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Phylogeny and biogeography of rainbowfishes (Melanotaeniidae) from Australia and New Guinea

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ABSTRACT

The family Melanotaeniidae (rainbowfishes) represents the largest monophyletic group of freshwater fishes found in Australia and New Guinea. The family consists of seven genera and a total of 81 species, which are broadly distributed throughout the region. We conducted a phylogenetic analysis of Melanotaeniidae based on nearly complete taxonomic sampling of all species. We sequenced seven protein coding mitochondrial genes and the first two introns of the nuclear S7 gene, for a total of 6827 base pairs. Our goal was to use the phylogeny to infer the biogeographic history of rainbowfishes in this region, to provide a framework for the timing of divergence within the family, and to test for possible introgression between species. We found strong support for the monophyly of Melanotaeniidae. Three species-poor genera-*Cairnsichthys*, *Rhadinocentrus* and *Iriatherina*-were all resolved as early branching lineages within the family. The three species-rich genera-*Melanotaenia*, *Chilatherina* and *Glossolepis*-did not form a single monophyletic group, but instead formed three monophyletic groups endemic to discrete geographic regions: western New Guinea, northern New Guinea, and southern New Guinea plus Australia, respectively. All three geographic regions also contained three to four additional lineages that were separated by large genetic divergences and were frequently sympatric (except in western New Guinea). Our molecular clock results provide much older estimates of divergence than some aspects of the present geological setting. For instance, the formation of the present day Central Highlands, the integration of the Birds Head region with the rest of New Guinea, and the present proximate position of Waigeo and Batanta islands relative to the Birds Head, are all younger than the rainbowfishes living there based on our molecular clock estimates. We also identified ten species that have likely experienced historical introgression. Most introgression events were between different groups within the northern New Guinea lineage and the Southern New Guinea/Australian lineages. Finally, we identified nearly 20 undescribed species within Melanotaeniidae, demonstrating that much work remains in describing freshwater fish diversity in this region.

1. Introduction

Australia and New Guinea contain the world's most depauperate freshwater fish fauna, but one of the most unique at a continental scale. Ostariophysan fishes (primarily consisting of minnows, characins and catfishes) dominate freshwater environments in most of the world with greater than 9600 species (Eschmeyer and Fong, 2012). Yet, they are almost completely absent from Australia and New Guinea—the only exception being two families of catfishes, which uniquely among osatriophysans include some marine species. Instead, the majority of freshwater fishes (around 80%) on the Australian continent are acanthopterygians. Acanthopterygians are the dominant fish group in marine environments, with some species also occurring in freshwater on all continents. This atypical pattern of faunal composition is largely thought to be a result of the long isolation of the Australian continent that prevented ostariophysans from colonising Australia. Instead, a number of more derived families with marine affinities established and diversified in freshwater habitats, probably throughout the Tertiary and potentially earlier in some cases.

The freshwater fish fauna of Australia and New Guinea consists of approximately 40 families, excluding species that only occasionally occupy freshwater. The total number of freshwater species is difficult to estimate, but is likely in excess of 500 (Allen, 1991; Allen et al., 2002), especially when considering that molecular examination of most groups shows that current taxonomy likely underestimates species richness (e.g., Jerry and Woodland, 1997; McGuigan et al., 2000; Hammer et al., 2007; Unmack et al.,

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2011). Of those 500 plus species, three families account for over 50% of the total species richness: Eleotridae (~100 spp.), Gobiidae (~90 spp.) and Melanotaeniidae (~81 spp.); and of these families, only Melanotaeniidae represents a monophyletic group endemic to Australia and New Guinea (Sparks and Smith, 2004). In fact, Melanotaeniidae represents the most speciose monophyletic radiation of freshwater fishes in the region.

Melanotaeniidae, commonly known as rainbowfishes, consists of seven genera, of which four (*Cairnsichthys*, *Iriatherina*, *Pelangia*, *Rhadinocentrus*) are currently recognised as being monotypic. The most speciose genera include *Melanotaenia* (57 spp.), *Chilatherina* (11 spp.) and *Glossolepis* (9 spp.) (Tappin, 2011; Allen and Unmack, 2012; Kadarusman et al., 2012). Despite approximately 35 species of freshwater fishes being shared between northern Australia and southern New Guinea (Allen et al., 2008), only two rainbowfishes are shared: *Iriatherina werneri* and *M. maccullochi*. Two additional species pairs have close relationships: *M. splendida* and *M. rubrostriatus*; and *M. trifasciata* and *M. goldiei* (Allen et al., 2008). Along with gobiids and eleotrids, rainbowfishes are among the most abundant and widespread families in Australia and New Guinea. In Australia they are found almost everywhere that water is present, excluding extremely arid regions (which are mainly devoid of fishes) and the southernmost regions, which are too cold (Fig. 1). In New Guinea, most localities that contain obligate freshwater fishes have rainbowfishes present. The only areas they are absent is the rugged northeastern portion of New Guinea east of the Markham River (which otherwise has several freshwater limited groups present) and at higher elevations (Fig. 1), with most native fishes being absent over ~1000 m and none found over 2000 m (Allen, 1991; Allen et al., 2008).

