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Trans-oceanic and endemic origins of the small minnow mayflies (Ephemeroptera, Baetidae) of Madagascar

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We investigated the relative importance of dispersal and vicariance in forming the Madagascar insect fauna, sequencing approximately 2300 bp from three rRNA gene regions to investigate the phylogeny of Afrotropical small minnow mayflies (Ephemeroptera: Baetidae). Six lineages contained trans-oceanic sister taxa, and variation in genetic divergence between sister taxa revealed relationships that range from very recent dispersal to ancient vicariance. Dispersal was most recent and frequent in species that spend the larval stage in standing water, adding to evidence that these evolutionarily unstable habitats may select for ecological traits that increase dispersal in insects. Ancestral state likelihood analysis suggested at least one Afrotropical lineage had its origin in Madagascar, demonstrating that unidirectional dispersal from a continental source may be too simplistic. We conclude that the Malagasy mayfly fauna should be considered in a biogeographical context that extends beyond Madagascar itself, encompassing trans-oceanic dispersal within multiple lineages.

Keywords: Africa; ancestral state; biogeography; dispersal; genetic; phylogeny

1. INTRODUCTION

Madagascar is one of the most biologically diverse areas on Earth and is well recognized as a discrete, globally important centre of evolution (Goodman & Benstead 2003; de Wit 2003). While large parts of the fauna and flora have evolved over more than 80 million years of isolation from all other landmasses (Lourenço 1996; Goodman & Benstead 2003), an increasing number of studies highlight the important impact of more recent colonizations (Cibois *et al.* 1999; Douady *et al.* 2002; Nagy *et al.* 2003; Yoder *et al.* 2003; Sparks 2004). In particular, recent molecular phylogenetic studies of reptiles (Raxworthy *et al.* 2002; Vences *et al.* 2003) and birds (Jansa *et al.* 1999; Groombridge *et al.* 2002) have begun to establish a closer link of Malagasy lineages with groups elsewhere, where trans-marine migrations occur within geographically widespread radiations. To date, these studies have been confined to vertebrates.

Insects comprise a large proportion of faunal biodiversity in Madagascar (Paulian & Viette 2003). Insects are highly diverse in their ecological attributes affecting dispersal propensity, and therefore their ability to colonize islands, but we are aware of no formal studies of the age and origin of Malagasy insect groups. The global biogeography of many insect groups is attributed to vicariance processes (e.g. Gauld & Wahl 2002; Sanmartin & Ronquist 2004). Single dispersal events are sometimes invoked to link speciose allopatric lineages, e.g. Tricoptera (Johanson 1998) and families of Coleoptera (Sequeira & Farrell 2001; Davis *et al.* 2002), but wide-ranging and repeated dispersal is thought to occur for only the more vagile groups, such as Lepidoptera (de Jong 2003; Zakharov *et al.* 2004). Nonetheless, many Malagasy insect groups are taxonomically more similar to Africa than Asia or Australia (e.g. Cassola 2003; Donnelly & Parr 2003). This suggests that geographical proximity and trans-oceanic dispersal may be an important determinant of the Malagasy insect fauna.

Mayflies (Ephemeroptera) are well suited for biogeographical studies because of their ancient origins, global distribution, limited dispersal powers and strict larval habitat affinity (Sartori *et al.* 2000). Mayfly fossils date from the Carboniferous (*ca* 300 Ma; Hubbard & Kukalova-Peck 1980), thus pre-dating Gondwanan vicariance. Their global diversification has been thought to be the result of ancient continental separations (Edmunds 1972, 1975), including their presence on islands (e.g. Gerlach 2001). Long before theories of continental drift were well established, the presence of mayflies on the Seychelles was taken as evidence that the islands were of continental origin (see Scott 1932; Perkins 1933). Mayfly dispersal is thought to be very limited (Brittain 1982). A number of

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studies of small minnow mayflies (Baetidae) suggest that dispersal is largely limited to the same or nearby water bodies (Hershey *et al.* 1993; Monaghan *et al.* 2002; Caudill 2003; Hughes *et al.* 2003). This is partly due to the fact that most species live only a few hours as winged adults, relying for energy in the adult stage entirely on reserves built up during the larval stage. However, some ovoviviparous females may live up to two weeks in the adult phase (Gillies 1949). Larvae of most species are restricted to either lentic (e.g. lakes, ponds) or lotic (streams, rivers) freshwaters.

