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Phylogenetic relationships and historical biogeography of melanotaeniid fishes in Australia and New Guinea

K. McGuigan[A], *D. Zhu*[A], *G. R. Allen*[B] *and C. Moritz*[A]

[A]Cooperative Research Centre for Tropical Rainforest Ecology and Management, Department of Zoology and Entomology, University of Queensland, Brisbane, Qld 4072, Australia.
email: kmcguigan@zen.uq.edu.au
[B]West Australian Museum, Francis Street, Perth 6009, Western Australia, Australia

Abstract. Phylogenetic analysis of melanotaeniid mtDNA cytochrome b and $tRNA^{Pro}$-control region sequence is broadly consistent with the current taxonomy. However, the molecular phylogeny supports the elevation of *M. s. australis* to full species status and indicates either that it is a composite species or has introgressed with sympatric *Melanotaenia* species. Phenotypically cryptic mtDNA diversity in north-eastern Australia possibly represents an undescribed species. Six major monophyletic clades present in the phylogeny were strongly supported by morphological data. The clades represent three biogeographic regions. Fish from northern New Guinea form a monophyletic clade, within which *Melanotaenia* and *Glossolepis* are polyphyletic. The divergence of this clade from those in southern New Guinea is consistent with the final uplift of the Central Highlands 5 million years BP. North-western New Guinea and associated islands represent another highly divergent, monophyletic clade of a similar age to that in northern New Guinea. The remaining four clades form a monophyletic assemblage restricted to southern New Guinea and Australia: one in northern Australia, one with a disjunct distribution in north-western and eastern Australia, one widespread throughout Australia and southern New Guinea, and one in southern New Guinea with an outlying species in northern Australia. The phylogenetic relationships between Australia and southern New Guinea are consistent with episodic connection via the freshwater Lake Carpentaria during periods of low sea level.

Introduction

The freshwater fishes of Australia and New Guinea are closely affiliated with one another, but distinct from nearby Asia from which they are separated by deep oceanic trenches. The Australia–New Guinea area is subdivided into two major biogeographic regions: northern New Guinea and southern New Guinea/Australia (Whitley 1959; Munro 1974; Allen and Cross 1982; Filewood 1984). Northern and southern New Guinea are separated from one another by the Central Highlands, a series of parallel mountain ranges in excess of 2000 m. Despite the short distances that separate the headwater streams of the northern and southern catchments, they share only three species of freshwater fish (Allen and Coates 1990). In contrast, southern New Guinea and Australia, separated by ocean, share 32 species of freshwater fish (Allen and Coates 1990). Paleo-ecological evidence indicates that Australia and New Guinea were, for most of the Quaternary, joined by a land bridge that provided a substantial freshwater connection via Lake Carpentaria (Torgersen *et al.* 1985). The two major regions have been subdivided by some researchers (e.g. Eastern Australia: Munro 1974; Eastern New Guinea: Filewood 1984), but the subdivisions are not consistently adhered to. Lake's (1971) division of Australia based on major drainage basins is one of the more consistently used classifications (e.g. Merrick and Schmida 1984; Allen 1989).

The above classification of biogeographic regions has been based on phenotypically inferred taxonomic relationships, and distribution records of species. Recent advances in molecular phylogenetics improve the reconstruction of the historical biogeography of taxa, leading to greater insight into evolution of both the taxa and the region (Avise 2000).

The family Melanotaeniidae (order Atheriniformes) contains relatively small (usually <10 cm), often brightly coloured freshwater fish. Rainbowfish are found throughout the island of New Guinea below 1600 m elevation, as well as on several large islands of the western half of the mainland, including Yapen, Salawati, Waigeo, Misool and the Aru Group. They also occur throughout northern Australia and in eastern coastal drainages south to the Hastings River and in much of the extensive Murray–Darling system (Fig. 1). Within this range rainbowfish are locally abundant and occupy the full spectrum of freshwater habitats ranging from arid-zone waterholes to rainforest streams, making them an important component of the relatively depauperate freshwater fauna of the region. They are generalist omnivores, feeding on algae as well as on aquatic and terrestrial insects and small crus-

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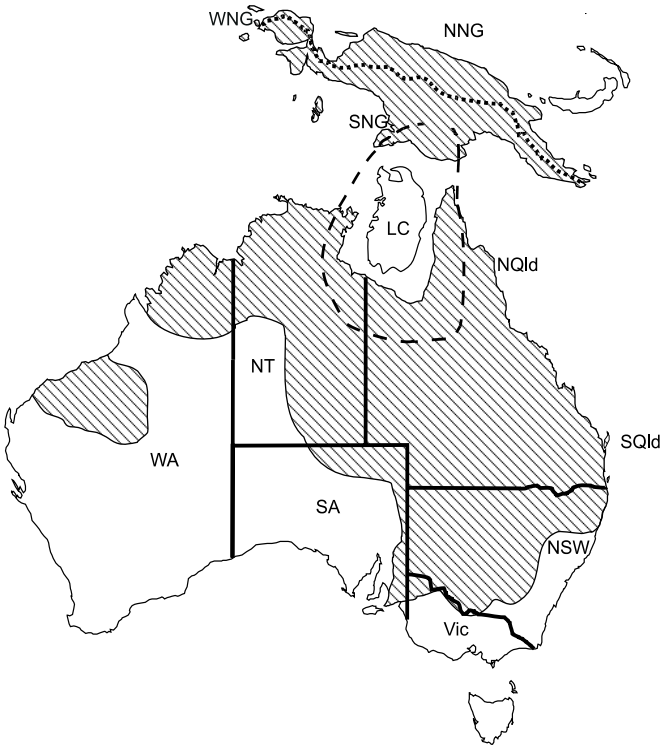

Fig. 1. Coarse-scale geographic distribution of Melanotaeniidae, the position of the Central Highlands of New Guinea (dotted line) and the hypothesized position of Lake Carpentaria and its drainage basin (dashed line) (adapted from Torgersen *et al*. 1985). LC, Lake Carpentaria; WNG, western New Guinea; NNG, northern New Guinea; SNG, southern New Guinea; WA, Western Australia; NT, Northern Territory; SA, South Australia; Vic, Victoria; NSW, New South Wales; SQld, southern Queensland; NQld, northern Queensland.

taceans, and are themselves prey to birds, fish and people (Coates 1990; Arthington 1992; Allen 1995; Pusey *et al.* 1995). Like other species of Australian freshwater fishes, rainbowfish show little ecological or morphological specialization (Roberts 1978; McDowall 1981).