Previous molecular research on the rainbowfishes is limited. Sparks and Smith (2004) examined the family within the context of Atheriniformes and hypothesised a sister group relationship to Bedotidae, with Telmatherinidae and Pseudomugilidae being their sister group. Several studies have also examined morphological data, including Dyer and Chernoff (1996) who found the same relationship among families as revealed by the molecular data (Sparks and Smith, 2004), while other morphological studies have found a close relationship among various combinations of these families (Saeed et al., 1994; Ivantsoff et al., 1997; Aarn and Ivantsoff, 1997; Aarn et al., 1998). Our current understanding of phylogenetic relationships among rainbowfish species is based on two molecular systematic studies (Zhu et al., 1994 and McGuigan et al., 2000). McGuigan et al. (2000) examined all 12 recognised *Melanotaenia* species from Australia, plus 21 species from New Guinea representing both *Melanotaenia* and *Glossolepis*, but lacking *Chilatherina*. Their molecular sampling consisted of 351 bp of the mitochondrial cytochrome *b* gene for all individuals, plus 331 bp of control region for a subsample of species. This previous research revealed six lineages within the family, which were mostly well resolved, although some relationships within lineages had only modest support. These six lineages corresponded to three geographic groups within rainbowfishes: southern New Guinea/Australia, northern New Guinea and western New Guinea.

One puzzling fact of rainbowfish biology is that despite extensive sympatry between species in the wild, there is little evidence for hybridisation between species. In fact, both interspecific and intergeneric hybridisation in the wild was historically thought to be exceedingly rare, with only two F1 hybrids (between *M. affinis* and *C. campsi*) ever being identified morphologically (Allen and