For the mayflies, taxonomic similarity among former parts of Gondwanaland appears more related to present-day geographical distance rather than to vicariant history (Sartori *et al.* 2000). Of 24 Baetidae genera in Madagascar, 16 are shared with Africa (separated 165 Ma). Of these 16 shared genera, only three occur in India (separated 88 Ma), and two in Australia (separated 110 Ma) (see Gattolliat & Sartori 2003). All species of mayfly in Madagascar are endemic, except for *Cloeon smaeleni* (Gattolliat & Rabeantoandro 2002; Elouard *et al.* 2003). The Malagasy fauna of mayflies therefore constitutes a puzzle in that affinities with the African continent are apparent, but their seemingly poor dispersal ability would argue against trans-ocean exchange. Testing the origin and relatedness of Malagasy lineages, and their sister relationships elsewhere, would be a major step towards the understanding of global diversity of the Ephemeroptera.

Here we investigate the relative importance of trans-oceanic exchange, vicariance and endemic radiations in the formation of the Malagasy fauna, using a group of insects thought to have limited powers of dispersal. We conducted a phylogenetic analysis of all major Malagasy groups of Baetidae and their African counterparts, to include representatives of 26 genera. Our specific goals were to determine the number of lineages shared between Madagascar and Africa and whether lineage origins could be attributed to either area. The results show a complex pattern, from very recent dispersal to events of ancient vicariance, and indicate that a traditional scenario of unidirectional dispersal from a continental source is too simplistic.

2. MATERIAL AND METHODS

(a) *Sampling*

Of the 24 Malagasy Baetidae genera, eight are endemic, 13 are restricted to the Afrotropical region, one is pantropical (*Cloeodes*) and two are cosmopolitan (*Labiobaetis*/*Pseudocloeon* and *Cloeon*) (Gattolliat & Sartori 2003). We focussed on 10 genera found in both Madagascar and Africa, and on six Malagasy endemic genera. Ten additional Afrotropical genera, as well as the Palaearctic and Afrotropical *Baetis* were added to the analysis to examine basal relationships among lineages of Baetidae. *Labiobaetis*/*Pseudocloeon* from Borneo and New Guinea were included in the analysis, as were *Cloeon* from Europe. Samples were collected in May–June 2003 by the authors or taken from collections of the Museum of Zoology in Lausanne. DNA was extracted from thoracic muscles using a Qiagen Dneasy Tissue Kit. Mitochondrial 12S and 16S ribosomal subunits were amplified using primers 12Sai and 12Sbi (Simon *et al.* 1994), and 16Sar (Simon *et al.* 1994) and 16S2 (Giessler *et al.* 1999). Two fragments of nuclear 18S rRNA were amplified using 18S5′, 18Sb5.0, and 18S1.0, 18Sbi, 18S2.0, and 18S3′ (Whiting *et al.* 1997; Shull *et al.* 2001). Both strands were sequenced using PCR primers and analysed with an ABI 3700 automated sequencer. For the choice of outgroup taxa, molecular evidence to date indicates Baetidae are the sister taxon to all mayflies, and that Odonata are the most closely related extant insects (Hovmöller *et al.* 2002; Ogden & Whiting 2003). Thus, four mayfly species from the family Tricorythidae were analysed for this study and sequences from the dragonfly *Libellula saturata* (Odonata, Libellulidae) were taken from Genbank (accession numbers: 12S AY282562; 16S AF037181; 18S AY338717). Multiple individuals of 38 in-group species were sequenced to test for errors, contamination, and mislabelling, but removed from final phylogenetic analysis. All specimens were given a unique number for the study and extracted DNA is stored at the Natural History Museum, London in the frozen collection database (BMNH 704056–704132 and BMNH 704630–704678).