Within Melanotaeniidae there are currently seven recognized genera. Three (*Chilatherina*, *Glossolepis* and *Pelangia*) are restricted to New Guinea, two (*Cairnsichthys* and *Rhadinocentrus*) to eastern Australia and the remaining two (*Iriatherina* and *Melanotaenia*) occur in both regions. *Melanotaenia* is the most specious and widespread genus, frequently co-occurring with the other more geographically restricted genera. Allen (1980) proposed generic relationships within Melanotaeniidae based on various skeletal attributes. He recognized *Melanotaenia*, *Glossolepis* and *Chilatherina* as the most derived groups within the family and concluded that these closely related genera can be distinguished on the basis of scale characteristics and jaw morphology.

There are 65 currently recognized Melanotaeniid species: 13 from Australia and 55 from New Guinea, with three taxa (*I. werneri*, *M. maccullochi* and *M. splendida*) being shared by both regions (Allen and Renyaan 1998). Extensive field investigations have enabled extensive advances in alpha taxonomy in recent years, with 31 new species described since 1980 (reviewed by Allen and Renyaan 1998). However, in contrast to the generic relationships, the phylogenetic relationships amongst species are poorly characterized. Both Allen (e.g. 1995) and Schmida (1997) adopted holistic approaches, suggesting various species groups based on unquantified, phenotypic similarity (Table 1). This same approach has also been used to suggest sister taxon relationships (e.g. *M. lacustris* and *M. mubiensis*: Allen 1996). Crowley *et al.* (1986) used evidence from allozymes and morphology to elevate *M. s. fluviatilis* to full species status and to reinstate *M. duboulayi* as legitimate species. Crowley and Ivanstoff (1991) examined the allozyme characteristics and morphology of the supposedly extinct *M. eachamensis* and concluded that it was a population of *M. s. splendida*. However, subsequent analysis of *M. eachamensis* and *M. s. splendida* morphology contradicted these findings (Pusey *et al.* 1997), as did an analysis based on molecular markers (mtDNA sequence and microsatellites) and morphology (Zhu *et al.* 1998). This contradiction was probably the result of misidentification of populations and limited power due to small sample sizes in Crowley and Ivanstoff's (1991) study.

Zhu *et al*. (1994) presented the most comprehensive study of phylogenetic relationships, analysing 15 species and subspecies of *Melanotaenia* and one species of *Glossolepis*. They observed that the divergence between *M. affinis* and *G. incisus* was less than that between *M. affinis* and other *Melanotaenia* species (Zhu *et al.* 1994). They also observed close relationships between Australia and New Guinea, rendering them a single phylogeographic unit (Zhu *et al*. 1994). However, no morphological comparisons were made, and the results contradicted some species groupings proposed by Allen (1995) or Schmida (1997) (e.g. *M. splendida australis* in Western Australia and western Northern Territory *v.* the eastern Northern Territory).

Although fish taxonomy is traditionally based on morphology, molecular markers are being increasingly used to resolve systematic relationships amongst taxa (Kocher and Stepien 1997). Neutral molecular markers may be particularly useful in resolving phylogenetic relationships when morphological traits are subject to selection and convergence. The effect of environmental factors, such as food availability, water flow and temperature on the morphology of rainbowfish is unknown. Understanding the relationships amongst currently described species, and defining the role of morphology in the systematics of these taxa, will facilitate future species descriptions as well as comparative studies of their ecology and evolution. The recent acceleration of species descriptions within this family, and the ongoing introductions of large piscivores for recreational angling or to provide a source of protein to people (Werry 1998), make this resolution urgent.

Table 1. Species name, abbreviation, collection localities and phenotypic species group assignment (after Schmida 1997) for species included in this study

Locality abbreviations as in Fig. 1. Source of morphological data for discriminant functions analysis indicated as superscript in species column ([1]Allen and Cross 1982, [2]Allen 1989, [3]Allen 1990, [4]Allen 1996, [5]Allen and Renyaan 1996, [6] Allen 1997, [7]Allen and Renyaan 1998)

