
	Santiago,RS-BR (17)	H47R5M	29° 12′ S, 54° 53′ W
D. seriema	Mucugê,BA-BR (3)	D62C4B	23° 51′ S, 45° 55′ W
	Grão Mogol,MG-BR (4)	D54M	23° 51′ S, 45° 55′ W
	Serra do Cipó,MG-BR (5)	D40F1	19° 18′ S, 43° 55′ W
	Morro do Chapéu,BA-BR (1)	D71C1B	11° 38′ S, 41° 01′ W
D. borborema	Milagres,BA-BR (2)	1281.0	12° 51′ S, 39° 53′ W
	Milagres,BA-BR (2)	1281.2	12° 51′ S, 39° 53′ W
D. buzzatii	Sertãozinho,SP-BR (10)	H34G5	21° 10′ S, 48° 05′ W
	Grão Mogol,MG-BR (4)	D54F5	16° 34′ S, 42° 54′ W
	San Juan-Argentina (19)	H98	31° 35′ S, 68° 31′ W
D. koepferae	Famatina-Argentina (18)	B26D2	29° 06′ S, 67° 10′ W

[a] The numbers inside the parentheses are related to the localities shown in Fig. 2.
SP—São Paulo State; BR—Brasil; BA—Bahia State; MG—Minas Gerais State; GO—Goias State; RS—Rio Grande do Sul State; PR—Paraná State.

terase patterns. However, these data sets are useful to species identification but contain no phylogenetic information (Baimai et al. 1983, Lapenta et al. 1998).

The effects of Quaternary climatic changes on a previously continuously distributed species have helped to explain the distribution and diversification pattern of *D. buzzatii* cluster species (Sene et al. 1988). This ancestral species would have had its range broken up by the isolation of host cactus populations because of the expansion of more mesic vegetation types during the moister interglacial periods of the Quaternary. This hypothesis assumes that isolation of host cacti and flies occurred simultaneously.

In this study we analyzed all species of the *D. buzzatti* cluster from several localities by using mtDNA COI gene sequences to investigate phylogenetic relationships among its species as well as to clarify relationships between populations of different species. The data are discussed together with previously available evidence to infer evolutionary events that may have played a role in the differentiation of species.

Materials and Methods

Specimens. We analyzed 28 individuals (fresh, dried, or kept in 70% ethanol) from different geographic localities within the ranges of the different species of the *D. buzzatii* cluster (Table 1). Two species from the other clusters of the *D. buzzatii* complex, *D. richardsoni* Vilela (La Parguera, Puerto Rico, code number 1421 from the Bowling Green Stock Center) and *D. martensis* Wasserman & Wilson (Barquieimeto, Venezuela, code number 1321 from Bowling Green Stock Center, OH) were analyzed as outgroups.

DNA Preparation and Sequencing. Total DNA was extracted from fresh, dried or ethanol preserved flies using Qiagen DNA extraction kits (Chatsworth, CA) and the methods described by Beckenbach et al. (1993). The template for the mitochondrial DNA from almost the entire COI gene was isolated by polymerase chain reaction (PCR) amplification using the primers TY-J-1460, C1-J2195, C1-N-2329 and L2-N-3014 described in Simon et al. (1994). PCR products were isolated by electrophoresis in 1% agarose gel and used for a second round of PCR amplification; the products were purified by acrylamide gel electrophoresis followed by passive elution and ethanol precipitation. The DNA was resuspended in 12 μl of water and used as a template in a sequencing reaction using the Circum Vent Cycle Sequencing kit (New England Biolabs, Beverly, MA) and 35SdATP as a label. We produced full sequences of both strands for all individuals studied and the DNA sequences were manually aligned unambiguously. *Drosophila yakuba* Burla sequences were used as standards (GenBank accession number X03240, J01400, J01402, J01403, J01406, J01408, V01521, and X 00563).

Phylogenetic Analysis. The sequence data set was tested for phylogenetic information using *G*-statistics (Hillis and Huelsenbeck 1992) performed in PAUP 3.1 (PAUP 1991). Phylogenetic analysis of the COI gene sequences was performed by maximum parsimony

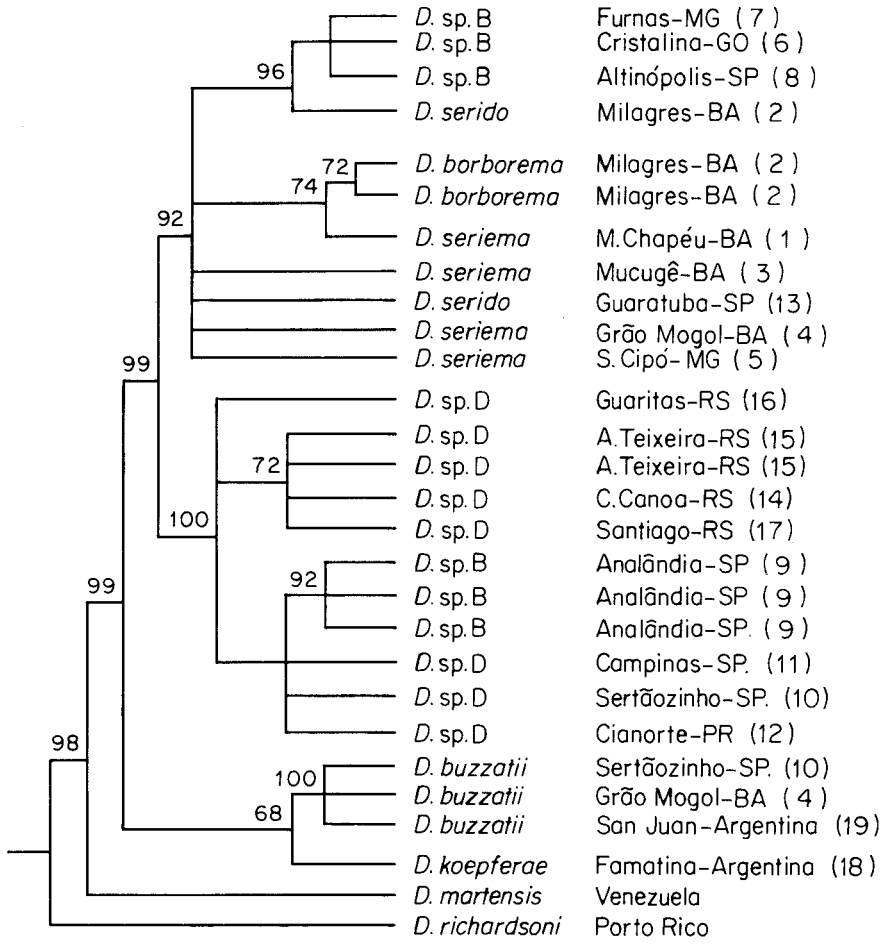

Fig. 3. Strict consensus tree for 100 bootstraps inferred from mtDNA COI for the *Drosophila buzzatii* cluster species. *D. richardsoni* and *D. martensis* were used as outgroup. Numbers on branches are bootstrap values above 50% based on 100 replicates.

methods using the branch and bound algorithm in PAUP 3.1 (PAUP 1991). To determine the confidence limits on tree nodes, the data were bootstrapped (Felsenstein 1985) 100 times.