(b) *Phylogenetic Analysis*

All four gene fragments were length-variable (two 18S fragments combined: 1444–1449 bp; 12S: 326–338 bp; 16S: 498–510 bp) and thus we used three general approaches to phylogeny reconstruction: direct optimization as implemented in Poy v. 3.0.11 (Gladstein & Wheeler 1999) and parsimony and maximum likelihood searches of static multiple alignments. For direct optimization, an initial alignment was performed manually for 18S regions and the sequences were separated into one conserved fragment and two variable fragments for ease of analysis. Poy searches were performed under equal weight of all character changes including indels, conducting 10 replicates and holding a maximum of 10 trees each replicate. Alternative gap cost parameters were explored with no substantial effects on topology. Complete command lines and implied alignments can be obtained from the corresponding author. Bremer support was calculated using a heuristic procedure implemented in Poy on the best output tree.

For parsimony and likelihood searches, we first examined gap opening penalties (from 1 to 15) for congruence of length-variable regions using an incongruence length difference (ILD) test (Farris *et al.* 1994, 1995). Multiple alignments were assembled using ClustalW (using web servers provided by Major Linux and Institut Pasteur). Tree searches were conducted with Paup* v. 4.0b10 (Swofford 2002) using random addition sequences, 1000 replicates and gaps coded as a fifth character state, holding 50 trees at each replicate. The tree with lowest ILD was found using gap penalties of 10 for all three ribosomal markers. Parsimony tree searches on this alignment (2385 characters; 12S: 365 bp, 16S: 554 bp, 18S: 1466 bp) were conducted with Paup* as above, with the multi-trees option. Congruence of mtDNA and nDNA markers was examined by comparing each partition with the total evidence tree. Mapped on the total evidence tree, mtDNA tree length increased by 0.1% (from 7076 to 7084) and nDNA by 7% (597–641), and there was no incongruence within the seven well supported lineages (see below). Based on these results, we present only the total evidence tree. Data were bootstrapped (1000 replicates) with Paup*. Inferred indels were treated as distinct character states (Phillips *et al.* 2000), but coding them as missing data had no effect on tree topologies within well supported lineages. We constructed a maximum likelihood topology under a GTR+I+G model (selected in MODELTEST 3.06; Posada & Crandall 1998) with all parameters estimated from the data using PHYML (Guindon & Gascuel 2003).

(c) *Ancestral state likelihood*

Using likelihood methods (Pagel 1999; Belshaw *et al.* 2000), we estimated the ancestral state for each well resolved clade joining African–Malagasy sister taxa. Geographic area was treated as a multi-state character (Africa, Madagascar, Seychelles, Asia, Europe) and we used MULTISTATE v. 0.8 to calculate tree likelihoods (10 replicates) with single nodes fixed at either Africa or Madagascar as a state. Using the highest log-likelihood calculated for each state, differences greater than 2.0 were considered significant (Schluter *et al.* 1997), with the higher likelihood considered to be the most well supported ancestral state.

3. RESULTS

(a) *Phylogenetic analysis*

Direct optimization produced a single tree (figure 1). This topology differed from that of the shortest parsimony tree search on static alignments by only a single node within the *Cloeon* lineage (clade B, figure 1). Six well supported clades (figure 1, clades A, B, D, E, F, G) contained both Malagasy and African taxa and a seventh (figure 1, clade C) was composed entirely of Malagasy species. Support for deeper nodes within the tree was weaker than at the tips, with very low support among the seven major clades. Maximum likelihood recovered the same seven lineages with only a single change within clade B: Afrotropical *Cloeon* was monophyletic, with *Procloeon* and the two European *Cloeon* species basal to the Afrotropical clade (figure 2*b*). The likelihood topology was different from direct optimization and parsimony at deeper nodes, notably by separating Baetidae into two basal sister groups (see figure 1 inset).