Abbreviation	Species	Locality (No. of samples if >1)	Species group
M. aff.	*Melanotaenia affinis* [1]	I. Buvu Ck, NNG II. Gogol R., NNG	'goldiei'
M. bat.	*M. batanta* [7]	Batanta I. WNG	
M. cae.	*M. caerulea* [4]	I. Kopi Chevron, SNG II. Kikori R., SNG III. Ofake Ck, SNG	
M. cat.	*M. catherinae* [1]	I. Batanta I., WNG II. Eastern Waigeo I., WNG III. Western Waigeo I., WNG	'goldiei'
M. dub.	*M. duboulayi* [2]	Robertson Ck, SQld (n = 2)	'australis'
M. eac.	*M. eachamensis* [1]	I. NQld species, Hanson stock II. NQld species, Walkamin Station	'australis'
M. exq.	*M. exquisita* [1]	South Alligator R., NT (n = 2)	'nigrans'
M. flu.	*M. fluviatilis* [2]	NSW (n = 2)	'australis'
M. fre.	*M. fredericki* [3]	Salawati I., WNG	'goldiei'
M. gol.	*M. goldiei* [1]	I. Timika, WNG II. Isaba Ck, SNG III. Lake Yamur, SNG	'goldiei'
M. gra.	*M. gracilis* [1]	Drysdale River, WA	'nigrans'
M. her.	*M. herbertaxelrodi* [1]	I. SNG species bred in NSW (n = 2) II. Lake Tebera, SNG	'goldiei'
M. jap.	*M. japenensis* [1]	Yapen I., NNG	'goldiei'
M. kam.	*M. kamaka* [5]	Lake Kamaka, WNG	'goldiei'
M. lac.	*M. lacustris* [1]	Lake Kutubu, SNG, bred in Qld (n = 2)	'goldiei'
M. lak.	*M. lakamora* [5]	I. Lake Lakamora, WNG II. Lake Aiwasa, WNG	'goldiei'
M. mac.	*M. maccullochi* [1]	I. Jardine R., NQld (n = 2) II. Fly R., SNG III. Finnis R., NT (n = 2)	'maccullochi'
M. mon.	*M. monticola* [1]	Moro Chevron, SNG	'goldiei'
M. mub.	*M. mubiensis* [4]	Ofake C k, SNG	
M. nig.	*M. nigrans* [1]	I. Jardine R., NQld II. Mary R., NT	'nigrans'
M. ogi.	*M. ogilbyi*	Timika, SNG	
M. par.	*M. parkinsoni* [1]	SNG species, bred in Vic	'splendida'
M. pyg.	*M. pygmaea* [1]	WA species bred in SQld (n = 1)	'nigrans'
M. sex.	*M. sexlineata* [1]	Fly R., SNG, bred in SQld (n = 2)	'goldiei'
M. sp.	*M.* species	I. Utchee Ck, Johnstone R., NQld II. Fishers Ck, Johnstone R., NQld (n = 2) III. Ithica Ck, Johnstone R., NQld	'australis'
M. s. aus.	*M. splendida australis*	I. Gibb R., WA II. Bellary Ck, WA (n = 3) III. South Alligator R., NT (n = 2) IV. Blackmore R., NT	'australis'
M. s. ino.	*M. s. inornata* [1]	I. Stans Lake, NQld	'splendida'
M. s. rub.	*M. s. rubrostriata* [1]	I. SNG species, bred in Vic II. SNG species, bred in SQld	'splendida'
M. s. spl.	*M. s. splendida* [1]	I. Davies Ck, NQld (n = 2) II. Burdekin R., NQld (n = 2) IX. Tinaroo Channel, NQld X. Daintree R., NQld (n = 3)	'splendida'
M. s. tat.	*M. s. tatei* [1]	I. Coongie Lakes, SA II. Ellery Ck, NT (n = 2)	'splendida'
M. syl.	*M. sylvatica* [6]	Sapoi R., SNG	
M. tri.	*M. trifasciata* [1]	I. Archer R., NQld II. Cohen R., NQld III. Gunshot Ck, NQld (n = 2) V. Pascoe R., NQld VI. Goyder R., NT (n = 2)	'goldiei'
G. inc.	*Glossolepis incisus*	Lake Sentani, NNG	
G. mul.	*G. multisquamatus*	Brahman, NNG	
G. ram.	*G. ramuensis*	Gogol R., NNG	
C. s. ful.	*Craterocephalus stercusmuscarum fulvus*	North Pine R., SQld	

In this study, mtDNA cytochrome b and tRNA^{Pro}-control region sequence was used to investigate the phylogenetic relationships among species of *Melanotaenia* and *Glossolepis*. The geographic distribution of phylogenetic groupings was used to make inferences about speciation and dispersal. The concordance of morphological and genetic data was tested to determine whether morphological and molecular diversification is congruent.

Materials and methods

Molecular analysis

Ethanol-preserved whole fish, caudal fin clips or frozen whole fish were obtained from throughout Australia and New Guinea (Table 1, Fig. 1). This collection represents all Australian *Melanotaenia* species, 18 of 35 New Guinean *Melanotaenia* and three of seven *Glossolepis* species. Whole genomic DNA was extracted by either lysis of cells in the presence of Chelax (Bio-Rad) (Zhu *et al.* 1994), or use of phenol–chloroform (Sambrook *et al*. 1989).

On the basis of observed patterns of sequence evolution and phylogenetic utility (Zhu *et al.* 1994), cytochrome b mtDNA was chosen to investigate relationships among species. Although mtDNA control region is inappropriate for resolving deep divergences, it is informative for estimating shallow relationships (Zhu *et al.* 1994), and was used in combination with cytochrome b to enhance resolution at the tips of branches.

Both cytochrome b and tRNA^{Pro}-control region sequences were generated following either manual (as in Zhu *et al*. 1994) or automated (Perkin–Elmer ABI PRISM kit) protocols using the same primers and initial amplification conditions as described in Zhu *et al.* (1994). Automated sequences were obtained by means of old dye technology on a 373A Applied Biosytems DNA Sequencer. Heavy and light strands of each sample were sequenced, resulting in complete overlap of the fragment used in analyses. Traces of automated gels were checked visually to ensure accuracy of base calls. A subset of manually sequenced samples were also sequenced following automated protocols to confirm consistency across techniques. Sequences were aligned by use of Clustal W (Thompson *et al.* 1994). Ambiguous segments of the alignment were deleted as described in Zhu *et al.* 1994.

The large number of individual sequences and constraints on computing time dictated the use of neighbour-joining algorithms to construct the phylogeny. A bootstrapped (1000 pseudoreplicates) neighbour-joining phylogeny based on Kimura 2-parameter distances between cytochrome b haplotypes was constructed with PAUP* (Swofford 1998). Addition sequence was random. The tree was outgroup rooted using an atherinid, *Craterocephalus stercusmuscarum fulvus*.

The partition homogeneity test in PAUP* (Swofford 1998) was used to determine the congruence of the cytochrome b and tRNA^{Pro}-control region sequences, following removal of invariant characters (see Cunningham 1997). The test was conducted with 100 replications of a heuristic search with two replicates of random addition and branch rearrangements by tree-bisection rejoining. Because of the reduction of the number of taxa in the combined data set relative to the cytochrome b data set, it was possible to use maximum parsimony to construct the phylogeny. A heuristic bootstrap analysis with 500 pseudoreplicates was performed on the combined fragments using PAUP* (Swofford 1998). Addition sequence was random, with 50 addition replicates.

Maximum-likelihood analyses with and without molecular clock enforced were conducted with PHYLIP v 3.5c (Felsenstein 1995). A likelihood-ratio test was used to determine whether there was heterogeneity in the rate of evolution amongst lineages. Because of constraints on computing time, only a subset of taxa were analysed. Three taxa from each clade were included, with taxa selected to represent the extremes of variation within the clade. The transition:transversion ratio was set at 4:1 (Zhu *et al.* 1994).