To estimate mutation rates for the mtCOI gene, we used 6.1 Myr as the divergence date between *D. melanogaster* Meigen and *D. yakuba* estimated from studies of the gene Adh (Thomas and Hunt 1991, Russo et al. 1995). We inferred a mutation rate of 6.85×10^{-9} per site per year for mtDNA considering the number of substitutions detected between the same mtDNA in the latter two species.

Results

We obtained 1495 bases of the mtCOI gene (base number 1495–2990 in the *D. yakuba* mitochondrial sequence) from 28 individuals, 306 of which were phylogenetically informative (20.47%, see *Appendix 1*). The tree length distribution of 5,000 randomly sampled trees indicated that the data contained significant phylogenetic information ($G = -0.6435$; $P < 0.01$). The strict consensus tree emerging from the analysis of the mtDNA COI haplotypes is shown in Fig. 3. The bootstrap values were generally high. This phylogenetic tree supports the monophyly of the *D. buzzatti* cluster (99% bootstrap).

Among the *D. buzzatii* cluster species two main branches were observed (Fig. 3). A low bootstrap value supported one branch including *D. koepferae* and *D. buzzatii*. The other branch, encompassing the remaining species of the cluster, was supported by high bootstrap values. This branch was further divided in two clades, with a bootstrap value of 99%. Individuals representing populations of the species *D.* sp. D and three individuals from one population of species *D.* sp. B comprise the first clade (100% bootstrap, Fig. 3). The internal branches within this group are poorly resolved, but two groups can be detected, one formed by the populations of Santiago-RS (locality 17, Fig. 2) and from the contact zone described between the species *D. serido* and *D.* sp. D (localities 14 and 15, Fig. 2) and the other composed of populations from localities 9, 10, 11, 12, and 16 (Fig. 2). The second clade is composed of *D. borborema*, *D. serido*, *D. seriema*, and

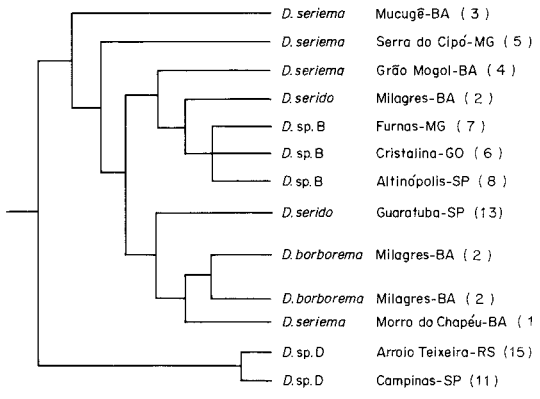

Fig. 4. Shortest tree obtained from mtDNA COI data for *Drosophila serido*, *D. seriema*, *D. borborema* and *D.* sp. B. *D.* sp. D was used as the outgroup.

D. sp. B (92% bootstrap, Fig. 3), which occur mainly in north and northeastern Brazil, as well as the Brazilian coast (Fig. 2). Inside this clade, branching is less well supported, with the exception of the group containing one individual of *D. serido* and individuals from *D.* sp. B (96% bootstrap). Possible relationships between these species can be evaluated in Fig. 4, representing the shortest established tree for this clade. *Drosophila seriema*, with great divergence between its populations, is paraphyletic and individuals of *D. serido* were allocated in different parts of the cladogram.

Plotting the cladogram obtained (Fig. 3) with the geographical distribution of the species (Fig. 2) shows a divergence sequence pattern suggestive of a geographical isolation model of mtDNA (Avise 1989) (Fig. 5). The first dicotomy separates individuals from the Gulf of Mexico, belonging to *D. martensis* and *D. stalkeri* clusters, *D. martensis* and *D. richardsoni*, respectively, and from individuals belonging to the *D. buzzatti* cluster. Within the *D. buzzatii* cluster, haplotypes characteristic of groups from the Chaco Domain, *D. koepferae* and *D. buzzatii* species, diverge from those of the remaining species. The latter species are divided in two clades, one composed of populations of *D.* sp D and one population of *D.* sp. B, in southeast South America, and the second composed of *D. serido*, *D. seriema*, *D. borborema*, and *D.* sp B in northern and eastern South America.

Using a mutation rate of 6.85×10^{-9} per site per year for mtDNA, the basal split separating *D. koepferae* and *D. buzzatti* from other species in the *D. buzzatii* cluster was estimated to have occurred approximately 6–12 Myr, whereas the split between the two main clades formed by other species of the cluster was estimated to have happened circa 3–6 Myr.

Discussion

The phylogenetic hypothesis established here from sequencing the mitochondrial COI gene for the *Drosophila buzzatti* cluster species is similar to those relationships established by chromosome inversions (Ruiz and Wasserman 1993). The hypothesis generated from the COI genealogy suggests that the *D. buzzatti* cluster is a well supported monophyletic group, phylogenetically close to the *D. martensis* cluster, with *D. richardsoni* (*D. stalkeri* cluster) as a sister species. This result is consistent with inversion data indicating a basal position for species belonging to the *D. stalkeri* cluster within the *D. buzzatti* complex (Ruiz and Wasserman 1993).

The relationship among *D. buzzatti* complex clusters was not completely elucidated when only part of the mitochondrial COI gene was analyzed (Spicer 1995). Spicer (1995) worked with 408 bp of mitocondrial COI (*D. yakuba* position 1783–2190) with 87 phylogenetically informative sites and always found support for the monophyly of the *D. buzzatii* complex; however, it was not possible to define the relations among the clusters and of the species within the clusters because bootstrap support was very poor except for a few nodes. As we know, different parts of the gene have different substitution rates (Simon et al. 1994). Therefore, the part of the COI gene used by Spicer (1995) may not be phylogenetically informative for this evolutionary problem.