The *Labiobaetis*/*Pseudocloeon*/*Baetis* lineage (clade A) was the most geographically widespread and our results highlight the taxonomic uncertainty of the group. Historically, adults with no hind wing and double intercalary veins on the forewing were assigned to the cosmopolitan genus *Pseudocloeon*. Waltz & McCafferty (1985) restricted the concept of *Pseudocloeon* to the type species, and Lugo-Ortiz *et al.* (1999) assigned all *Labiobaetis* to *Pseudocloeon*. Malagasy species described subsequently were assigned to *Labiobaetis* (Gattolliat 2001*a*). To avoid confusion, we hereafter refer to this lineage as *Labiobaetis*/*Pseudocloeon*. African and Malagasy members of *Labiobaetis*/*Pseudocloeon* appeared polyphyletic, with each well supported clade containing two Malagasy and one African species. Asian (New Guinea, Borneo) *Labiobaetis*/*Pseudocloeon* clustered together weakly but were not supported as sister to an Afrotropical clade. Likelihood branch lengths suggest a wide range of genetic divergence between over-ocean sister taxa within the group (figure 2*a*).

The *Cloeon* and *Procloeon* lineage (clade B, figure 1) displayed three clear instances of trans-oceanic sister relationships, including very closely related *C. smaeleni* in both Africa and Madagascar. For the taxa studied, Africa–Madagascar divergence, based on branch length differences, appeared less than Seychelles–Madagascar divergence (figure 2). This pattern does not correspond with geological age (165 Ma and 65–80 Ma, respectively). A third major clade (clade C) consisted entirely of Malagasy species. Eight Malagasy species of *Afroptilum*, *Dicentroptilum* and *Xyrodromeus* occurred within clade C, and the fact that their African congeners occurred in other clades makes these genera paraphyletic. The four smaller lineages (figure 1, clades D, E, F, G) each contained African and Malagasy sister taxa. Malagasy species within these clades always were monophyletic, and branch lengths revealed a wide range of trans-oceanic genetic divergence (figure 2).

(b) *Ancestral distribution*

Ancestral state reconstructions were conducted separately for nine nodes within the likelihood tree where species from Madagascar and Africa occurred in lineages (figure 2). Likelihood estimations testing for a significantly higher probability for Africa or Madagascar as the ancestral state found that only the *Cloeodes* (clade F) lineage clearly discriminated between both possibilities, with Madagascar as the more likely ancestral state (figure 2). No significant differences were found between likelihoods in analyses of the remaining lineages.

4. DISCUSSION AND CONCLUSIONS

Most species of Baetidae in Madagascar could be grouped into seven well supported lineages. One lineage was entirely endemic, four were Afrotropical (i.e. composed of Malagasy and African mainland species), and two included Asian and European species. Phylogenetic support was inconclusive for basal relationships among the seven lineages; nonetheless, a number of inconsistencies with proposed species complexes are clear from the data. *Bugilliesia* (Lugo-Ortiz & McCafferty 1996), *Centroptiloides* (Lugo-Ortiz & McCafferty 1998*a*) and *Cloeodes* (Lugo-Ortiz & McCafferty 1998*b*) species complexes were all polyphyletic based on our molecular reconstruction. Likelihood analysis recovered two major lineages within the family and provided support for the hypothesis that the Afrotropical Baetidae is composed of two subfamilies, Baetinae and Cloeoninae (Gillies 1991). The inclusion of Asian and European taxa in these two proposed subfamilies indicates they may represent a deep subdivision of the Baetidae globally. Several genera were polyphyletic, suggesting that taxonomic revision is needed. This was particularly the case for the *Labiobaetis*/*Pseudocloeon* lineage and for species within the Malagasy endemic lineage that are assigned to African genera (see below).