Net sequence diversity within clades and divergences between clades and geographic regions were calculated by use of DNAsp (Rozas and Rozas 1999) on the basis of cytochrome b haplotype Kimura 2-parameter distances calculated in PAUP* (Swofford 1998).

Morphological analysis

Maxima and minima for 16 meristic or morphometric characters (Fig. 2) were obtained from the published literature for species (and subspecies) of *Melanotaenia* for which cytochrome b sequence data were available (Table 1). Only one genus was used in this analysis to avoid potential confounding effects of genus-level differences. *Melanotaenia* was selected because it represents the greatest proportion of the phylogenetic data and, unlike *Glossolepis*, is not geographically restricted. The data were normalized and standardized by log transformation. Species were categorzed into clades on the basis of the results of the phylogenetic analysis. A discriminant functions analysis was performed using SPSS (Anon. 1999), with clade as the discriminator. Maxima and minima were analysed separately.

Results

Sequence characteristics

Cytochrome b sequence was obtained for 71 haplotypes representing 36 species or subspecies, including one outgroup taxon[1]. Of the 351 bp sequenced, 123 characters were both variable and parsimony informative. Kimura 2-parameter sequence divergences among haplotypes within described species ranged from 0.00% in several taxa to 13.84% between *M. s. australis* from Western Australia and the Northern Territory. The divergence between *M. s. australis* populations was unusually high, with the next highest divergence within *M. goldiei* (6.60%). Divergences between described species ranged from 0.29% between haplotypes of *M. kamaka* and *M. lakamora* to 20.78% between haplotypes of *M. s. australis* and *M. japenensis*.

tRNA^{Pro}-control region sequence was obtained from samples representative of intraspecific cytochrome b diversity, with the focus on southern New Guinean and Australian *Melanotaenia* species for which relationships were less well resolved by cytochrome b alone. A total of 41 haplotypes from 25 species or subspecies were sequenced[2]. Alignment of the tRNA^{Pro}-control region sequence identified the same two areas of alignment ambiguity observed by Zhu *et al.* (1994). These regions were excluded from further analysis. Of the remaining 331 bp, 104 characters were variable and parsimony informative. Numerous insertions and deletions were observed, with the largest, a 40 bp deletion, being observed in all individuals of *M. trifasciata*, *M. herbertaxelrodi*, *M. goldiei*, *M. lakamora* and *M. mubiensis*.

[1]Individual sequences are available from EMBL nucleotide sequence database with accession numbers AJ401620-AJ401690 (http://www.ebi.ac.uk/embl/index.html); alignment available from http://www.zen.uq.edu.au/mzl/.

[2]Individual sequences available from EMBL nucleotide sequence database with accession numbers AJ400663-AJ400703 (http://www.ebi.ac.uk/embl/index.html).

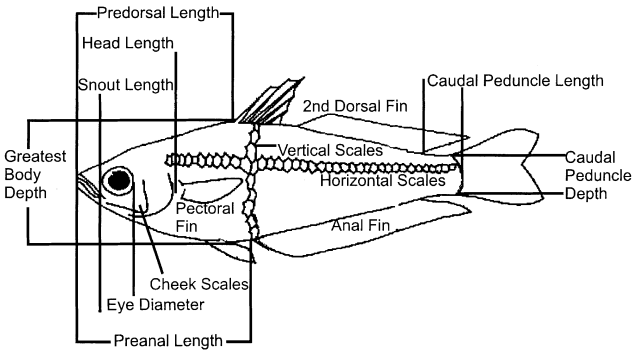

Fig. 2. Morphological traits included in discriminant functions analysis. Interorbital width (not shown) is the distance between the bony upper edges of the eye sockets. Predorsal scale count (not shown) is the number of horizontal scales between the origin of the first dorsal and the interorbital region. All morphometric traits are reported as percentages of standard length; meristic traits are reported as counts. Figure adapted from, and measurements defined in, Allen and Cross (1982).

Phylogenetic relationships

There was no significant incongruity between the two mtDNA fragments ($P = 0.31$)[3]. Six distinct clades were identified in the neighbour-joining phylogeny of cytochrome b sequences (Fig 3*a*). The topology was well supported, with all clades except B having bootstrap values of ≥70%. The combined cytochrome b and tRNA^{Pro}-control region phylogeny identified the same clades (Fig. 3*b*), with the exception of clade E for which there was no tRNA^{Pro}-control region sequence available because of repeated failure to amplify. Again, only clade B had bootstrap support of <70%. Notably, the bootstrap support for clade B in the combined analysis increased from 61% to 97% if *M. ogilbyi* was excluded (Fig. 3*b*). Owing to this lack of confidence in the relationships of *M. ogilbyi* it was not included in clade B for any further anal-

[3]Combined fragment alignment available from http://www.zen.uq.edu.au/mzl/.