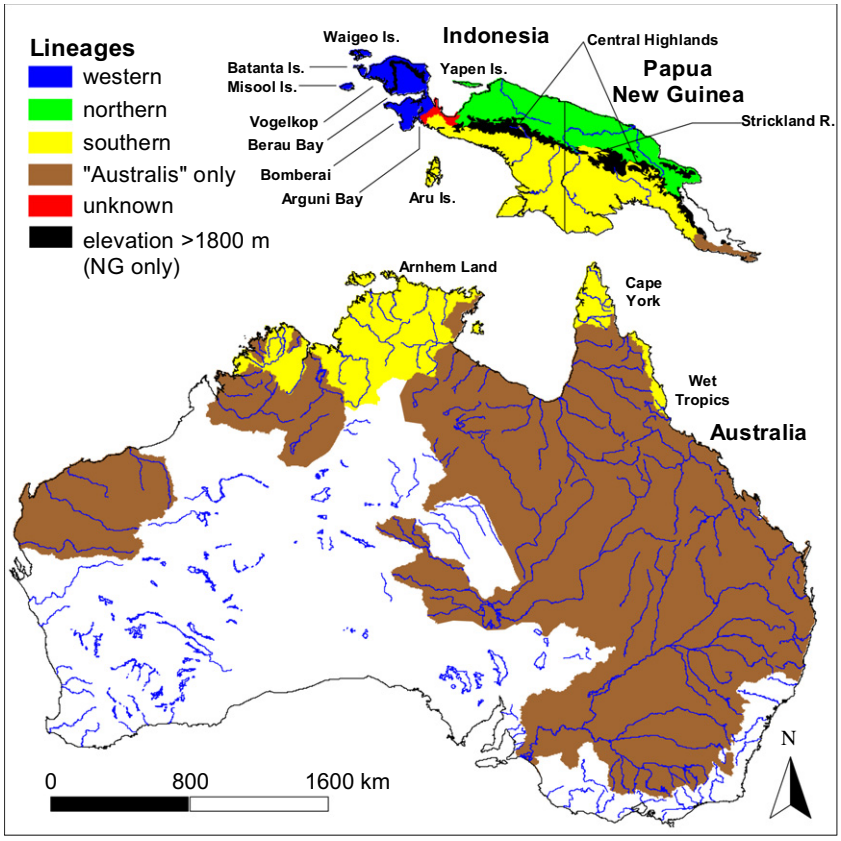

Fig. 1. Distribution of Melanotaeniidae species showing the range of each major lineage, along with place names described in text. Within the western lineage, the thick line shows the boundary between the "Northern" and "Southern Birds Head" groups. The extensive distribution of the "Australis" group relative to the rest of the southern lineage is also indicated. Areas over 1800 m are shown for New Guinea to indicate the Central Highlands and to represent highland areas devoid of native fishes. The region between the western and southern lineages is shown as unknown because more precise lineage distributions are yet to be determined across this area.

Cross, 1982). This situation is surprising given that most, if not all, rainbowfishes in the genera *Melanotaenia*, *Chilatherina* and *Glossolepis* are known to readily hybridize in captivity, and there are also reports of hybrids between *M. fluviatilis* and a *Bedotia* species (probably *B. madagascariensis*) (Allen and Cross, 1982; Allen, 1985). Molecular work on 33 rainbowfish species (Zhu et al., 1994; McGuigan et al., 2000) pointed to possible introgression of *M. australis* with both *M. gracilis* and *M. nigrans*. Although, rainbowfishes are known to hybridize readily in captivity, no research to date has tested the hypothesis that these species have experienced natural patterns of introgression.

Clearly, the geographic distribution of rainbowfish species, coupled with their biology, presents a unique opportunity to understand freshwater fish biodiversity in Australia and New Guinea. Here we present a new phylogeny of Melanotaeniidae based on nearly complete sampling of all described species in the family, using a far more comprehensive DNA data set than previous work (seven mitochondrial genes and the first two introns of the nuclear S7 gene). The purpose of this study is to test the monophyly of the family and each genus, examine the biogeographic history of rainbowfishes and to explore the timing of phylogenetic divergence within the family. Moreover, our broad taxonomic sampling, coupled with sequencing of both mitochondrial and nuclear markers, provides a framework for identifying possible introgression events.

2. Materials and methods

2.1. Study taxa and sampling

We sampled all but the following 10 valid described rainbowfish species (*M. ajamaruensis*, *M. corona*, *M. fasinensis*, *M. mairasi*, *M. maylandi*, *Pelangia mbutaensis*) and four rainbowfish species that were described after our analysis was complete (*M. arguni*, *M. urisa*, *M. veoliae* and *M. wanoma*; Kadarusman et al., 2012) (Table 1). We also included a number of undescribed forms, as well as multiple samples of widespread species with significant intraspecific divergence indicative of cryptic undescribed species. In addition, we included several mitochondrially-introgressed individuals that had divergent genotypes representing distinct lineages. Most species were sourced from wild populations, but in a few cases we were limited to captive populations. These were obtained from dedicated rainbowfish keepers (as indicated in Table 1). For captive populations, we usually sourced the same species from fish keepers from Australia, Europe and the USA in order to provide a higher level of quality control (in all cases the same results were obtained, but we only include data here from single representatives). Many of these collections can be directly linked back to the original wild collections (Tappin, 2011). For outgroups we included a number of species representing the families Pseudomugilidae, Telmatherinidae, Bedotidae and Atherinidae as multiple molecular and morphological studies have found one or more of these families to be closely related to Melanotaeniidae (Saeed et al., 1994; Dyer and Chernoff, 1996; Ivantsoff et al., 1997; Aarn and Ivantsoff, 1997; Aarn et al., 1998; Sparks and Smith, 2004). Trees were rooted with *Hypoatherina tsurugae* (Atherinidae).