Our results show that the taxa considered as species within the *D. buzzatii* cluster behave as two distinct units. The first is composed of *D. koepferae* and *D. buzzatti*, and the second by the species *D. borborema*, *D. seriema*, *D. serido*, *D.* sp. B, and *D.* sp. D. The definition of *D. buzzatti* and *D. koepferae* as sister species contradicts the definition of the *D. serido* superspecies taxon which divides the cluster into two phyletic lineages based on aedeagus morphology (Tidon-Sklorz and Sene 1995a). *Drosophila buzzatii* has a wide geographical distribution, occurring all along the cluster distribution. We sequenced three individuals of *D. buzzatti* originating from isolated populations representing geographically distant populations in the southmost (locality 19), middle (locality 10), and northmost (locality 4) parts of its distribution. However, the haplotypes obtained from these populations do not show the geographic brake observed in other haplotypes obtained for the cluster (Fig. 5). Considering the hypothesis that *D. buzzatii* originated in the Chaco Domain, its genetic homogeneity suggests a recent dispersion into Brazilian territory or gene flow among its populations. Genetic homogeneity for this species has been observed elsewhere in South America for chromosomal inversions and metaphase chromosomes (Baimai et al. 1983, Barker et al. 1985, Figueiredo and Sene 1992). In some localities in Brazil, *D. buzzatti* is sympatric to *D. meridionalis* Wasserman and *D. serido*. In these areas, the latter two species exhibit interpopulational variation in their metaphase chromosomes, whereas *D. buzzatti* did not show any differentiation (Barker et al. 1985). Another explanation for this genetic homogeneity pattern, if we consider available data, is that *D. buzzatii* responds differently to selective pressures when compared with other species of the *D. buzzatii* cluster.

Our haplotype data suggests that *D. koepferae* could be considered a sister species of *D. buzzatii*, compris-

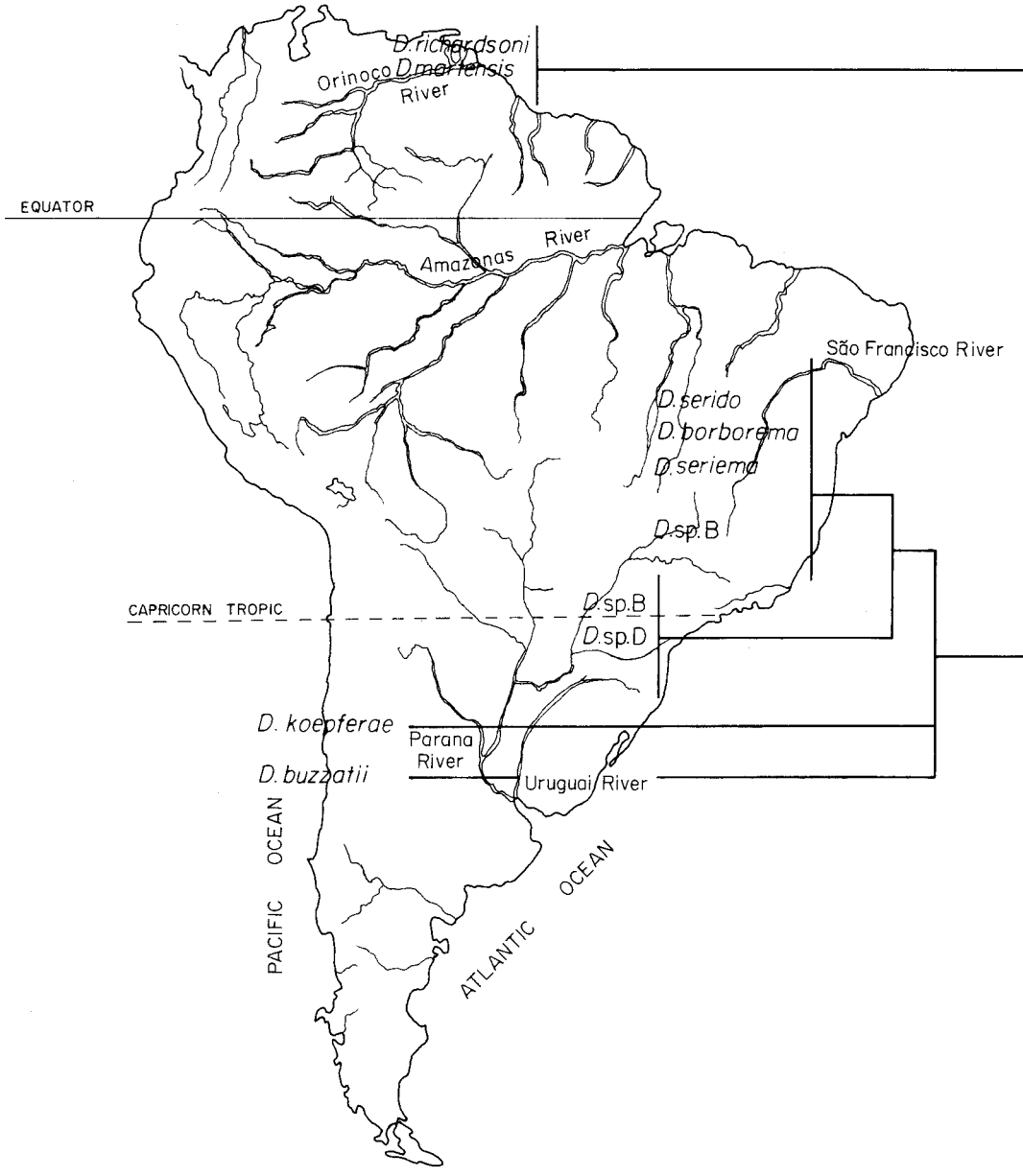

Fig. 5. Phylogeographic structuring of mtDNA haplotypes dividing the *Drosophila buzzatii* cluster populations into three distinct lines. *Drosophila buzzatii* and *D. koepferae* haplotypes in the Chaco Domain characterize the basal group. The *D.* sp. D haplotype characterizes the Paraná-Paraguai River basin group. The other species haplotypes characterize the northeast group of the cluster. Phylogeny was obtained as a parsimonious tree (See Fig. 3 for the phylogeny using mtDNA for the *D. buzzatii* cluster).