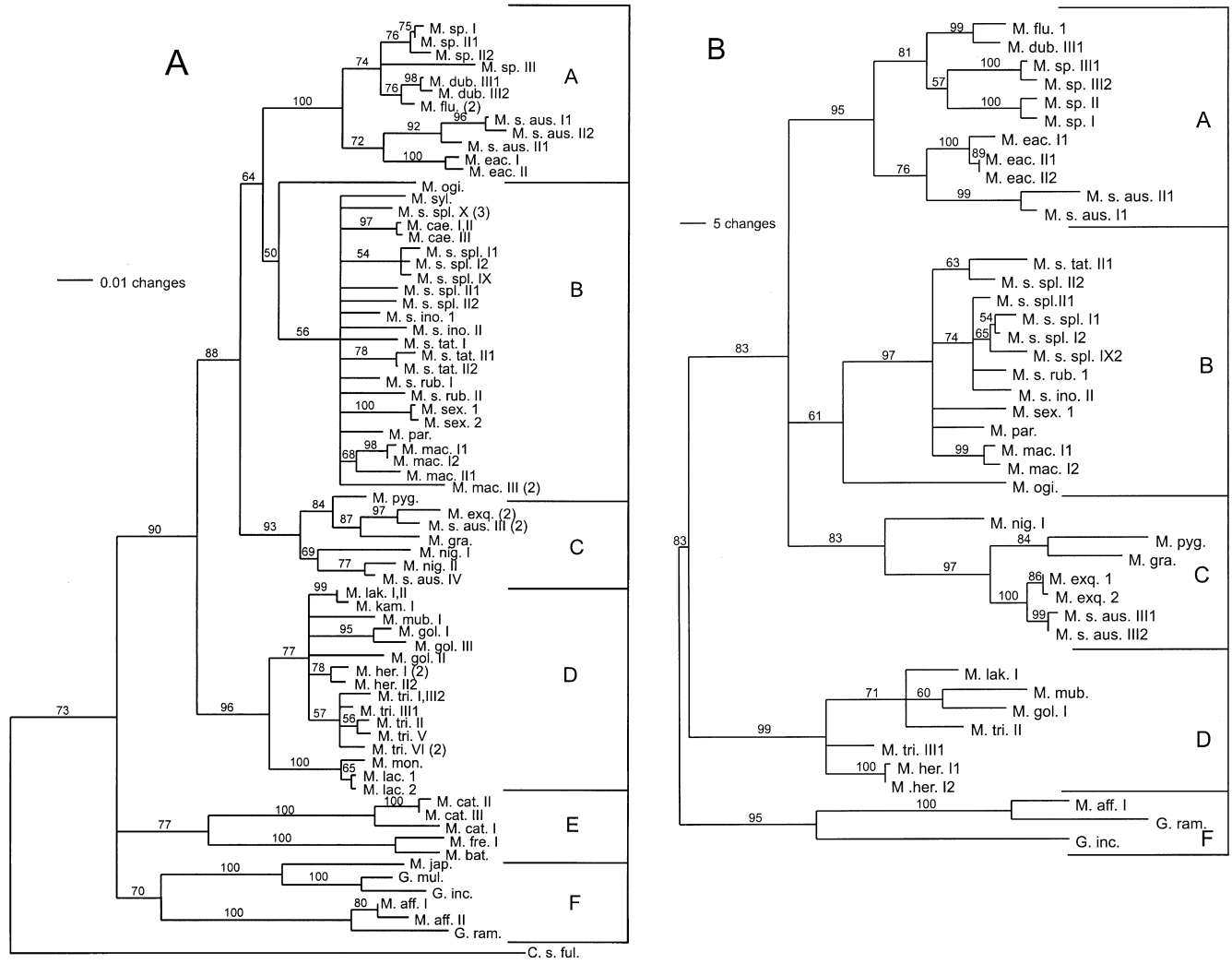

Fig. 3. Molecular phylogenetic relationships amongst Melanotaeniidae haplotypes. Species abbreviations and locality identifiers defined in Table 1. Numbers above branches indicate bootstrap support and all nodes with <50% bootstrap support collapsed. Major clades are identified and labelled. *(A)* Bootstrapped neighbour-joining phylogeny using Kimura 2-parameter distances based on cytochrome b sequence; *(B)* Bootstrapped maximum-parsimony phylogeny based on combined cytochrome b and tRNA^{Pro}-control region sequence.

yses. Net nucleotide divergences between clades ranged from 8.10% to 15.43% (Table 2). A log-likelihood-ratio test comparing the maximum-likelihood trees inferred with and with and without a molecular clock revealed significant heterogeneity in rates of change among lineages $\chi^2 = 27.77$, df 16, $P < 0.05$).

The extent to which relationships were resolved within clades varied. Both clades E and F were well resolved, with most internal nodes having bootstrap values of 100% and all of at least 80%. Clades A and C were also well resolved with >70% bootstrap support for most internal nodes. In contrast, clades B and D were poorly resolved with many nodes having <50% bootstrap support. Average divergences between haplotypes within clades B and D were also lower than in the other clades (Table 2). The low average divergence within clade B was contributed to by the inclusion of multiple haplotypes of *M. s. splendida*. However, the lack of phylogenetic resolution justified their inclusion, and reducing this replication did not markedly increase the average divergence (compare 3.38% divergence with six haplotypes of *M. s. splendida* with 3.63% divergence with one *M. s. splendida* haplotype).

Most described taxa represented in the data set by more than one individual were monophyletic. However, *M. s. australis* appeared diphyletic, with Western Australian populations in clade A and Northern Territory populations in clade C. The net divergence between these two groups of *M. s. australis* was 11.98%. In the cytochrome b analysis, Northern Territory *M. s. australis* was further polyphyletic with *M. nigrans* and *M. exquisita* (Fig. 3*a*).

Specimens from the Johnstone River, North Queensland, were identified during collection as either *M. eachamensis* (Localities I and II) or *M. s. splendida* (Locality III). However, they have been labelled M. sp. (*Melanotaenia* sp.) in the analyses because they were phylogenetically distinct from both those species and more closely related to southern clade A taxa; these appear to represent an undescribed species (McGuigan unpublished).

Clades E and F were both geographically restricted and isolated, occurring only in western and northern New Guinea respectively (Fig. 4). In contrast, southern New Guinea was occupied by two clades (B and D) with broadly overlapping distributions. Both of these clades were also present in northern Australia, where clade B is widespread and D occurs in disjunct patches. The remaining clades (A and C) were restricted to Australia. Clade C occurred mainly in the Northern Territory and adjacent northern Western Australia but had an outlying population on Cape York Peninsula. Its distribution overlapped with clades A, B and D. Clade A overlapped with clades B and C only slightly, and was unusual because of the geographic disjunction in its distribution caused by the inclusion of Western Australian *M. s. australis* (Fig. 4). The net nucleotide divergences between species from northern New Guinea (clade F), western New Guinea (clade E) and southern New Guinea (clades B and D) were all >12% (Fig. 5). In contrast, the divergences between New Guinean and Australian species/populations in clades B and D were <7% (Fig. 5).

