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COMPARATIVE PHYLOGEOGRAPHY OF A SIBLING PAIR OF RAINFOREST *DROSOPHILA* SPECIES (*DROSOPHILA SERRATA* AND *D. BIRCHII*)

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Abstract.—*Drosophila serrata* and *D. birchii* are presumed sibling species; the former is a widespread generalist and the latter is restricted to rainforests. Comparison of the mtDNA sequence phylogeographies revealed two highly divergent, geographically distinct lineages of *D. serrata* that are as distinct from each other as either is from *D. birchii*. However, diversity in *D. birchii* is low and unstructured. The low diversity in *D. birchii* corresponds with a late-Pleistocene-Holocene contraction of lowland rainforests. We suggest that future studies of speciation and adaptation should compare the two lineages of *D. serrata* to each other as well as to *D. birchii*.

Key words.—Biogeography, *Drosophila*, mtDNA, ND5, phylogeny, rainforest, selection.

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Since the 1950s, species of *Drosophila* have been used as a model system for studies of speciation and adaptation (Dobzhansky and Mather 1961; Rice and Hostert 1993). To interpret differences (e.g., in morphology, physiology, reproductive compatibility) found by such studies within or between species, it is important to know the history and relationoships of the taxa involved (Harvey and Pagel 1991). In particular, analyses of adaptation benefit from identification of evolutionarily independent lineages and the relationships among these.

Two species used extensively in studies of speciation and adaptation are *D. birchii* and *D. serrata* (e.g., Ayala 1965a,b; Hoffman 1991; Blows and Hoffman 1993, Blows 1998). The advantage of using this species pair lies in the difference in their geographic range, physiological tolerances, and habitat preferences (Ayala 1965a,b; Parsons 1982; Hoffman 1991). *D. birchii* appears to be restricted to patches of warmer, wet tropical rainforest between New Guinea and Eungella (mid-eastern Queensland; Fig. 1) and is sensitive to low temperature and humidity. By contrast, *D. serrata* has a more cosmopolitan habitat distribution from New Guinea down the east coast of Australia to southern New South Wales and has a higher tolerance of environmental extremes. Interpretation of differences between *D. serrata* and *D. birchii* has been based on a sibling and presumed sister species relationship. They are nearly identical in their morphology and lack post-mating isolation (Dobzhansky and Mather 1961; Ayala 1965a,b), although there is strong premating isolation between *D. serrata* and *D. birchii*, and evidence for partial isolation between certain *D. birchii* populations (Ayala 1965a,b).

The purpose of the present study was to investigate geographic-genetic structure within and relationships between Australian populations of *D. serrata* and *D. birchii*. With respect to geographic variation, we predicted that the more rainforest-restricted species, *D. birchii*, should show stronger phylogeographic structure than the habitat generalist, *D. serrata*, because of current disjunctions and historical vicariance of rainforests, as observed in several species of vertebrate from this area (Joseph and Moritz 1994; Joseph et al. 1995; Schneider et al. 1998). To test this prediction, we analyzed mtDNA sequence variation at part of the ND5 gene among populations from the two species across their respective ranges in eastern Australia (Fig. 1). The ND5 gene seemed ideal for this purpose because of the high variability it has shown in previous studies (Rand et al. 1994; Rand and Kann 1996). Tests for neutrality were also carried out in this study, because the effect of selection can confound historical patterns (Rand et al. 1994; Ballard and Kreitman 1994).

MATERIALS AND METHODS

Field-caught flies were collected from fermenting bananas which were left in the field in buckets for three days. *Drosophila serrata* and *D.birchii* males, used in molecular genetic analyses, were identified following Dobzhansky and Mather's (1961) description. The flies were temporarily preserved in a solution of 20% dimethyl-sulfoxide (DMSO) in saturated NaCl. *Drosophila serrata* was collected from six locations between Brisbane and Mossman Gorge (Brisbane, Eungella, Paluma, Kirrama, Cairns and Mossman, Fig. 1). *Drosophila serrata* strains from Bulli, Forster, and Coffs Harbour were also obtained from isofemale lines maintained by N. Jenkins at LaTrobe University. Wild *D. birchii* were collected from Eungella, Paluma, Kirrama, Cairns, and Mossman.