2.2. DNA isolation, amplification, and sequencing

We extracted genomic DNA from muscle tissue from each specimen using the DNeasy Tissue Kit (QIAGEN Inc., Chatsworth CA) or by phenol–chloroform extraction. Seven of the 13 mtDNA protein coding genes (ND1, ND2, ND4L, ND4, ATPase6/8, cyt *b* and partial sequence from COIII) were amplified, representing over a third of the mitochondrial genome (5696 bp). Single and nested PCR amplification strategies were used to obtain product for different gene combinations. We also included the first two introns and the second exon of the nuclear S7 gene. We did not include any outgroup species for S7 due to difficulty in aligning the introns between them. All nuclear sequences were obtained by nested PCR. Details of the primers and nesting combinations are in Supplementary document 1. For nested PCR the first reaction size was 10 μL. This first PCR reaction was then diluted to 1:49, and 1 μL of this product was added to a second 25 μL reaction. All other single reactions were 25 μL. Final concentrations for PCR components were as follows: 25 ng template DNA, 0.25 μM of each primer, 0.625 units of Taq DNA polymerase, 0.1 mM of each dNTP, 2.5 μL of 10× reaction buffer and 2.5 mM $MgCl_2$. Amplification parameters were as follows: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 60 s (in the first nested reactions this was increased by 1 min per each thousand bp), and 72 °C for 7 min. PCR products were examined on a 1% agarose gel using SYBR safe DNA gel stain (Invitrogen, Eugene, OR, USA) and purified using a Montage PCR 96 plate (Millipore, Billerica, MA, USA). Sequences were obtained via cycle sequencing with Big Dye 3.0 dye terminator ready reaction kits using 1/16th reaction size (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were run with an annealing temperature of 52 °C, but otherwise followed the ABI manufacturer's protocol. Product was cleaned using Sephadex columns in MultiScreen 96 well assay plates (Millipore, Billerica, MA, USA), and then dried. Most sequences were obtained using an Applied Biosystems 3730 XL automated sequencer at the Brigham Young University DNA Sequencing Center. All sequences obtained in this study were deposited in GenBank, accession numbers XXXXXX–XXXXXX and the sequence alignment was deposited in Dryad, http://dx.doi.org/10.5061/dryad.qq846.

2.3. Analysis of sequence data

Sequences were edited using Chromas Lite 2.0 (Technelysium, Tewantin, Queensland, Australia) and imported into BioEdit 7.0.5.2 (Hall, 1999). Sequences coding for amino acids were aligned by eye and checked via amino acid coding in MEGA 5.05 (Tamura et al., 2011) to test for unexpected frame shift errors or stop codons. S7 sequences were aligned using the online version of MAFFT 6.822 (Katoh and Toh, 2008) using the slow iterative refinement FFT-NS-i algorithm with the scoring matrix for nucleotide sequences set to 1PAM/K = 2, a gap opening penalty of 1.53 and an offset value of 0.1. This was compared to an alignment created in Muscle 3.7 (Edgar, 2004) on the CIPRES cluster at the San Diego Supercomputer Center (Miller et al., 2010), which differed in its alignment, but produced the same maximum likelihood tree topology. The final dataset consisted of a total of 6827 base pairs, 5696 bp of mtDNA and 1131 bp of S7. Separate comparisons of tree topology for mtDNA and S7 (data not shown) demonstrated that several samples had different relationships for each marker, with species coming out in different lineages. In all cases the nuclear S7 data was consistent with previous morphological interpretations. In order to show these different relationships we included these individuals with different relationships as two operational taxonomic units, one based on only mtDNA, the other based only on S7. In one case (*M.* sp. Bindoolah, Table 1), we only included S7 data as the mtDNA was otherwise essentially identical to one *M. australis* sample (Isdell, Table 1). Combined phylogenetic analyses were performed with a likelihood approach using GARLI 2.0 (Zwickl, 2006). For our maximum likelihood (ML) analysis we identified the best-fitting model of molecular evolution using the Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada and Crandall, 1998) using PAUP* 4.0b10 (Swofford, 2003). For the

Table 1
Locality data for all Melanotaeniidae samples and outgroups examined. Locality column provides the name of the river system or lake, followed by the abbreviation for Australian states or the country (NSW = New South Wales, NT = Northern Territory, QLD = Queensland, VIC = Victoria, WA = Western Australia, IND = Indonesia, PNG = Papua New Guinea). Locality names preceded with **AS** represent samples obtained from reliable aquarium sources with their original wild collection locality unless unknown. The lineage, group column is based on their phylogenetic relationships (Fig. 2) and their distributions are shown in Fig. 1.