Table 2. Net nucleotide divergences (%) in cytochrome b sequence between clades (below diagonal) and average divergence within clades (on diagonal)

	A	B	C	D	E	F
A	4.42					
B	8.45	3.38				
C	9.38	8.10	4.64			
D	10.46	9.41	9.53	3.60		
E	15.43	14.56	14.51	13.90	8.36	
F	15.02	14.53	14.04	12.52	14.50	9.78

Fig. 4. Geographic distribution of the phylogenetic clades identified in Fig. 3. Dashed line, Central Highlands of New Guinea.

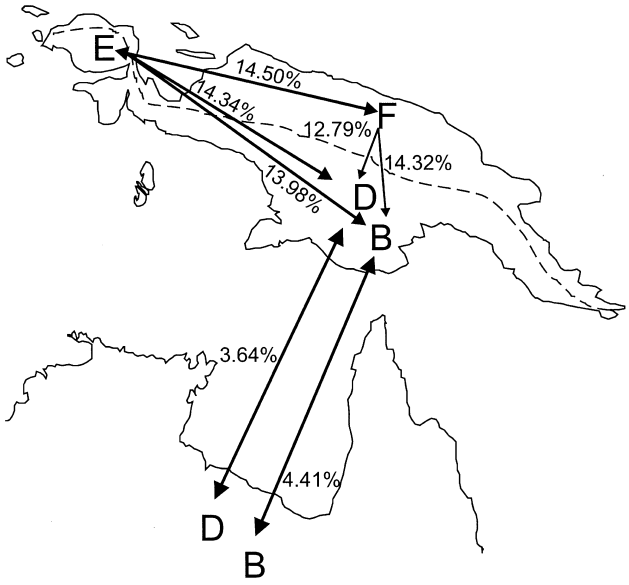

Fig. 5. Net cytochrome b sequence divergence between regionally restricted phylogenetic clades identified in Fig. 3. Dashed line, Central Highlands of New Guinea.

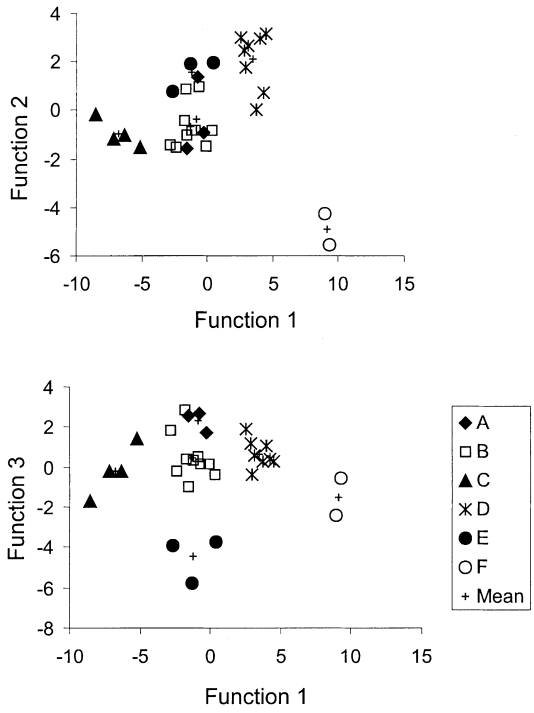

Fig. 6. Discriminant scores for each species, grouped by Discriminator (phylogenetic clade as identified in Fig. 3), on first three Functions from the discriminant functions analysis.

Morphology

Analyses of maxima and minima gave qualitatively similar results – as expected, given that they are both measurements of the same trait. In both cases, the overall model did provide statistically significant discrimination amongst phylogenetic clades (P <0.001). However, for analysis of maxima, only the first discriminant function accounted for a significant amount of interclade variation. In contrast, the first three functions of the minima analysis accounted for a significant portion of the variation (P <0.001, P = 0.006 and P = 0.027 respectively). In both analyses, the same traits contributed most to the discrimination, so only the results from the analysis of minima were presented.

Function 1 was dominated by dorsal, anal and pectoral ray and cheek scale counts. Clade C was at one extreme with low counts for these traits while Clade F was at the other extreme with high counts for these traits (Fig. 6, Table 3). The contributions of individual traits to the other functions were not as clear-cut. Function 2 was dominated by predorsal length, and pectoral and anal ray counts. Again, clade F was at one extreme of function 2, with clade D at the other (Fig. 6). Positive contributions from caudal length and eye diameter and negative contributions from anal and dorsal ray counts dominated function 3. Clade E was the most distinct on this function (Fig. 6).

Clades A and B are the only clades that did not form distinct clusters on any of the first three functions (Fig. 6). However, the difference between these two clades on function 3 was statistically significant (one-way ANOVA with planned comparisons: P = 0.004).

Table 3. Mean ± s.e. of traits that contribute most to discrimination amongst clades

For definition of traits see Fig. 2. Eye diameter, caudal length and predorsal length represented as percentages of standard length (see Allen and Cross 1982). All traits except eye diameter are significant in univariate comparisons of means across clades (P <0.05)

	A (n = 3)	B (n = 10)	C (n = 4)	D (n = 8)	E (n = 3)	F (n = 2)
Dorsal rays	9.00±0.58	8.80±0.39	7.50±0.29	11.63±0.53	12.33±0.67	14.00±1.00
Anal rays	15.67±0.67	16.20±0.53	15.25±0.63	18.63±0.56	22.67±1.86	22.00±4.00
Pectoral rays	11.33±0.33	12.10±0.28	12.00±0.00	13.13±0.23	12.67±0.33	12.50±0.50
Cheek scales	8.33±0.67	9.40±0.43	6.25±1.11	13.25±0.96	11.33±0.33	17.00±4.00
Eye diameter	8.17±0.48	7.83±0.26	7.68±0.49	8.34±0.46	7.60±0.25	7.05±0.15
Caudal length	16.63±0.18	14.82±0.87	15.60±0.63	14.46±0.53	12.87±0.44	10.65±0.45
Predorsal length	40.60±1.59	44.66±0.68	46.65±1.60	46.98±0.53	48.77±1.53	43.10±5.40

Discussion

Phylogenetics, morphology and taxonomy

The level of cytochrome b sequence divergence observed within described species of rainbowfish, 0.0% to 13.84%, falls outside the range observed in other fish species (0.4–8.4% Avise *et al*. 1998). However, if *M. s. australis* is not considered, the maximum within-species divergence, 6.60%, falls within the range. The divergence between sister species of 0.29–20.78% is consistent with levels observed between other fish sister species (<1%–40% Johns and Avise 1998).