ing a distinctive evolutionary lineage from the *D. serido* superspecies. Nevertheless, bootstrap support (68% bootstrap, Fig. 2) is below the trust limit of the phylogenetic reconstitution (Hillis and Bull 1993). Available data show that *D. koepferae* and *D. buzzatii* share only the tergite pattern and are lighter in color when compared with other *D. buzzatii* cluster species (Fontdevila et al. 1988). The latter character, however, may be considered subjective and may present great inter- and intrapopulational variation. We analyzed only one individual from *D. koepferae* in this study. This fact limits our discussion about the species, especially considering that this species exhibits an incipient racial differentiation among its populations from Argentina and Bolivia as evidenced by inversion polymorphism and genetic distance (Fontdevila et al. 1988). Ethological isolation between Argentinian populations of *D. koepferae* and *D. serido* is complete, whereas Bolivian *D. koepferae* is only partially isolated from *D. serido* (Fontdevila et al. 1988). The analysis of

the haplotype data, with other available data, does not allow us to define the evolutionary relationships between *D. koepferae* and other species within the *D. buzzatii* cluster.

The second group defined by COI haplotypes inside the *D. buzzatti* cluster is well supported and is comprised of *D. serido*, *D. borborema*, *D. seriema*, and *D.* sp. B and *D.* sp. D. Inside this evolutionary lineage, species are divided into two well-supported clades. Individuals representing populations of *D.* sp. D, which shared the fixed inversion $2x^7$, basically comprise the first. Apparently, this clade is associated with the cactus *C. hildmannianus* (Monteiro and Sene 1995) found in mesophytic or galley forests of the valleys of the Paraná-Paraguai river drainages. Morphological analysis of the aedeagi of *D.* sp. D does not allow discrimination among populations, suggesting the existence of gene flow along the river valley corridors. There is also a latitudinal cline in aedeagus characteristics (Monteiro and Sene 1995). The hypothesis we present here for *D.* sp. D implies subdivision of this species due to its geographic distribution. There is a group composed of the populations from the upper Paraná River Basin (localities 10, 11, and 12; Fig. 2), a branching of the population from a montain area called Guarita, at the south of the depression of Rio Grande do Sul state in the Uruguay River Basin (locality 16; Fig. 2), and the populations from the depression and coast of Rio Grande do Sul state in Brazil (localities 14, 15, and 17; Fig. 2). Populations from localities 14 and 15 are described as hybrid populations between *D.* sp. D and *D. serido* that occur in a geographical region formed by old sand dunes, the meeting point of the Rio Grande do Sul depression with the Atlantic coast, forming a continuum with the coastal populations of *D. serido.*

The aedeagus, host plant, and inversion data from the population from locality 9 (Fig. 2) indicate that it formally belongs to species *D.* sp. B. Our data, however, allocated the individuals analyzed from this locality within the populations from the upper Paraná River Basin of *D.* sp. D clade (localities 10, 11, and 12; Fig. 2). In this case, there is a lack of agreement between the genealogy of the COI haplotype and the evolutionary relationships of the individuals. This fact could be explained, among other possible causes, by the secondary contact between these two species, which would be possible due to populational expansion during alterations of the glacial cycles. The population from locality nine of *D.* sp. B is found in the southern end of this species distribution, which is also the northern limit of the distribution of *D.* sp. D. In this region, populations of the cacti *C. hildmaniannus* and *P. machrisis* grow close together within about a 60 km west-east corridor geologically formed by the limits of sandstone and basalt formations with limestone outcrops, seemingly the end of the geologic formation from the Espinhaço range, which is a region endemic for cacti of the genus *Pilosocereus* (Zappi 1994).

The facts reported above suggest secondary contact between species of the *D. buzzatti* cluster, at the limits of their distributions, and a differential introgression of mitochondrial genomes—in this case unidirectional gene flow from *D.* sp. D to *D.* sp. B. In cases of hybridization, mtDNA tends to introgress faster then nuclear markers (Arnold 1993), characterizing the distinct behaviors of mtDNA and nuclear markers that are being observed in hybrid zones, the so-called cytonuclear disequilibrium.

The second group designed by COI haplotypes is defined by the populations of *D. borborema*, *D. seriema*, *D.* sp. B, that shared the fixed inversion $2e^8$, and *D. serido* with the fixed inversion $2x^7$. This group is found in eastern and northeastern Brazil, a region where the Espinhaço mountain range is prominent. This range is a long north-south oriented rock formation, a region endemic for several groups of insects and plants (Giulieti et al. 1987, Zappi 1994). *Drosophila seriema* inhabits the Espinhaço range in isolated populations and is paraphyletic because of divergence among its populations. However, Tidon-Sklorz and Sene (1995b) and Kuhn et al. (1996) suggest that this species would be a practically monomorphic evolutionary lineage having a single polymorphic inversion and no significant variation in aedeagus mophology. Also, these authors suggested a recent origin of *D. seriema.* If we accept this hypothesis the pattern exhibited by the haplotypes of mtCOI for this species could be explained by ancestral polymorphism. However, several hypotheses could be used to explain the pattern exhibited by the haplotypes of mtCOI for *D. seriema.* This pattern could be the result of a restricted gene flow causing the accumulation of independent changes between the populations, because populations of *D. seriema* occur as isolated populations above 1,000 m (Tidon-Sklorz and Sene 1995b) or could also be explained by introgressive hybridization of mtDNA from other species, which obscures any historical patterns maintained in the nuclear genome. This is possible because *D. seriema* is sympatric with other species of the *D. buzzatii* cluster throughout its range, and hybridization experiments have shown different degrees of reproductive isolation with populations of *D. buzzatii* cluster species, but there is no know case of complete isolation (Bizzo 1983, Moraes 1992, Madi-Ravazzi et al. 1997).

Little information exists on *D. borborema.* This species is found in the Caatinga Domain and rocky fields, where it is sympatric with *D. seriema.* Our data suggest that a haplotype of the latter species could be ancestral to that of *D. borborema;* however, these species possess distinct metaphase karyotypes and patterns of polymorphic inversions (Kuhn et al. 1996). Our mtDNA data suggest that *D. seriema* and *D. borborema* are closely related and that the latter species is a monophyletic group.