A 398-bp 5′ segment of the ND5 gene was obtained for individuals of *D. serrata* and *D. birchii* from each geographic location sampled for these species. The resulting alignment of these sequences is available from the European Bioinformatics Institute site (ftp//ftp.ebi.ac.uk/pub/data bases/embl/align/) with accession number ds38750.

Template DNA for the polymerase chain reaction (PCR) was extracted from single flies by boiling in a chelex solution. The ND5 fragment was sequenced using the 2838L-2299R primer pair, where the number represents the 3′ position of the primer and the letter represents the direction of chain elongation—left (L) or right (R)—in relation to the light

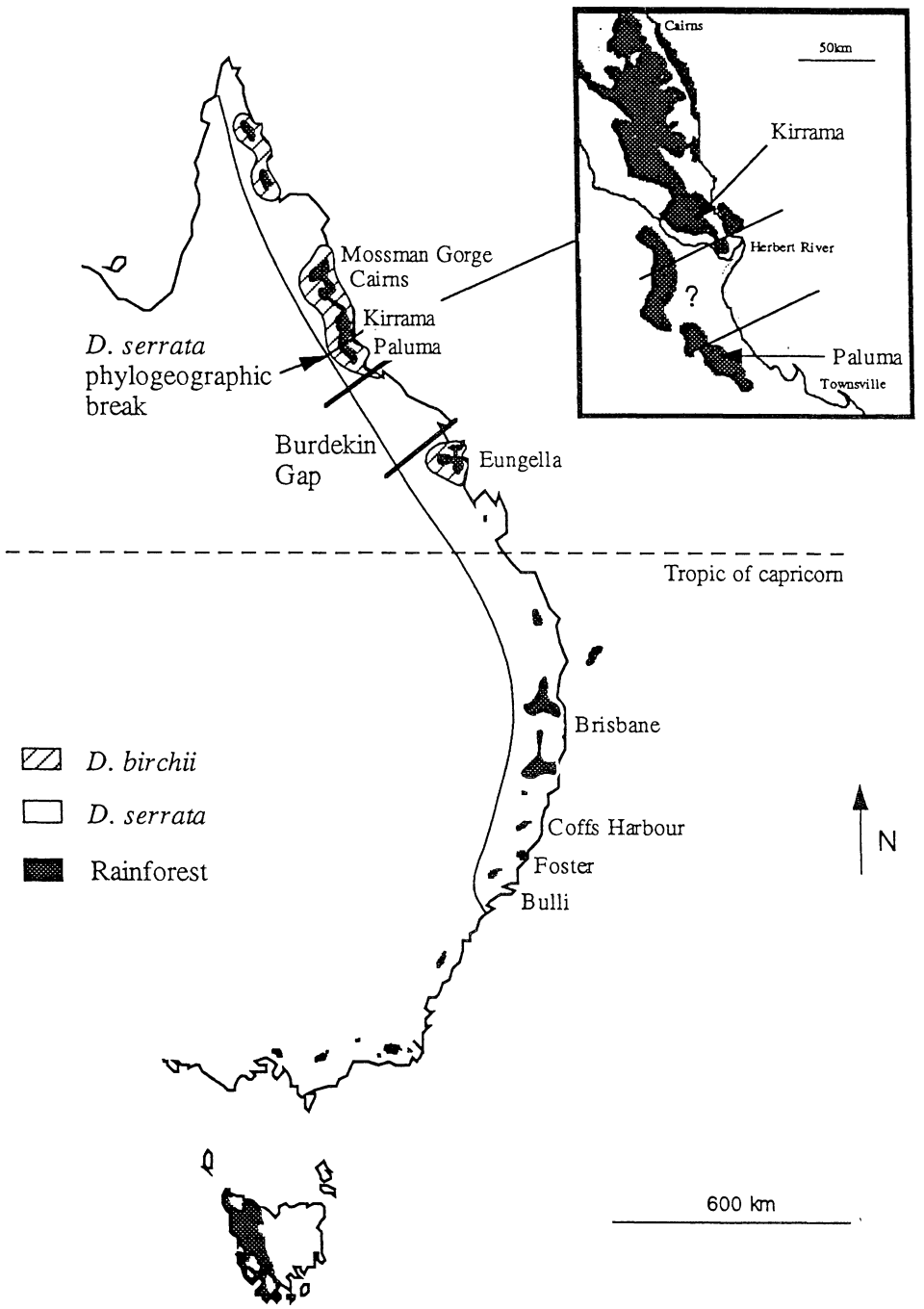

FIG. 1. Location of sampling sites for *Drosophila serrata* and *D. birchii* in eastern Australia. Both species were sampled from sites where they co-occur. Labeled localities indicate sampling sites (see Table 1).

strand of the mtDNA published by Garesse (1988). The primer 2299R is published in Rand and Kann (1996). 2838L (5′CAATTTGTAGAATTAGATTTGT3′) was designed based on preliminary sequences of the complete ND5 gene from a few representative *D. serrata* and *D. birchii* individuals.