The phylogeny inferred in this study is consistent with the preliminary results of Zhu *et al*. (1994), but extends them considerably. There are some inconsistencies between the molecular phylogeny and the species groups identified phenotypically by Allen (1995) and Schmida (1997). The inconsistencies are largely a result of pooling into single phenotypic species groups species that are differentiated molecularly into clades E, F, B and D. However, the discriminant functions analysis of morphometric data indicated that morphology strongly supports the phylogenetic clades, indicating that there is phylogenetic signal in morphology. Given the molecular paraphyly of *Glossolepis* and *Melanotaenia* in northern New Guinea, it might have been useful to include *Glossolepis* in the morphological analyses to increase the sample size of clade F. However, the two genera are very different in a number of characters, typified by the high counts for horizontal, vertical and predorsal scales rows in *Glossolepis* species. Inclusion of *Glossolepis* without the removal of these characters might have artificially increased the differentiation of clade F, which was already well discriminated on the basis of two *Melanotaenia* species.

The morphological characters that contributed most to discrimination between clades were second dorsal, anal and pectoral fin ray counts, cheek scale counts, eye diameter and predorsal and caudal-peduncle lengths. This suggests that whereas these characters reflect the phylogenetic history of the group, the other characters measured are affected more by *(i)* strong selection for particular trait values in different environments, *(ii)* phenotypic plasticity, or *(iii)* lack of stabilizing selection, resulting in extensive diversifying drift.

Some specific deficiencies of the current classification of rainbowfish are demonstrated by this study. As already observed by Zhu *et al*. (1994), *M. s. australis* is represented by two distinct lineages (clade A versus clade C), which are up to 13.84% divergent in cytochrome b. These lineages are geographically restricted to Western Australia and the Northern Territory respectively. The phylogeny does not support the inclusion of either lineage within *M. splendida*. *M. s. australis* was not included in the analysis of morphology because data from the two regions are pooled in the literature, and hence species groupings could not be assigned *a priori*. However, Schmida (1997) distinguishes eastern Northern Territory *M. s. australis* (unassigned) from those of the Kimberly and Pilbarra regions of Western Australia and western Northern Territory (‘*australis*’ group: Table 1).

The differentiation of *M. s. australis* lineages in this study could *(i)* indicate the presence of two distinct species, *(ii)* be the result of introgression of the Northern Territory population with sympatric *M. nigrans* or *M. exquisita*, or *(iii)* reflect retention of ancestral polymorphisms. This third possibility is unlikely because the polymorphisms would need to be present in the ancestor of clades A, B and C. To distinguish between hypotheses (i) and (ii), *M. s. australis*, *M. nigrans* and *M. exquisita* would need to be characterized molecularly (mtDNA and nuclear) and morphologically from across their geographical range. This would also help to determine the cause of the polyphyly in cytochrome b of Northern Territory *M. s. australis*. If hypothesis (i) is true, then the name *M. solata* (Taylor 1964) could be applied to the Northern Territory populations following re-description. Irrespective of the situation in the Northern Territory, West Australian populations of *M. s. australis* should be accorded species status.

Populations from Fishers, Utchee and Ithica Creeks in the Johnstone River drainage of North Queensland (M. sp.) have variously been identified as *M. eachamensis* (Pusey *et al*. 1997), *M. s. splendida* (Allen and Cross 1982) or *M. trifasciata* (Leggett and Merrick 1987), but are 3.82–13.04% divergent from each of these species in cytochrome b sequence. They are most closely related to *M. duboulayi* and *M. fluviatilis* from southern Australia. The Johnstone River populations are represented by two distinct lineages that show a high level of divergence from one another (3.50% divergence in cytochrome b, compared with, for example, 0.89% between *M. duboulayi* and *M. fluviatilis*). Evidence from microsatellites and morphology suggest that these lineages represent an undescribed species (F. Jones and K. McGuigan, unpublished). Fish from Utchee Creek have previously been suggested to represent an undescribed species (Allen and Cross 1982), and are sold in the aquarium trade as Utchee Creek rainbows, a distinct colour type of *M. s. splendida*. Also, Schmida (1997), using nape stripe and fin shape characteristics, classified this population as related to, but distinct from, *M. eachamensis*.

The cytochrome b phylogeny revealed strongly supported (100% bootstrap) polyphyly of *Melanotaenia* and *Glossolepis* genera north of the New Guinea highlands. This polyphyly is also observed in mtDNA 12S rRNA (Zhu 1995). The observation of intergeneric hybrids between *Melanotaenia* and *Glossolepis* (Allen and Cross 1982) is less surprising given these results. *Melanotaenia* and *Glossolepis* are distinguished from one another on the basis of scale characteristics (Allen 1980). The function of these characters is undetermined. *Glossolepis* has been identified as primarily lacustrine (Allen and Cross 1982), and independent specialization to this habitat with morphological convergence rather than shared common ancestry may have resulted in the observed similarities. Although *Glossolepis* and *Melanotaenia* differ greatly in the number (and shape) of horizontal, vertical and predorsal scale rows, none of these are important in discriminating between

Melanotaenia clades, suggesting a lack of phylogenetic signal in those traits. Examination of mtDNA and morphology of all Melanotaeniidae species north of the New Guinea highlands is necessary to determine the true status of current genera.

Biogeography

The results reported here support the recognition of three biogeographic provinces for rainbowfish: northern New Guinea, western New Guinea, and southern New Guinea/Australia.

Mountain ranges running east to west through the centre of New Guinea sharply delineate watersheds into northern and southern catchments. These mountain ranges are the result of a major period of uplift during the Miocene–Pliocene, approximately 8–3 myr [million years] BP (Loffler 1977; Hill and Gleadow 1989). Additionally, New Guinea south of the Central Highlands is part of the Australian craton, whereas the land north of the highlands is composed of many terranes of different origins, some of a composite nature (Pigram and Davies 1987). These terranes began docking in the late Oligocene and this continued until the Pliocene (5–2 myr BP) (Pigram and Davies 1987).