Populations of *D.* sp. B, with the exception of that from locality 9, do not form a well-supported clade, having the haplotype of *D. serido* as ancestral. These populations (localities 6, 7, and 8, Fig. 2) occur in the vicinity of the distribution limits of the species, on isolated hills, associated to the cactus *P. machrisis.*

Haplotypes of *D. serido* suggest that this species is not monophyletic. This hypothesis is based on a single

specimen from each of the two localities studied, and so this conclusion should be considered preliminary. However, metaphase chromosomes and polymorphic inversions divide *D. serido* in two groups, which agree with the haplotype data; there is a group in northeastern Brazil and a coastal group (Baimai et al. 1983, Tosi and Sene 1989). More sampling, especially from localities between these two regions, could eventually define the taxonomic status of these populations.

Populations of *D. serido* and *D.* sp. D shared the fixed inversion $2x^7$ but the hypothesis elaborated from mtCOI gene allocated their individuals in different clades of the cladogram with high bootstrap support (99%). Considering their geographical distribution and the hypothesis of hybrid population in the coast of Brazil, we suggest that *D.* sp. D species could be the result of a vicariant differentiation process of a group of populations of the *D. serido* and *D.* sp. D common ancestral species.

The geographic distribution of the COI haplotypes we found shows a pattern of sequence divergence among geographically isolated taxa (Avise 1989). Three mitochondrial groups are basically distinguished (Fig. 5). One group may be considered as characteristically from the Chaco Domain and includes the haplotypes of *D. buzzatti* and *D. koepferae*, which is basal to the *D. buzzatii* cluster. Another group occurs in southeastern South America, in the southern depression of the Brazilian Plateau and in the Paraná-Paraguai river basin and includes the haplotypes of *D.* sp. D and a population of *D.* sp. B. Finally, a third group includes the species *D. serido*, *D. borborema*, *D. seriema* and *D.* sp. B in northeastern Brazil and Atlantic coastal regions. The discontinuity we observed could be evidence of long-established zoogeographical barriers preventing gene flow and allowing the accumulation of genetic differences. This suggests vicariant differentiation of these three groups inside the *D. buzzatii* cluster.

Considering the replacement rates of 6.85×10^{-9} per site per year for mtDNA, the estimated age for the separation of the clades comprised by *D. koepferae* and *D. buzzatti* from the five remaining *D. buzzatti* cluster species is approximately 6–12 Myr, whereas separation of the clades composed of *D.* sp. D and *D. seriema*, *D. borborema*, *D. serido*, and *D.* sp. B, is estimated at 3–6 Myr.

The distribution pattern of haplotypes of mtCOI and the proposed divergence ages, calculated on the basis of molecular data, suggest pre-Quaternary vicariant events for the establishment of regional divisions inside the *D. buzzatti* cluster. This contradicts the hypothesis suggested by Sene et al. (1982, 1988) who proposed that all species except *D. buzzatii* in the cluster had differentiated simultaneously in a mosaic fashion from isolated populations of a previously continuously distributed species. Also, they suggested breakup of this continuity occurred during the paleoclimatic cycles in the Quaternary Period.

Other studies have shown that perhaps many events of population differentiation that were thought to have occurred at the end of the Quaternary Period occurred as early as the Pliocene (Klincka and Zink 1997). In Amazonia, for instance, where many Quaternary events have been invoked to explain patterns of differentiation of plants and animals (Haffer 1969, Vanzolini and Williams 1970, Prance 1973), some hypotheses have been challenged, including pre-Quaternary estimates of differentiation (Cracraft and Prum 1988, Brower 1994, Bush 1994).

There are also ecological factors that may be directly connected to *D. buzzatii* cluster differentiation, such as host affiliations. The phylogeny of host cacti and the affinity of the flies to their host plants in these South American species group are not well studied. Little host plant data exist, preventing an understanding of the possible constraints and pathways by which this association could have influenced *D. buzzatti* cluster differentiation. However, host plant specificity has been observed in four species of the *D. mulleri* complex estimated from differences in larval fitness on different host cacti (Ruiz and Heed 1988). In the Argentinean Chaco Domain, it has been observed that *D. buzzatti* breeds mainly on fermenting *Opuntia* cladodes, yet columnar cacti are the main breeding and feeding resources of *D. koepferae* (Hasson et al. 1992, Fanara et al. 1999). In Brazil, in the areas where the cacti *Cereus* and *Opuntia* are sympatric, *D. buzzatti* emerge mainly from *Opuntia*, whereas *D.* sp. D uses *C. hildmannianus* (Ruiz et al. 1996).

Our data, while supporting the monophyly of the *D. buzzatii* cluster and its division in three distinctive filetic lineages, clearly reveals that the Quaternary glaciation cycles have not played a role in the early differentiation of the *D. buzzatii* cluster. Our findings indicate that speciation has not been simultaneous. Thus, it seems that evolution of the open vegetation *Drosophila* fauna may have happened against a more complex set of historical and ecological factors than previously supposed.

Acknowledgments

We thank Mario de Vivo for a critical reading of the manuscript. This work was supported by Coordenação de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundação de Amparo a Pesquisa do Estado de São Paulo.

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Received for publication 14 July 2000; accepted 2 February 2001.

Appendix 1

Alignments of variable and informative sites of mtDNA COI sequences for 28 individuals of species of *D. buzzatii* cluster. The number above the sequences are the positions of the variable and informative sites of the 1495 base pair sequenced in this work.