PCR was performed with an initial denaturation of 94°C for 1 min and cycle parameters of 45-sec denaturation at 94°C, 45-sec annealing at 45°C, and 1-min extension at 72°C, for 32 cycles. The 25 μL reaction mixture consisted of 13.15 μL of autoclaved milliQ water, 2.5 μL of 10X ProMega PCR buffer, 0.15 μL of 10 μM dNTPs, two each of 0.5 μL of 10 μM primer, 3 μL of 25 μM ProMega $MgCl_2$, 1 unit of ProMega Taq DNA polymerase, and 5 μL of DNA extract. The PCR product was gel purified and sequenced using an automated sequencing procedure. ABI prism termination dye was incorporated into the DNA chain in a Perkin Elmer DNA thermal cycler 480, using cycling conditions and reagent concentrations recommended by the supplier.

The DNA sequences were aligned by eye, using the codon frame for ND5. Measures of silent and replacement poly-

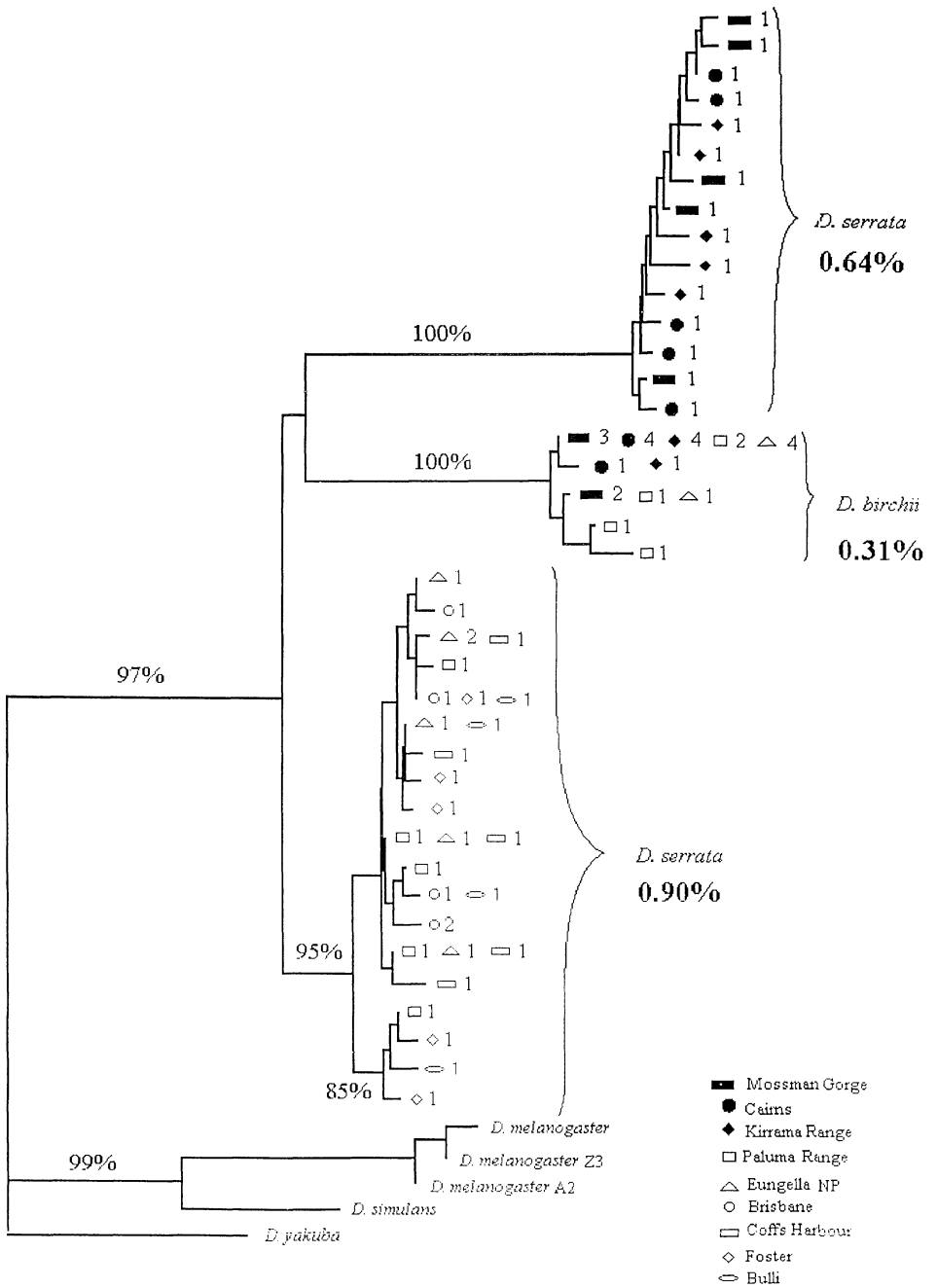

FIG. 2. Neighbor-joining tree representing the geographic distribution of *Drosophila serrata* and *D. birchii* mtDNA haplotypes. Locations are mapped in Figure 1; solid symbols indicate localities from Kirrama north and open symbols from Paluma south. Values indicate the number of individuals displaying that haplotype. Numbers below species names are within-linage nucleotide diversities and numbers on major branches are the percent bootstrap support.

morphisms and diversity were given by the DNAsp (Rozas and Rozas 1997) program. Molecular divergence among alleles was estimated using Jukes-Cantor distances from DNAsp. Values of nucleotide diversity within species/clades were obtained from the Arlequin 1.0 (Schneider et al. 1996) program. The hierarchical distribution of sequence variation among populations and regions was examined through analysis of molecular variance (Excoffier et al. 1992) as implemented in Arlequin 1.0 (Schneider et al. 1996). The pattern of within- and between-lineage polymorphisms was used to test for selection on the 5′ segment of ND5. Tajima's *D* measure (Tajima 1989) was used for within-species/clade tests in Arlequin 1.0 (Schneider et al. 1996) and the McDonald-Kreitman test (McDonald and Kreitman 1991) for between-lineage comparisons in DNAsp (Rozas and Rozas 1997).