Species	Locality	Lineage, group
Cairnsichthys rhombosomoides	Behana Ck, QLD	*Cairnsichthys*
Chilatherina alleni	Siriwo R, IND	Northern, "Chilatherina"
Chilatherina axelrodi	**AS**, Pual R, PNG	Northern, "Chilatherina"
Chilatherina bleheri	**AS**, L Holmes, IND	Northern, "Chilatherina"
Chilatherina bulolo	Ramu R, PNG	Northern, "Chilatherina"
Chilatherina campsi	Markham R, PNG	Northern, "Chilatherina"
Chilatherina crassispinosa	Markham R, PNG	Northern, "Chilatherina"
Chilatherina fasciata I	Mamberano R, IND	Northern, "Chilatherina"
Chilatherina fasciata II	Tor R, IND	Northern, "Chilatherina"
Chilatherina fasciata III	Hewa R, IND	Northern, "Chilatherina"
Chilatherina fasciata IV	Ramu R, PNG	Northern, "Chilatherina"
Chilatherina lorentzi	Sermowai R, IND	Northern, "Glossolepis"
Chilatherina pagwiensis	Sepik R, PNG	Northern, "Chilatherina"
Chilatherina pricei	Wapoga R, IND	Northern, "Chilatherina"
Chilatherina sentaniensis	**AS**, L Sentani, IND	Northern, "Chilatherina"
Chilatherina sp. Gidomen	Mamberano R, IND	Northern, "Chilatherina"
Glossolepis dorityi	L Nenggwambu, IND	Northern, "Glossolepis"
Glossolepis incisus	L Sentani, IND	Northern, "Glossolepis"
Glossolepis kabia I	Sepik R, PNG	Northern, "Glossolepis"
Glossolepis kabia II	Ramu R, PNG	Northern, "Glossolepis"
Glossolepis kabia III	Markham R, PNG	Northern, "Glossolepis"
Glossolepis leggetti	Wapoga R, IND	Northern, "Glossolepis"
Glossolepis maculosus	**AS**, Markham R, PNG	Northern, "Affinis"
Glossolepis multisquamata	Mamberano R, IND	Northern, "Glossolepis"
Glossolepis pseudoincisus	L Ifaten, IND	Northern, "Glossolepis"
Glossolepis ramuensis	Golgol R, PNG	Northern, "Affinis"
Glossolepis sp. Gidomen	Mamberano R, IND	Northern, "Glossolepis"
Glossolepis wanamensis I	**AS**, L Wanam, PNG	Northern, "Glossolepis"
Iriatherina werneri I	Cadell R, NT	*Iriatherina*
Iriatherina werneri II	Jardine R, QLD	*Iriatherina*
Melanotaenia affinis I	**AS**, Blue Hole, IND	Northern, "Affinis"
Melanotaenia affinis II	Golgol R, PNG	Northern, "Affinis"
Melanotaenia ammeri	Trib to Arguni Bay, IND	Western, "southern BH"
Melanotaenia angfa	**AS**, Yakati R, IND	Western, "southern BH"
Melanotaenia arfakensis	**AS**, Prafi R, IND	Western, "northern BH"
Melanotaenia australis I	Fortescue R, WA	Southern, "Australis"
Melanotaenia australis II	Isdell R, WA	Southern, "Australis"
Melanotaenia australis III	Mitchell R, WA	Southern, "Nigrans"
Melanotaenia batanta	Warmon Ck, Batanta Is, IND	Western, "northern BH"
Melanotaenia boesemani	**AS**, IND	Western, "northern BH"
Melanotaenia caerulea	Kikori R, PNG	Southern, "Maccullochi"
Melanotaenia catherinae I	Kali Raja, Waigeo Is, IND	Western, "Waigeo"
Melanotaenia catherinae II	Wei Sam Ck, Waigeo Is, IND	Western, "Waigeo"
Melanotaenia duboulayi I	Richmond R, NSW	Southern, "Australis"
Melanotaenia duboulayi II	Granite Ck, QLD	Southern, "Australis"
Melanotaenia eachamensis	Dirran Ck, QLD	Southern, "Australis"
Melanotaenia exquisita	Umbrawarra Gorge, NT	Southern, "Nigrans"
Melanotaenia fluviatilis	Broken R, VIC	Southern, "Australis"
Melanotaenia fredericki I	**AS**, IND	Western, "northern BH"
Melanotaenia fredericki II	**AS**, Sth of Sorong, IND	Western, "northern BH"
Melanotaenia goldiei I	Aru Is, IND	Southern, "Goldiei"
Melanotaenia goldiei II	Aru Is, IND	Southern, "Goldiei"
Melanotaenia goldiei III	Yamur L, IND	Southern, "Goldiei"
Melanotaenia goldiei IV	Timika, IND	Southern, "Goldiei"
Melanotaenia goldiei V	Timika, IND	Southern, "Goldiei"
Melanotaenia goldiei VI	Pulau R, IND	Southern, "Goldiei"
Melanotaenia goldiei VII	Fly R, PNG	Southern, "Goldiei"
Melanotaenia goldiei VIII	Kikori R, PNG	Southern, "Goldiei"
Melanotaenia goldiei IX	Lakekamu R, PNG	Southern, "Goldiei"
Melanotaenia goldiei X	**AS**, Angabunga R, PNG	Southern, "Goldiei"
Melanotaenia goldiei XI	Laloki R, PNG	Southern, "Goldiei"
Melanotaenia gracilis	Drysdale R, WA	Southern, "Nigrans"
Melanotaenia herbertaxelrodi	**AS**, L Tebera, PNG	Southern, "Goldiei"
Melanotaenia irianjaya	**AS**, Fruata village, IND	Western, "southern BH"
Melanotaenia iris	Fly R, PNG	Northern, "Affinis"
Melanotaenia japenensis	Yapen Is, IND	Northern, "Glossolepis"
Melanotaenia kamaka	L Kamakawaiar, IND	Southern, "Goldiei"
Melanotaenia kokasensis	Kokas village, IND	Western, "southern BH"
Melanotaenia lacustris	**AS**, L Kutubu, PNG	Southern, "Goldiei"
Melanotaenia lakamora	L Lakamora, IND	Southern, "Goldiei"