		11	1111111111	1111111112	2222222222
	1112233344	5666789900	1122223344	4566778880	0122334455
	0291606957	0369070925	1401365814	7635183691	7325140925
D. martensis	aagatttttg	cgtcctttca	tatgtctttt	tcgttatata	ctttaataac
D. richardoni	.g.....	.agt.a....	c.a.....a.	.t......at	t.......tt
D. serido					
H49Flm	??????????	???tt..ctt	.....tcca.	at.a....ag	t...g...t.
1431.3	.g...c.c..	.aatt..ctt	.....tcca.	ataa....a.	t...g.....
D. sp. B					
H32.9	.g.....	.?att.cc.t	..c..tcca.	a..a....ag	t.........
H32M	.g.....	.aatt.cc.t	..c..tcca.	a..a....ag	t.........
H32.1	.g.....	.aatt.cc.t	..c..tcca.	a..a.g..ag	t.........
H24S3	.g...c.c..	.aatt..ctt	.....tcca.	at.a....a.	t...g.....
H35	.g...c.c..	.aatt..ctt	.....tcca.	at.a...ca.	t...g.....
H6G6	.ga.ac.c.a	.aatt..ctt	.....tcca.	at.a....a.	t...g.....
D. sp. D					
H42R2M	.g.....	.aatt.cc.t	..c..tcca.	a..a....ag	t.......tt
H34G8	.g.....c.t	.aatt.cc.t	.gc..tccaa	a.aa....ag	t.........
D93S6M	.g.....c..	.tatt.cc.t	..c..tcca.	a.aa....ag	t.........
H44R2M	????????..	.aatt....t	.t...tcca.	a..ag..tag	t......g.t
H41S1	.g....a...	..att.cc.t	..c..tcca.	at.a....ag	t........t
H41S2	?????????.	.aatt.cc.t	..c..tcc..	at.a....ag	t........t
H48F1M	??????????	?aatt...tt	.....tcca.	a..a...tag	t.......t.
H47R5M	.g.....	.aatt.cc.t	..c..tcca.	a..a....ag	t........t
D. seriema					
D62C4B	.g.....c..	.tatt..ctt	.....tcca.	at.a...ca.	t...g.....
D54M	.g.....c..	.aatt..ctt	.....tcca.	a..a....a.	t...gg....
D40F1	.g.....c..	.aatt..ctt	.....tcca.	at.a....a.	tc..g.c...
D71C1B	.g.....c..	.aatt..ctt	.....tcca.	at.a....a.	t...g.....
D. borborema					
1281.0	.g.....c..	.aatt..ctt	.....tc.a.	at.a....a.	t...g.....
1281.2	.g.....c..	.aatt..ctt	.....tcca.	at.a....a.	t...g...t.
D. buzzatii					
H34G5	.g.....cc.	.aatt...tt	...ac...a.	a..a......	t.aa....t.
D54F5	.g.....cc.	.aatt...tt	...ac...a.	a..a......	t.aa......
H98	tg.....cc.	.aatt...tt	...ac...a.	a..a......	t.aa......
D. koepferae					
B26D2	.g.t......	taatt...tt	........a.	atta..a.a.	t.......t.
	2222222223	3333333333	3333333333	3444444444	4444445555
	6778888990	0112233344	6667778889	9001222233	5567890000
	769256143	9481736928	3692581470	3287067928	3681021457

D. martensis	tatatctcac	ataagcataa	acacagtctt	atatattatc	attatattct
D. richardsoni	.tc.....t	..tttt..gt	ttgt...t.a	t.......at	.a.g.....
D. serido					
H49F1M	.t....atta	..ct.t...g	tt.tct.ac.	ta.ctcctct	.ac.ct.ata
1431.3	.t.g..atta	..ct.t...g	t..tct.ac.	ta.ctcctct	.a..ct.ata
D. sp. B					
H32.9	.t....att.	..ct.t...g	tt.tct.a..	ta.ct.ctct	.ac.ct.ata
H32M	.t....att.	..ct.t...g	tt.tct.a..	ta.ct.ctct	.ac.ct.ata
H32.1	.t....att.	..ct.t...g	tt.tct.a..	ta.ct.ctct	.ac.ct.ata
H24S3	.t.g..atta	..ct.t...g	t..tctcac.	ta.ctcctct	.a..ct.ata
H35	.t.g..atta	..ct.t...g	t..tct.ac.	ta.ctcctct	.a..ct.ata
H6G6	.t.g..atta	..ct.t...g	tt.tct.ac.	ta.ctcctct	.a..ct.ata
D. sp. D					
H42R2M	.t....att.	..ct.t...g	tt.tct.a..	ta.ct.c.ct	.ac.ct.ata
H34G8	.t....att.	..ct?t.a.g	tt.tct.a..	ta.ct.ctct	.ac.ct.ata
D93S6M	.t....att.	..ct.t...g	tt.tct.a..	ta.ct.ctct	.ac.ct.ata
H44R2M	.ta.a.att.	..t.ttt..t	tt.ttt.a..	ta.ct.c.ct	.ac.ctcata
H41S1	.t....att.	..ct.t...g	tt.tct.a..	ta.ct.c.ct	.ac.ct.ata
H41S2	.t....att.	..ct.t...g	tt.tct.a..	ta.ct.c.ct	.ac.ct.ata
H48F1M	.t....att.	..ct.t...g	?t.tct.a..	ta.ct.ctct	.ac.ct.ata
H47R5M	.t....a.t.	..ct.t...g	tt.tct.a..	ta.ct.c.ct	.ac.ct.ata
D. seriema					
D62C4B	.t....atta	..ct.t...g	t..ttt.a..	ta.ctcctct	.ac.ct.ata
D54M	.t.g..atta	..ct.t...g	t..tct.ac.	ta.ctcctct	.a..ct.ata
D40F1	.t....atta	..ct.t...g	t..tct.a..	ta.ctcctct	.ac.ct.ata
D71C1B	.t....atta	..ct.t...g	tt.ttt.a..	ta.ct.ctct	gac..t.ata
D. borborema					
1281.0	.t....atta	..ct.t...g	tt.tct.a..	tagct.ctct	.ac..t.ata
1281.2	.t....atta	..ct.t...g	tt.tct.a..	ta.ct.ctct	.ac..t.ata
D. buzzatii					
H34G5	ct..atatta	g.tt.t....	t..ttt.a..	t..ctc..ct	gac..t.ata
D54F5	ct..atatta	gctt.t...t	t..ttt.a..	t..ctc..ct	gac..t.ata
H98	ct..atatta	g.tt.t....	t..ttt.a..	t..ctc..ct	gac..t.ata
D. koepferae					
B26D2	.t..atatta	g.tt.t....	ct.ttt.a..	t..ctc...t	.a...t.ata
	5555555555	5555555556	6666666666	6666677777	7777777777
	1112334555	6666778880	0112233444	5568901222	3334556668
	0368476235	1278032586	9281439258	4767027069	2587092580
D. martensis	ttataaaata	ttccgacatt	taacctaaaa	tactgtaaca	atttcagagg
D. richardsoni	.a.gct.t..	..atat..aa	...ttc....	..tcta..t.	....t.a.aa
D. serido					
H49F1M	.a.a.tt.cc	..tta...a.	.t.tt...tg	ct..a...tg	....t....a
1431.3	.aga.tt...	..tta..ga.	.t.ttc..t.	ctt.t..gtg	....
D. sp. B					
H32.9	ca.gttt.c.	..tta.t.a.	.t.ttc..c.	c.......t.	.cc.t.a..a
H32M	ca.gttt.c.	..tta.t.a.	.t.ttc..c.	c.......t.	.cc.t.a..a
H32.1	ca.gttt.c.	..tta.t.a.	.t.ttc..c.	c.......t.	.cc.t.a..a
H24S3	.aga.tt...	..tta..ga.	.t.ttc..t.	ctt.a...t.	....
H35	.aga.tt...	..tta..ga.	.t.ttc..t.	ctt.a...t.	....
H6G6	.aga.tt...	..tta..ga.	.t.ttc..t.	ctt.a...t.	....
D. sp. D					
H42R2M	.a...tt..t	tcacaa....	.t.tt...t.	c...a...t.	.cc.t.a..a
H34G8	ca.gttt.c.	..tta.t.a.	.t.ttc..c.	c.......t.	.cc.t.a..a
D93S6M	ca.gttt.c.	..tta.t.a.	.t.ttc..c.	c.......t.	.cc.t.a..a
H44R2M	ca.g.tt.c.	..tta.tga.	.a.g.tt.c.	...tta.tga	.t.tt...t.
H41S1	ca.a.tt.c.	..tta.tga.	.t.tt...t.	c...a...t.	.cc.t.a..a
H41S2	ca.g.tt.c.	..tta.tga.	.t.tt...t.	c...a...t.	.cc.t.a..a
H48F1M	ca.gttt.c.	..tta.t.a.	.t.ttc..c.	c.......t.	.cc.t.a..a
H47R5M	ca.g.tt.c.	..tta.tga.	.t.tt...t.	c...a...t.	.cc.t.a..a
D. seriema					
D62C4B	.a.a.tt.c.	..tta..ga.	.t.ttc..tg	ct..a...t.	......a...
D54M	.aga.tt...	..tta..ga.	.t.ttc..t.	c...a..ttg	..c.t.....
D40F1	.a.a.tt.c.	..tta..ga.	.t.ttc..t.	ct..a...t.	..c......a
D71C1B	.a.a.tt.cc	..tta...a.	.t.ttc..tg	ct..a...t.	..c......a
D. borborema					
1281.0	.a.a.tt.cc	..tta...a.	.t.ttc..t.	ct......tg	..c......a
1281.2	.a.a.tt.cc	..tta...a.	t.ttc..t.	ct......tg	..c......a
D. buzzatii					
H34G5	ca....t..g	cctta...a.	...ttcg...	....a..ct.	g..c..a.a.
D54F5	ca....t..g	cctta...a.	c..ttcg...	....a...t.	g..c..a.a.
H98	ca....t..g	cctta...a.	c..ttcg...	....a..ct.	g..c..a.a.
D. koepferae					
B26D2	.a.c.tt...	..ttata...	...ttc.c..	a...a...t.	....tg.g.a
	7777788888	8888888888	8888888999	9999999999	9999999999
	8899900011	2223444455	6677799011	1223344445	5556666777
	3425845767	2584023928	1703614325	8146923581	2470139058