An intraspecific gene tree was constructed using the neighbor-joining method (Saitou and Nei 1987) in the PAUP* program (Swofford 1998). The Tamura-Nei algorithm (Tamura

TABLE 1. Total divergence (% d_{xy}, shown upper right) and among-population sequence divergence (ϕ_{ST}, shown lower left) in comparisons of the 5′ sequences of the ND5 gene from populations of *Drosophila serrata*. The values in italic indicate comparisons between clades. Nucleotide diversity, π (Jukes-Cantor) is shown across the diagonal in bold. M, Mossman Gorge; C, Cairns; K, Kirrama Range; P, Paluma Range; E, Eungella National Park (Finch Hatton Gorge); B, Brisbane; Co, Coffs Harbour; F, Forster; Bu, Bulli Woolongong). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

	M	C	K	P	E	B	Co	F	Bu
M	**0.76**	0.63	0.76	*7.27*	*7.01*	*7.39*	*7.16*	*7.28*	*7.42*
C	−0.009	**0.51**	0.56	*7.13*	*6.90*	*7.27*	*7.02*	*7.11*	*7.28*
K	0.103	0.004	**0.61**	*7.20*	*7.01*	*7.36*	*7.09*	*7.21*	*7.36*
P	*0.905***	*0.916****	*0.911****	**0.92**	0.77	0.84	0.80	1.07	0.90
E	*0.921****	*0.932****	*0.928****	0.032	**0.59**	0.75	0.64	1.08	0.91
B	*0.912****	*0.924***	*0.920****	0.029	0.025	**0.76**	0.85	1.11	0.89
Co	*0.911***	*0.923****	*0.918***	−0.044	−0.059	0.086	**0.76**	1.11	0.99
F	*0.878***	*0.888***	*0.883***	−0.079	0.097	0.042	0.056	**1.38**	1.10
Bu	*0.891**	*0.904***	*0.899****	−0.111	−0.022	−0.094	−0.017	−0.159	**1.19**

and Nei 1993) was used to construct a distance matrix and the pattern of substitution rates along the gene was assumed to follow a gamma distribution with a shape parameter of 0.4 (see Tamura 1992; Spicer 1995). Confidence values for the branches were obtained using Rzhetski and Nei's (1993) bootstrapping method, again in PAUP*.