Table 1 (*continued*)

Species	Locality	Lineage, group
Melanotaenia maccullochi I	**AS**, PNG	Southern, "Maccullochi"
Melanotaenia maccullochi II	Burster Ck, QLD	Southern, "Maccullochi"
Melanotaenia maccullochi III	Etty Bay, QLD	Southern, "Maccullochi"
Melanotaenia misoolensis I	Waitama, Misool Is, IND	Western, "northern BH"
Melanotaenia misoolensis II	Waitama, Misool Is, IND	Western, "northern BH"
Melanotaenia monticola	Purari R, PNG	Southern, "Goldiei"
Melanotaenia mubiensis	Kikori R, PNG	Southern, "Goldiei"
Melanotaenia nigrans I	Adelaide R, NT	Southern, "Nigrans"
Melanotaenia nigrans II	Burster Ck, QLD	Southern, "Nigrans"
Melanotaenia ogilbyi	Timika, IND	Southern, "Maccullochi"
Melanotaenia oktediensis	Fly R, PNG	Southern, "Goldiei"
Melanotaenia papuae	Laloki R, PNG	Southern, "Maccullochi"
Melanotaenia parkinsoni I	**AS**, Kemp Welsh R, PNG	Southern, "Australis"
Melanotaenia parkinsoni II	**AS**, Eastern PNG	Southern, "Australis"
Melanotaenia parva	**AS**, L Kurumoi, IND	Western, "southern BH"
Melanotaenia pierucciae	Trib to L Kamakawaiar, IND	Southern, "Goldiei"
Melanotaenia praecox	Mamberano R, IND	Northern, "Chilatherina"
Melanotaenia pygmaea	**AS**, Prince Regent R, WA	Southern, "Nigrans"
Melanotaenia rubripinnis	Wapoga R, IND	Northern, "Glossolepis"
Melanotaenia rubrostriatus I	Aru Is, IND	Southern, "Australis"
Melanotaenia rubrostriatus II	Fly R, PNG	Southern, "Australis"
Melanotaenia rubrostriatus III	Kikori R, PNG	Southern, "Australis"
Melanotaenia salawati	Kali Doktur, Salawati Is, IND	Western, "northern BH"
Melanotaenia sexlineata I	Fly R, PNG	Southern, "Maccullochi"
Melanotaenia sexlineata II	Fly R, PNG	Southern, "Maccullochi"
Melanotaenia sp. Dekai	Pulau R, IND	Southern, "Maccullochi"
Melanotaenia sp. NT	Finnis R, NT	Southern, "Maccullochi"
Melanotaenia sp. Pianfon	Trib to L Pianfon, IND	Northern, "Glossolepis"
Melanotaenia sp. Rawarra	Sebyar R, IND	Western, "southern BH"
Melanotaenia sp. Suswa	Karabara R, IND	Western, "northern BH"
Melanotaenia splendida splendida	Deepwater R, QLD	Southern, "Australis"
Melanotaenia s. inornata	Jardine R, QLD	Southern, "Australis"
Melanotaenia s. tatei	Finke R, NT	Southern, "Australis"
Melanotaenia sylvatica	Lakekamu R, PNG	Southern, "Maccullochi"
Melanotaenia synergos I	Wai Bin Ck, Batanta Is, IND	Western, "Waigeo"
Melanotaenia synergos II	**AS**, Warey R, Batanta Is, IND	Western, "Waigeo"
Melanotaenia trifasciata I	S Alligator R, NT	Southern, "Goldiei"
Melanotaenia trifasciata II	Blyth R, NT	Southern, "Goldiei"
Melanotaenia trifasciata III	Wenlock R, QLD	Southern, "Goldiei"
Melanotaenia trifasciata IV	Gap Ck, QLD	Southern, "Goldiei"
Melanotaenia utcheensis	Utchee Ck, QLD	Southern, "Australis"
Melanotaenia vanheurni	Mamberano R, IND	Northern, "Glossolepis"
Rhadinocentrus ornatus I	Marom Ck, NSW	*Rhadinocentrus*
Rhadinocentrus ornatus II	N Maroochy R, QLD	*Rhadinocentrus*
Rhadinocentrus ornatus III	Searys Ck, QLD	*Rhadinocentrus*
Rhadinocentrus ornatus IV outgroups	Byfield Ck, QLD	*Rhadinocentrus*
Bedotia madagascariensis	**AS**, sequences from M. Miya	
Bedotia leucopteron	**AS**, Madagascar	
Bedotia longianalis	**AS**, Madagascar	
Bedotia marojejy	**AS**, Madagascar	
Bedotia sp. Ankavia	**AS**, Madagascar	
Bedotia sp. Namorona	**AS**, Madagascar	
Rheocles alaotrensis	Madagascar	
Rheocles vatosoa	Madagascar	
Rheocles wrightae	Madagascar	
Pseudomugil gertrudae	**AS**, sequences from M. Miya	
Marosatherina ladigesi	**AS**, sequences from M. Miya	
Hypoatherina tsurugae	GenBank AP004420.1	