D. martensis	ataccataat	tttttacata	atttcctaac	atacaatgta	cccaaattca	
D. richardsoni	...ttg..t.	.a.a..t...	..a.aaa.ta	t.t....aa.	.at.gt..tt	
D. serido						
H49F1M	t..t...t.c	ca....t..t	tcacaaa..t	......ct.t	tatggt....	
1431.3	tc.tt....c	.a....t..t	tcacaaa..t	......ct.t	tatggt....	
D. sp. B						
H32.9	t.gt..c...	.a....t..t	tcacaa....	....t.ct.t	tat..t....	
H32M	t.gt..c...	.a....t..t	tcacaa....	....t.ct.t	tat..t....	
H32.1	t.gt..c...	.a....t..t	tcacaa....	....t.ct.t	tat..t....	
H24S3	tc.tt....c	.a....t..t	tcacaaa..t	......ct.t	tatggt....	
H35	tc.tt....c	.a....t..t	tcacaaa..t	......ct.t	tatggt....	
H6G6	tc.tt....c	.a....t..t	tcacaaa..t	......ct.t	tatggt....	
H42R2M	t..t.....	.a....t..t	tcacaa....	....t.ct.t	tat..t....	
D. sp. D						
H34G8	t.gt.....	.a....t..t	tcacaa....	....t.ct.t	tat..t....	
D93S6M	t.gt..c...	.a....tc.t	tcacaaa...	....t.ct.t	tat..t....	
H44R2M	c...a...t.	.cc.t.a..a	t.gt.....	....t.ct.t	tat..t....	
H41S1	t..t.....	.a....t..t	tcacaaa...	....t.ct.t	tat..t....	
H41S2	t.gt.....	.a....t..t	tcacaaa...	....t.ct.t	tat..t....	
H48F1M	t..t..c...	.a....t..t	tcacaa....	....t.ct.t	tat..t....	
H47R5M	t.gt.....	.a....t..t	tcacaa....	....tgct.t	tat..t....	
D. seriema						
D62C4B	t..t.....	.a.......t	tcacaaa..t	..g...ct.t	tat.gt.a..	
D54M	t..t.....c	.a....t..t	tcacaaa..t	..g...ct.t	tat.gt....	
D40F1	tc.t.....	.a....t..t	tca.aaa..t	......ct.t	tat.gt....	
D71C1B	tc.t.....c	.a....t..t	tcacaaa..t	......ct.t	tat.gtc...	
D. borborema						
1281.0	tc.t.....c	.a....t..t	tcacaaa..t	......ct.t	tat.gtc...	
1281.2	tc.t.....c	.a....t..t	tcacaaa..t	......ct.t	tat.gtc...	
D. buzzatii						
H34G5	t..t..c...	.ac.c.t..t	tca.aaag..	.a.t..ctct	ta...t....	
D54F5	.ac.c.t..t	tca.aaa...	.a.t..ctct	ta...t....	ca....tc..	
H98	.ac.c.t..t	tca.aaa...	...t..ctct	ta...t....	ca....tc.a	
D. koepferae						
B26D2	t.........	.ac.c.t.ct	tca.aaa..t	.a....ct.t	.at.gt..t.	
	1111111	1111111111	1111111111	1111111111	1111111111	
	9990000000	0000000000	0000011111	1111111111	1111111122	
	8990011123	3444555677	8889900111	1122333445	5566778801	
	8362812795	8457039524	0675814034	6928147092	5848395615	
D. martensis	tgtaatcttt	attattatct	cctttccctt	aaatacttct	cccttatttt	
D. richardsoni	.a.g..t...	..ct....ta	tt.cattt..	.t...taca.	ttt..t...c	
D. serido						
H49F1M	.a.g..t.aa	g.cta...ta	.t.a.tt..c	tt.act..a.	tt.c...c..	
1431.3	.a.tt.t.ga	gccta...ta	.t.a.tt..c	tt.act..a.	tt.c...c..	
D. sp. B						
H32.9	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt.....c..	
H32M	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt.....c..	
H32.1	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt.....c..	
H24S3	.a.g.ct.ga	g.cta...ta	tt.a.tt..c	tt.act..a.	tt.c...c..	
H35	.a.g.ct.ga	g.cta...ta	.t.a.tt..c	tt.act..a.	tt.c...c..	
H6G6	.a.g.ct..a	g.cta...ta	.t.a.tt..c	tt.act..a.	tt.c...c..	
D. sp. D						
H44r2M	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt........	
H34G8	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt.....c..	
D93S6M	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt.....c..	
H42R2M	.a.g..t..a	..cta..cta	tt.actt.cc	t..act..a.	tt....cc..	
H41S1	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt....cc..	
H41S2	.a.g..t..a	..cta..cta	tt.actt.cc	t??actc.a.	tt....cc..	
H48F1M	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt.....c..	
H47R5M	.a.g..t..a	..cta..cta	tt.actt.cc	t..actc.a.	tt....cc..	
D. seriema						
D62C4B	.a.g..t.ga	..cta.c.ta	.tga.tt..c	tt.act..a.	tt.....c..	
D54M	.a.g..t.ga	..cta.c.ta	.t.a.tt..c	tt.act..a.	tt.c...c..	
D40F1	.a.g..t.aa	..cta.c.ta	.t.a.tt..c	tt.act..a.	tt.c...c..	
D71C1B	.a....t.aa	..cta...ta	.t.a.tt..c	tt.a.t..a.	.t.c...c..	
D. borborema						
1281.0	.a....t.aa	g.cta...ta	.t.a.tt..c	tt.a.t..a.	tt.c...c..	
1281.2	.a....t.aa	..cta...ta	.t.a.tt..c	tt.a.t..a.	tt.c...c..	
D. buzzatii						
H34G5	ca....tc..	..ccac..ta	tt.a..tt.c	t..a.t..ac	tt..c.....	
D54F5	..ccac..ta	tt.a..tt.c	t..a.t..ac	tt..c.....	cctt..cc..	
H98	..ccac..ta	tt.a..tt.c	t..a.t..ac	t..t..c...	tt..c.....	
D. koepferae						
B26D2	.ac...tc..	g.cta...ta	tt...ttt.c	ttga.t..a.	tt...t..c.	
	1111111111	1111111111	1111111111	1111111111	1111111111	111111
	2222222222	2222223333	3333333333	3333344444	4444444444	444444