RESULTS

Drosophila serrata shows strong phylogeographic structure ($\phi_{CT} = 0.904$, $P = 0.008$) with 90% of the molecular variation distributed between two geographically defined and strongly supported clades distributed to the north of Kirrama and to the south of Paluma, respectively (Fig. 2). Average sequence divergences between populations from the two different clades are in the order of 7% (mean = 7.19%, SD = 0.15%), in comparison to 1% or less (northern *D. serrata* mean = 0.65%, SD = 0.1%; southern *D. serrata* mean = 0.92% SD = 0.15%) among populations within each of the two regions (Table 1). Nucleotide diversity is relatively low within both of these clades (0.63% in the north [SD = 0.13%] and 0.93% in the south [SD = 0.3%]). The diversity within the geographic clades (the remaining 10% of the molecular variation) is distributed almost entirely within populations with close to zero variation among populations ($\phi_{SC} = 0.07$, $P = 0.425$). This is reflected by the small divergence values for pairwise population comparisons within regions (Table 1), with negative values of ϕ_{ST} which indicate that there is more variation within the populations involved than between them.

TABLE 2. Nucleotide diversities, π (Jukes-Cantor) is shown across the diagonal bold italic, total divergence (d_{xy}, shown upper right), and among-population divergence (ϕ_{ST}, shown lower left) in comparisons of the 5′ sequences of the ND5 gene from populations of *Drosophila birchii*. M, Mossman Gorge; C, Cairns; K, Kirrama Range; P, Paluma Range; E, Eungella National Park (Finch Hatton Gorge).

	M	C	K	P	E
M	***0.30***	0.25	0.25	0.50	0.22
C	0.201	***0.10***	0.08	0.51	0.15
K	0.201	−0.250	***0.10***	0.51	0.15
P	0.052	0.135	0.135	***0.71***	0.48
E	0.136	0.001	0.001	0.026	***0.20***

Only five haplotypes were found among 25 *D. birchii* individuals in comparison to 34 among the 45 *D. serrata*. *Drosophila birchii* shows no significant geographic genetic structure ($\phi_{ST} = 0.068$, $P = 0.219$) and has low overall nucleotide diversity (mean = 0.28%, SD = 0.25%). One of the haplotypes is very common and widespread while two are confined to Paluma (Fig. 2). As a result, Paluma shows the highest within-population diversity, comparable to that within populations of *D. serrata* (Table 2). Variation within populations accounts for 93% of the nucleotide diversity, whereas only 7% occurs among populations.

Divergence between the major clades of *D. serrata* (mean $d_{xy} = 7.2\%$) is comparable to that between each of these and *D. birchii* ($d_{xy} = 8.4$ and 5.9% for the northern and the southern clades, respectively).

The only suggestion of a departure from neutrality for these mtDNA genomes is a significantly higher level of replacement polymorphism in the 5′ region of the ND5 gene within *D. birchii* relative to fixed differences between *D. birchii* and both the northern and southern *D. serrata* clades ($G = 8.89$, $P < 0.01$ and $G = 5.42$, $P < 0.05$, respectively; Table 3). On the other hand, no such evidence for selection in *D. birchii* was found using Tajima's D test ($D = -0.54$, $n = 30$, $P > 0.05$) for the same gene region.

DISCUSSION

It was expected that *D. birchii*, a habitat specialist confined to relatively warm rainforest areas, would show stronger phylogeographic structure on a macrogeographic scale than the more generalist *D. serrata* because of the current and historical discontinuous nature of rainforests. Contrary to this prediction, *D. birchii* showed very little diversity and no geographic structure even across the dry Burdekin Gap which currently separates Eungella from the rainforest areas to the north (Fig. 1) and which represents a major genetic disjunction in rainforest vertebrates (Joseph et al. 1993; Joseph and Moritz 1994). On the other hand, *D. serrata* showed a deep and geographically narrow phylogenetic break between the Kirrama and Paluma ranges with no overlap between the two clades. The pattern in *D. serrata* indicated historical isolation between the two major lineages (Avise et al. 1987), possibly due to vicariance, which was unexpected within a species

TABLE 3. McDonald-Kreitman test of neutrality. The *G*-value was obtained from the comparison of the fixed changes between species to the within-species polymorphism. D. bir, *Drosophila birchii*; n D. ser, northern *D. serrata* clade; s D. ser, southern *D. serrata* clade; repl., replacement; polym., polymorphisms; v, compared to. * $P < 0.05$, ** $P < 0.01$.