The phylogenetic reconstruction suggests that the Malagasy Baetidae fauna is not the result of simple vicariance or unidirectional mainland–island dispersal. In many instances, Malagasy species had closest relatives in Africa, and clades also included closely related Asian species (e.g. within clade A). The large number of trans-oceanic sister groups and the wide range of genetic relatedness is strong evidence that Madagascar is part of a larger geographical network of lineage evolution and exchange that includes the African continent (*sensu* Raxworthy *et al.* 2002), Indian Ocean Islands (Seychelles in our study; see Groombridge *et al.* 2002; Vences *et al.* 2003) and southern Asia. The wide range of genetic divergence between African and Malagasy sister taxa (estimated from likelihood branch length) indicates a lack of synchrony in divergence events and shows that trans-oceanic exchange has occurred repeatedly.

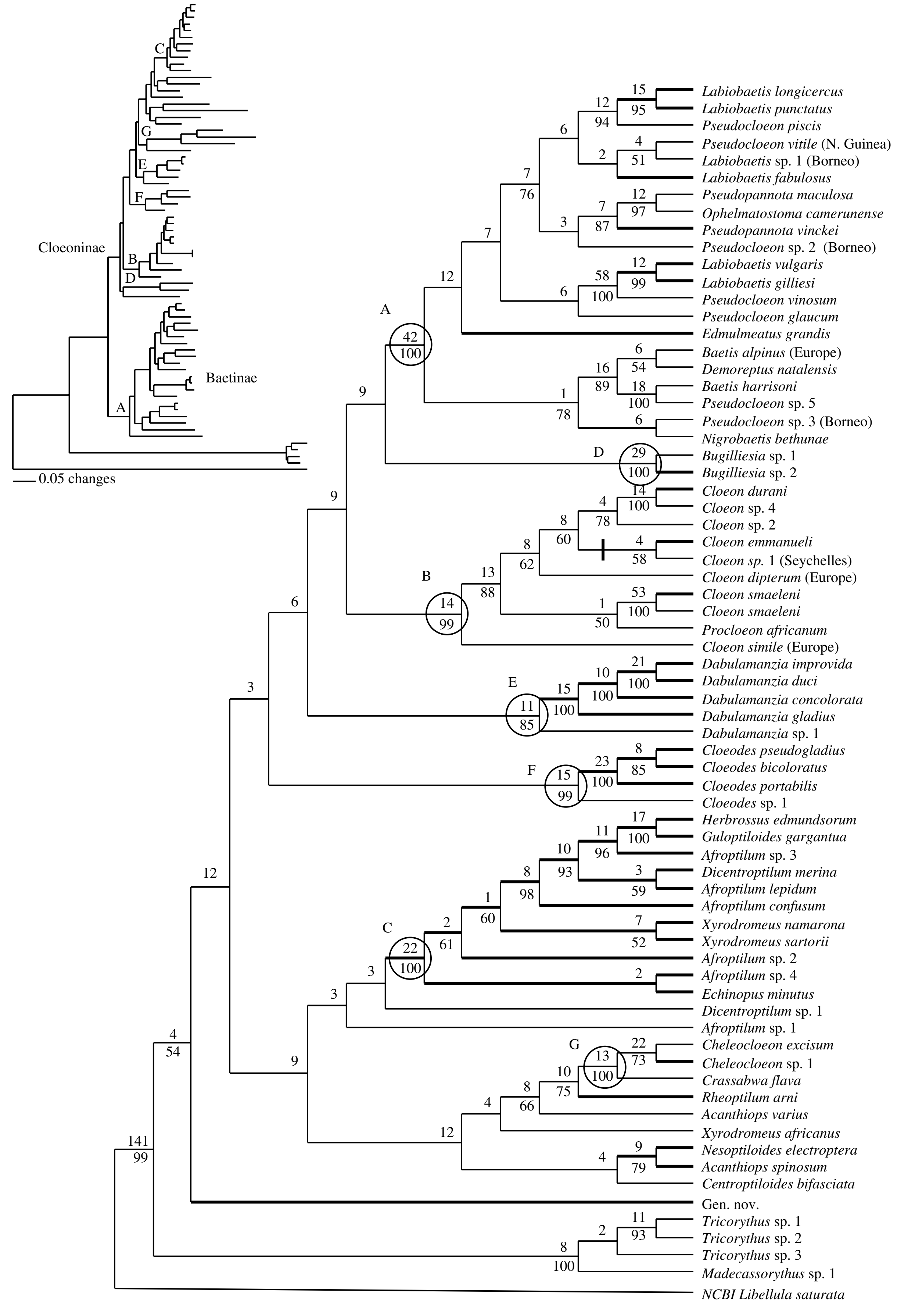

Figure 1. Phylogenetic reconstruction of Afrotropical Baetidae based on the single resulting tree from direct optimization of 12S, 16S, and two 18S rRNA gene regions using Poy. The vertical bar indicates the alternate placement of *Cloeon* sp. 2 using parsimony reconstruction. Values above branches indicate Bremer Support and values below branches indicate parsimony bootstrap percentage (if above 50%). Letters A–G indicate well supported lineages (see text). Branches are thickened for Malagasy species and the origin of non-Afrotropical taxa is in parentheses. The maximum likelihood topology is summarized in the upper left, with the corresponding lineages marked.