Rainbowfish north and south of the Central Highlands form separate monophyletic clades. The net cytochrome b sequence divergence between the north and the south ranges from 12.79% (clade D) to 14.32% (clade B), which is broadly consistent with a cessation of gene flow ~5 myr BP (assuming calibration for fish cytochrome b of 2.5% per myr by Meyer *et al.* 1990), although this should be interpreted cautiously given the observed heterogeneity in evolutionary rates within Melanotaeniidae. These results support previous recognition of northern New Guinea as a distinct biogeographic region for freshwater fishes (Whitley 1959; Munro 1974; Allen and Cross 1982; Filewood 1984). There is a relatively high level of genetic divergence within northern New Guinea (up to 9.78%), suggesting that the area has retained ancestral diversity, although this does not correspond to divergence between the two genera investigated (*Glossolepis* and *Melanotaenia*). The diversity may correspond to biogeographic barriers older than the Central Highlands, and may be a consequence of the patchwork origin of northern New Guinea (see Pigram and Davies 1987).

The second biogeographic region identified is western New Guinea, consisting of the Vogelkop Peninsula and the nearby islands of Waigeo and Batanta. Allen (1995) identified the Vogelkop Peninsula as a centre for rainbowfish diversity. Ten endemic species of *Melanotaenia* are known from the Vogelkop Peninsula and nearby islands, but only three were included in this study. The cytochrome b sequence divergence between western rainbowfish and those in northern New Guinea is 14.50%, and between western and southern clades it ranges from 13.98% (clade B) to 14.34% (clade D). These divergences equate to a disruption of gene flow ~5.5 myr BP. Again, there is relatively high divergence within this clade (up to 8.36%), indicating the survival of old lineages. In contrast, the divergence between *M. batanta* (Batanta I.) and *M. fredericki* (Salawati I.) is relatively small, and is consistent with recent speciation following isolation by Pleistocene rises in sea level.

Like northern New Guinea, western New Guinea is not part of the Australian craton (Pigram and Davies 1987). The western New Guinean terranes are unique in that they are primarily continental rather than oceanic in affinity, including one terrane that is thought to have originated from Gondwana (Pigram and Davies 1987). However, rainbowfish must have colonized western New Guinea after the docking of these terranes (late Oligocene through to the Pliocene: Pigram and Davies 1987), and the allochthonous nature of the landscape does not explain the lack of subsequent successful dispersal into, or out of, western New Guinea. The Vogelkop Peninsula is connected to the rest of New Guinea via a narrow, mountainous isthmus, and it is possibly this feature of the landscape that has resulted in isolation of watersheds. Investigation of the distribution of genetic diversity within and among other obligate freshwater taxa of this region would allow us to understand the true history of the region (e.g. Bermingham and Martin 1998).

The third biogeographic region identified consists of southern New Guinea and Australia. A close biogeographic relationship between northern Australia and southern New Guinea has been predicted on the basis of species relationships and distribution patterns (Whitley 1959; Munro 1974; Allen and Cross 1982; Filewood 1984). Unlike northern and western New Guinea, most of southern New Guinea is part of the Australian continent, and a drop in sea level of only 10 m would expose dry land (Keast 1981). Specifically, palaeological evidence indicates that the land connection between New Guinea and Australia was disrupted by rises in sea level 340–320, 130–115 and 7–0 thousand years BP (reviewed by Keenan 1994). Palaeological evidence also indicates the presence on the land bridge of an intermittently freshwater lake, Lake Carpentaria (Torgersen *et al.* 1985; De Deckker *et al* 1988; Fig. 1). Lake Carpentaria united the drainages of southern New Guinea and northern Australia. This freshwater connection would have facilitated dispersal of fish while the oceanic incursions would have disrupted gene flow and isolated populations, setting the scene for divergence and subsequent allopatric speciation. The close relationship between Australian and southern New Guinean species is consistent with this episodic freshwater connection.

Melanotaenia species in the region are divided into four monophyletic clades. These clades show extensive geographic overlap. Two clades (A and C) are endemic to Australia. Clade C is restricted to north-western Australia, with an outlying population of *M. nigrans* on Cape York Peninsula, providing further support for the role of Lake Carpentaria in linking all northern drainages.

Clade A spans much of the coastal range of Australian Melanotaeniidae and is characterized by large geographic

disjunction between sister groups: Western Australia to northern Queensland (western *M. s. australis* to *M. eachamensis*) and northern Queensland to southeastern Australia (*M.* sp. to *M. fluviatilis*). With the exception of the closely related southern species, *M. duboulayi* and *M. fluviatilis*, the sequence divergence among species is substantial (mean divergences 3.91% for clade A and 4.60% for clade C), suggesting old divergences. Alternatively, the phylogenetic distinctiveness of these species could be due to large-scale extinction of intervening populations if these taxa represent remnants of a formerly widespread ancestral taxon. The identification in clade A of cryptic diversity has important implications for the status of other phenotypically distinct populations in the region, such as those recognized through the aquarium trade.

The two clades shared between southern New Guinea and Australia, clades B and D, have lower divergences among taxa and relatively poorly resolved relationships. This pattern suggests recent speciation and range expansion. Clade B is more closely related to the Australian clades A and C than to clade D. This suggests that although the two clades show similar patterns, they originated independently. Noting the high frequency of recently separated species, we suggest that clades B and D represent excellent material for the study of the speciation processes in Melanotaeniidae.

The phylogenetic relationships of rainbowfish within Australia do not fit current biogeographic theory. Overall, the pattern suggests that there has been high connectivity amongst all drainages in the north, and that the present patterns are the result of several episodes of colonization and speciation, with stochastic variation affecting which drainages were colonized and when. A greater understanding of Australian freshwater biogeography may be gained by examining patterns within species groups (e.g. families) and then looking for concordance across these groups, with consideration of differences in ecology between groups.

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