Between-species	Fixed repl.	Fixed silent	Within-species	Repl. Polym.	Silent polym.	G
D. bir v. n D. ser	2	30	D. bir	4	2	8.890**
D. bir v. n D. ser	2	30	n D. ser	1	13	0.010
D. bir v. s D. ser	2	14	D. bir	4	2	5.417*
D. bir v. s D. ser	2	14	s D. ser	2	18	0.050
n D. ser v. s D. ser	2	19	n D. ser	1	13	0.053
n D. ser v. s D. ser	2	19	s D. ser	2	18	0.002

with such a broad habitat range and tolerance of environmental extremes relative to *D. birchii*.

Paleoclimatic modelling (Nix 1991) suggests that the relatively warm lowland rainforest environments preferred by *D. birchii* (Parsons 1982) were very restricted during the last glacial period of the Pleistocene. From studies of Holocene charcoal deposits, Hopkins et al. (1996) concluded that lowland rainforests in this region may have been restricted to small riparian coastal refugia and did not begin reexpanding until just over 1000 years ago. Recolonization of lowland eucalypt woodlands by rainforest species seems to have occurred in the last 200 years, perhaps due to the cessation of burning by indigenous communities.

It follows that the low diversity and lack of geographic structuring of mtDNA in Australian *D. birchii* may be a consequence of a recent range expansion, either from a restricted lowland rainforest refugium in the wet tropics or from southern Papua New Guinea. The latter is suggested by similarities in chromosome structure and reproductive compatibility between north Queensland and Daru (southern Papua New Guinea), but not other Papua New Guinean populations (Baimai 1969, 1970). Either of these scenarios suggests that the absence of geographic differentiation in *D. birchii* is due to recent range expansion rather than ongoing gene flow. In this context, the elevated replacement polymorphism within *D. birchii* might be due to relaxed selection against slightly deleterious mutations during a recent range expansion (e.g., Nachman et al. 1996).

The presence of a large phylogeographic break within *D. serrata* for mtDNA as well as its location are puzzling. One possibility is that the two lineages represent distinct, morphologically cryptic species. Previous crossing experiments (Ayala 1965a) between *D. serrata* from the south and Cooktown individuals indicated early stages of reproductive isolation, represented by asymmetric mating success. Another possibility is that the two lineages represent range expansions from separate southern and northern refugia, with the current break point representing secondary contact (Avise et al. 1987). The location of this contact between Paluma and Kirrama (Fig. 1) differs from phylogeographic and biogeographic evidence from other rainforest species that typically associate Paluma with areas to the north and Eungella with those in the south (Joseph et al. 1993; Joseph and Moritz 1994). Further, it is difficult to envisage a habitat barrier that would restrict gene flow across this break in *D. serrata* but not in the more specialized *D. birchii*. We are left with the rather unsatisfying hypothesis of historical contingency. Assuming that the ranges of both *D. serrata* and *D. birchii* contracted during Pleistocene glacial periods, it may be that *D. birchii* colonized the relatively small rainforest areas of Paluma and Eungella from the north, whereas *D. serrata* colonized Paluma from the south. Interestingly, Blows (1993) observed slight isolation by distance in reproductive compatibility within the southern lineage of *D. serrata*.

The molecular divergence between the two lineages of *D. serrata* (9.51%; L. Kelemen, unpubl. data) is similar to that between *D. yakuba* and *D. simulans* (9.3%, Rand et al. 1994) for a larger segment of the ND5 gene. Neither sequence (5′ ND5 and 16S rRNA; L. Kelemen, unpubl. data) nor RFLP band (Nikolaidis and Scouras 1996) phylogenetic analyses provide clear resolution of the relationships between the two lineages of *D. serrata* and *D. birchii* and other species of the *montium* subgroup.

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