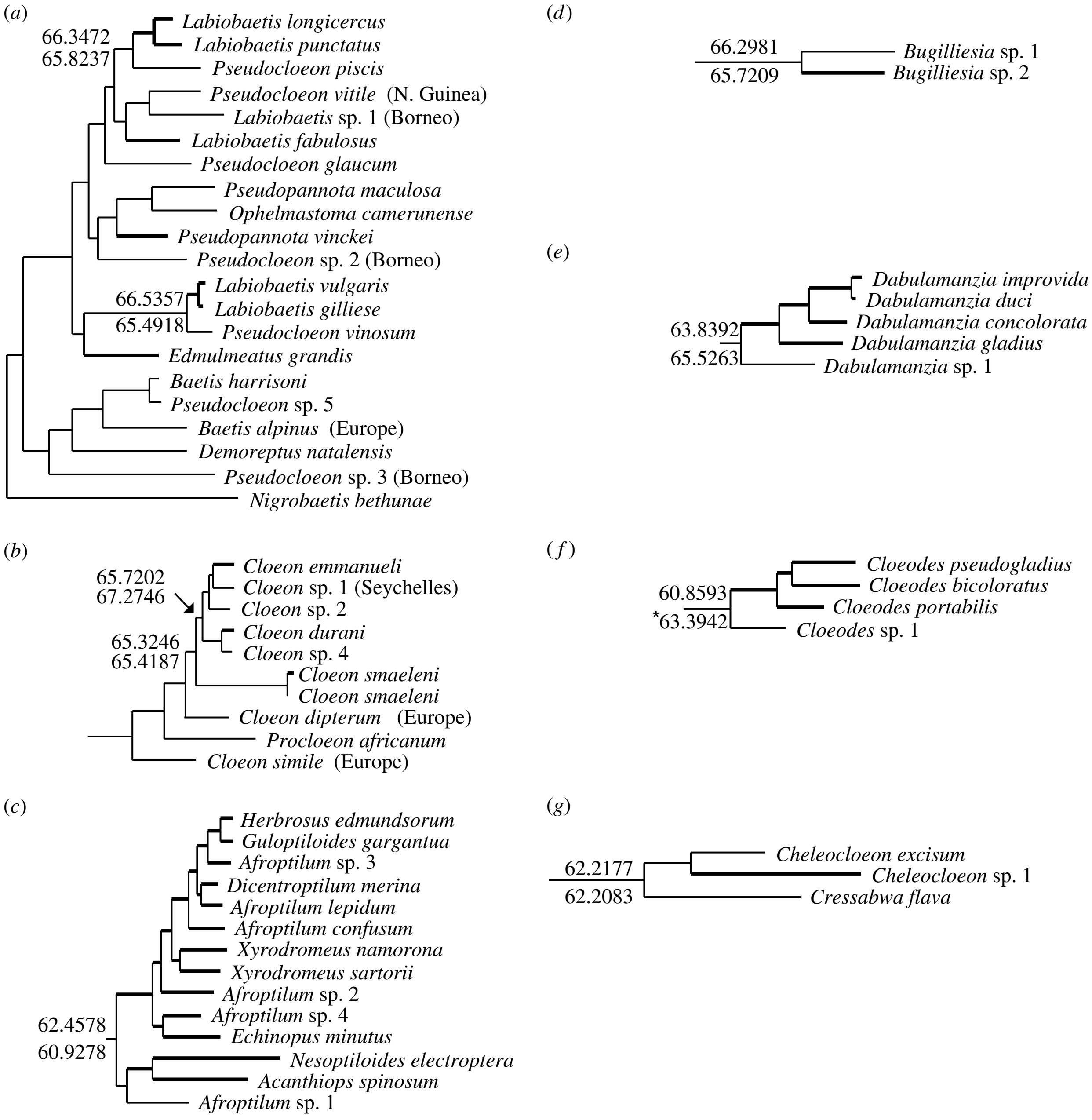

Figure 2. Likelihood tree topologies, branch lengths and ancestral state reconstructions for the seven well supported lineages within Afrotropical Baetidae (*a–g* corresponding to clades A–G in figure 1), taken from the maximum likelihood tree. Branch thickness and letters corresponding to nodes are as in figure 1. Ancestral state likelihoods are shown for Africa (above branch leading to node if interest) and Madagascar (below branch). Significantly higher likelihood is marked by asterisk.

It may be appropriate to consider the evolution of the Malagasy insect fauna in a biogeographical context that extends beyond Madagascar itself, and that trans-oceanic dispersal may be more common than thought for a broad range of insects (Trewick 2000). Zakharov *et al.* (2004) recently discussed genetic evidence that the highly vagile swallowtail butterflies (*Papilio* spp.) have made repeated trans-oceanic dispersal. Our estimates of ancestral states were largely inconclusive with regard to the origin of Malagasy lineages, except for *Cloeodes*, which our results indicate to be ancestrally a Malagasy lineage with descendents now confined to Africa. Further sampling of African taxa is required to confirm such a result (Emerson 2002), but it casts doubt on any hypothesis of unidirectional, mainland–island dispersal. The widespread nature of several lineages also requires a more thorough sampling and assessment of the Indian subcontinent and Asia as potential ancestral areas.

For the two most widespread lineages (*Cloeon* and *Labiobaetis*/*Pseudocloeon*), close trans-oceanic sister relationships preclude any significant substructure within the Afrotropical region, including Madagascar. For example, the very small genetic difference between trans-oceanic *C. smaeleni*, and its wide sub-Saharan and southern Arabian distribution (Gillies 1985) suggest a large, continuous range and frequent dispersal, even across the ocean. Species of *Cloeon* possess several attributes that may act alone or in combination to increase their dispersal success and therefore their range size. Eggs can reach complete development in the adult female and hatch upon contact with water (Gillies 1949), and unmated females can produce fully reproductive offspring (Harker 1997). Larvae can tolerate periods of anoxia and unusually high water temperature (e.g. Nagell 1980) as well as high salinity (Forbes 1968; Forbes & Allanson

1970). Not all species possess all of these characteristics, but singly or in combination, these traits are likely to increase the success of active or passive long-distance dispersal. We are unaware of any such mechanisms in *Labiobaetis*/*Pseudocloeon*, although some data suggest that the specific habitat type in which larvae live may be related to their range size (see below).