	1234556667	7788990001	1334555666	7888900112	2234445556	677789
	8735140490	5814032584	7081039025	7036947392	5813695896	703681
D. martensis	ttaactttat	taattccttt	taacacataa	ctcttatcat	ttctacaccc	ttttct
D. richardsoni	..t.t...t.	.t..att.cc	ag.t.tg.g.	..t....a..	.ata.t.ta.	aaa.ta
D. serido						
H49f1M	..tct.....	ag..a.ta.c	c.t..t.cg.	..t...aa.c	c.t.ttg...	.c..t.
H1431.3	..tt.....	.g..a.ta.c	c....t.cg.	..t...aa.g	c.t.t..taa	.ca.t.
D. sp. B						
H32.9	..tc.c....	.t..a.ta.c	c..t.t....	tct..taa..	c.t.ttg?..	.c..t.
H32m	..tc.c....	.t..a.ta.c	c..t.t....	tct..taa..	c.t.ttgt..	.c..t.
Hh32.1	..tc.c....	.t..a.ta.c	c..t.t....	tct..taa..	c.t.ttgt..	.c..t.
H24S3	..tt.....	.g..a.ta.c	c....t.cg.	..t...aa.c	c.t.t.....	....t.
H35	..tt.....	.g..a.ta.c	c....t.cg.	..t...aa.c	c.t.t.....	.c..t.
H6G6	..tt.....	.g..a.ta.c	c....t.cg.	..t...aa.c	c.t.t.....	....t.
H42R2M	..tc.c....	.t..a.ta.c	c..t.t....	tct...aa..	c.t.t.gt..	.c..t.
D. sp. D						
H34G8	..tc.c....	.t..a.ta.c	c..t.t....	tct..taa..	c.t.ttgt..	.c.at.
D93S6M	..tc.c....	.t..a.ta.c	c..t.t....	..t...aa.c	c.t.tt....	....t.
H44R2M	..tc.c....	.t..a.ta.c	...t.t....	tct...aa..	c.t.ttg...	.cact.
H41S1	..tc.c....	.t..a.ta.c	...t.t....	tct...aa..	c.t.ttg...	.c..t.
H41S2	..tc.c....	.t..a.ta.c	...t.t....	tct...aa..	c.t.ttg...	.c..t.
H48F1M	..tc.c....	.t..a.ta.c	c.tttt....	tct..taa..	c.t.ttgt..	.c..t.
H47R5M	..tc.c....	.t..a.ta.c	...t.t....	tct...aa..	c.t.ttg...	.c.ct.
D. seriema						
D62C4	..tc.....c	.t..a..a.c	c..t.t..gg	.ct...aa..	c.t.tt.t..	.c.ct
D54M	..tc.....c	.g..a..a.c	c....t.cgg	.ct...aa.?	c.t.t..t..	.c..t.
D40F1	..tt.....c	.g..a.ta.c	c....t.cg.	.ct.c.aa..	c.t.tt.t..	.c..t.
D71C1	..tc.....c	.g..a.ta.c	.....t.cg.	.ct...aa.?	c.t.t.....	.c..t.
D. borb.						
1281.0	..tct.....	.g..a.ta.c	c....t.cg.	..t...aa.c	c.t.t.....	.c..ta
1281.2	..tct.....	.g..a.ta.c	c....t.cg.	..t...aa.c	c.t.t.....	.c..t.
D. buzzatii						
H34G5	cctt..cc..	at..a..a.c	a.........	..t...aag.	c.t.....	.....
D54F5	cctt..cc..	atg.a..a.c	a.........	..t...aag.	c.t....t..	.....
H98	cctt..cc..	a...a..a.c	a.........	..t...aag.	c.t.....	..c...
D. koepferae						
B26D2	..tt..c...	a..aatt..c	a.c.....	..tc..ca..	..t..t....	.....