mtDNA data Modeltest identified GTR + I + G as the best model and for S7 TrN + G was the best model. For maximum likelihood (ML) analysis we ran GARLI with ten search replicates using the default settings with two partitions representing mtDNA and S7 with their respective models. For bootstrapping we ran 1000 replicates with the previous settings except that the options genthreshfortopoterm was reduced to 10,000 and treerejectionthreshold was reduced to 20 as suggested in the GARLI manual to speed up bootstrapping. The combined ML tree presented in this study was deposited in TreeBASE, accession number TB2:S13525, (http://purl.org/phylo/treebase/phylows/study/TB2:S13525). Average between and within lineage genetic distances were calculated with MEGA based on mitochondrial data only using the proportion of shared differences (*p*-distance).

2.4. Molecular clock analysis

To estimate molecular divergence times we used BEAST 1.7.2 (Drummond and Rambaut, 2007). Input files were generated using BEAUti 1.7.2. The analysis used an uncorrelated lognormal relaxed molecular clock with rate variation following a tree prior using the Yule model. For the mtDNA partition we used the GTR + I + G model while for S7 it was TrN + G model (identified using the AIC in Modeltest) and a random starting tree. MtDNA divergences based

on pairwise comparisons in this study were assumed to occur at 1.0% per million years which we fixed as the mean value for the rate. Rates for the S7 partition were estimated relative to the mtDNA partition. Other studies on teleost fishes have calibrated molecular clocks for protein coding mtDNA genes and obtained values between 0.68% and 1.66% pairwise divergence per million years (based on a summary of 11 studies by Burridge et al., 2008). Other studies on Australian freshwater fishes have used a rate of 1.0% (Unmack and Dowling, 2010), or obtained a similar rate (0.84%) based on biogeographic calibrations (Unmack et al., 2011). Although molecular clock estimates vary, and in many cases provide crude estimates of divergence times (Magallon, 2004; Pulquerio and Nichols, 2007; Donoghue and Benton, 2007), they can provide important insights into relative patterns of divergence. Hence, we interpret our molecular clock findings herein with an appropriate level of caution. Multiple shorter runs were conducted to check for stationarity and that independent runs were converging on a similar result. Final results from the BEAST analyses were based on four separate runs for 50 million generations each, with parameters logged every 1000 generations. Tree and logfile outputs were combined in LogCombiner 1.7.2 with a burn-in of 10% with the extra step of resampling trees every 5000 generations (due to computer memory limitations). Outputs from BEAST were examined in Tracer 1.5 while the age estimates were summarised using TreeAnnotator 1.7.2 with the mean values placed on the maximum clade credibility tree.

2.5. Identification and characterisation of introgression

Instances of introgression were inferred when we found major incongruence between the mitochondrial relationships versus those recovered by nuclear DNA. Furthermore, in all cases, nuclear DNA relationships were also supported by morphological identifications. Introgression can manifest itself across multiple time frames, which Near et al. (2011) categorised as 'recent,' 'intermediate' and 'deep.' Examples of recent introgression are characterised by the donor and recipient species with the same or very similar mitochondrial types. Intermediate introgression is characterised by older hybridisation events where the recipient and donor mitochondrial types have now diverged significantly from one another. Deep events are characterised by speciation within the recipient lineage post introgression. We adopt this framework (Near et al., 2011) to interpret potential introgression events here.