Only in one case (clade C) was a major radiation entirely composed of species endemic to Madagascar. It is an ecologically important group, because one or more members of this clade often are present in high abundance (e.g. Benstead *et al.* 2003) and members of the group are found in most rivers in Madagascar (authors', personal observation). Interestingly, every feeding behaviour known for Baetidae (collector–gatherer, scraper, predator) occurred in this lineage except for detritus 'shredding,' which is poorly represented in the tropics generally (Dudgeon 1999; Dobson *et al.* 2002). This lineage is most likely the result of a radiation from an ancestor that was a collector–gatherer such as *Afroptilum*. Both Malagasy *Xyrodromeus* exhibit a high degree of convergence to African *Xyrodromeus africanus*, with specialized mouthparts for scraping epilithic algae (Gattolliat & Sartori 2003). This character is also present in several other Malagasy genera outside of this lineage, including all species of *Rheoptilum* and *Scutoptilum* (Gattolliat 2001*b*, 2002), and in a single species each of *Cloeodes* and *Dabulamanzia* (Gattolliat & Sartori 2000; Gattolliat 2001*c*), indicating it has evolved repeatedly. Interestingly, Malagasy *Herbrossus* and *Guloptiloides* are predators, a relatively rare life strategy among mayflies (Gattolliat & Sartori 2001). These were recovered as sister taxa in our analysis, and members of the endemic clade; however, predation was paraphyletic in Madagascar based on the phylogenetic position of a third predator, *Nesoptiloides*. A fourth, African carnivore (*Centroptiloides*) appeared within yet another lineage, strongly suggesting predation has evolved independently several times.

The spatial scale of lineage evolution within the Baetidae supports the hypothesis that habitat type is an important predictor of aquatic insect range size (Ribera & Vogler 2000). For aquatic beetles (Coleoptera), Ribera *et al.* (2001, 2003) found that standing-water (lentic) species had larger ranges than species living in running waters, and hypothesized that standing water bodies of the size inhabited by most insects are short-lived at the scale of decades, and hence long-term persistence of populations is only possible through dispersal. Consistent with these predictions, the predominantly lentic clade of *Cloeon* was least structured geographically, and hence the most dispersive lineage in our study. *Labiobaetis*/*Pseudocloeon* was the only other lineage with closely related trans-oceanic sister taxa. Interestingly, larval *Labiobaetis*/*Pseudocloeon* species live in running waters, but many species are confined to slow-moving sections of rivers and are found in aquatic vegetation where water movement is slow (personal observation). Based on this finding, we hypothesize that the two other standing-water Afrotropical genera, *Demoulinia* and *Potamocloeon* (Gattolliat 2003), also have undergone recent trans-oceanic dispersal events.

In conclusion, our study contributes to recent evidence that dispersal has greatly affected the faunal composition of Madagascar, and proposes that the geographical extent of lineages may be predicted by ecological traits of organisms that are principally determined by their habitat type. The results demonstrate the high vagility of insects, even for mayflies (e.g. Johnson 1969), whose brief winged phase and strict habitat fidelity would seem to prohibit trans-oceanic dispersal (Brittain 1982; Brittain & Sartori 2003). It is also evident that dispersal is not necessarily unidirectional; our results show a high likelihood that one lineage originated in Madagascar, and an equal likelihood of a Malagasy or African origin for six other lineages. With such regularities in phylogenetic and biogeographical patterns emerging (e.g. Raxworthy *et al.* 2002), the challenge is now to determine what factors may promote dispersal. Mayflies associated with ponds and small standing water bodies had the widest range and showed the most recent, and presumably most frequent, trans-oceanic exchange, adding to a growing body of evidence that strategies for survival in evolutionarily unstable habitats may select for greater dispersal abilities in any taxonomic group (Ribera *et al.* 2001). As a predictive framework for identifying deeply separated, endemic lineages from those of more recent origin, habitat affinity also could be broadly used to set conservation priorities in the endangered fauna of Madagascar.

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