3. Results

3.1. Phylogenetic analyses

Sequence analysis of 139 OTUs (Table 1) yielded 3222 invariant characters, 415 variable but parsimony uninformative characters, and 3190 parsimony informative characters. ML analysis recovered one tree with a likelihood score of −106943.041982 (Fig. 2). Most nodes between deeper clades had strong support (100% of all bootstrap replicates). Dates obtained from the Beast analysis are presented based on their 95% highest posterior density (HPD) in Fig. 3; all estimates had effective sample sizes >338, most were >1000. Average pairwise between and within group genetic divergences (*p*-distance) are presented in Table 2.

Phylogenetic analysis of DNA sequences provided strong support for the monophyly of Melanotaeniidae as well as between the various lineages (Fig. 2). The three species-poor genera, *Cairnsichthys*, *Rhadinocentrus* and *Iriatherina*, all branched at basal positions within the phylogeny of the family. None of the three larger genera, *Melanotaenia*, *Chilatherina* or *Glossolepis* were monophyletic. Instead, species from these genera formed three monophyletic lineages, which are endemic to three discrete geographic regions, western New Guinea (Birds Head region), northern New Guinea and southern New Guinea plus Australia (Fig. 1). Interestingly, all three geographic regions contain an additional three or four monophyletic groups that are separated by large genetic divergences. In western New Guinea there are three groups that are strictly allopatric. The most divergent group is limited to Waigeo and Batanta islands (which we refer to as the "Waigeo" group), the remaining species separate in two groups, described here as "Northern Birds Head" and the "Southern Birds Head." These two groups separate on the southern side of Vogelkop Peninsula, with the northern group limited to drainages west of Berau Bay, as well as drainages in northern Vogelkop Peninsula (Fig. 1). The "Southern Birds Head" group includes all drainages that enter Berau Bay (and adjacent Bintuni Bay) and south through the Bomberai Peninsula to drainages in the vicinity of Arguni Bay. Northern New Guinea has three deeply divergent

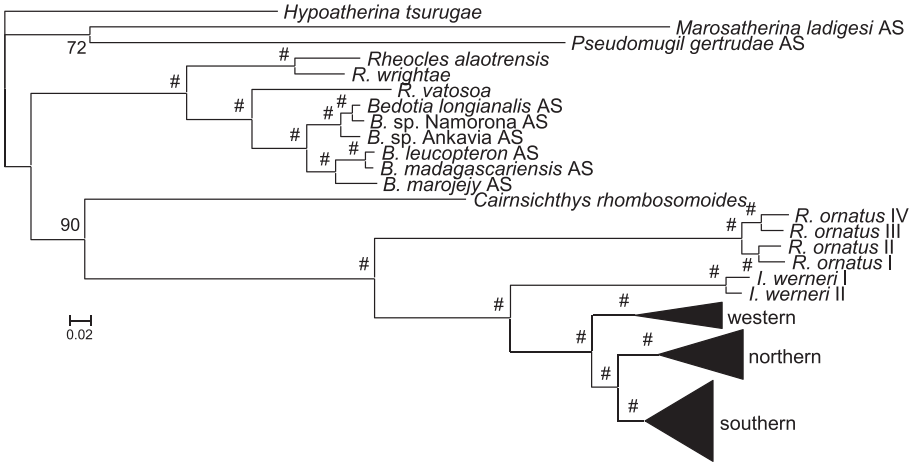

Fig. 2. Maximum likelihood tree for Melanotaeniidae based on analysis of combined mitochondrial DNA and the nuclear S7 gene. Panel A shows the complete tree with the three speciose lineages collapsed. Panel B shows the collapsed portion of the tree expanded. A label ending in MT or S7 indicates that the OTU is based on only that portion of the genetic data with MT representing an introgressed mitochondrial genome. Arrows point to the relative positions of introgressed mitochondrial genomes from their nuclear DNA position. A dashed line indicates that the mitochondrial data specific to that sample was not included, but it is closely related to the individual indicated. A label ending with AS indicates a specimen obtained from rainbowfish aquarium hobbyists. Bootstrap values are based on 1000 pseudoreplicates, a # symbol represents bootstrap values over 95 while an * represents values of 80–94. The tree is rooted with *Hypoatherina tsurugae*. Locality details are provided in Table 1.

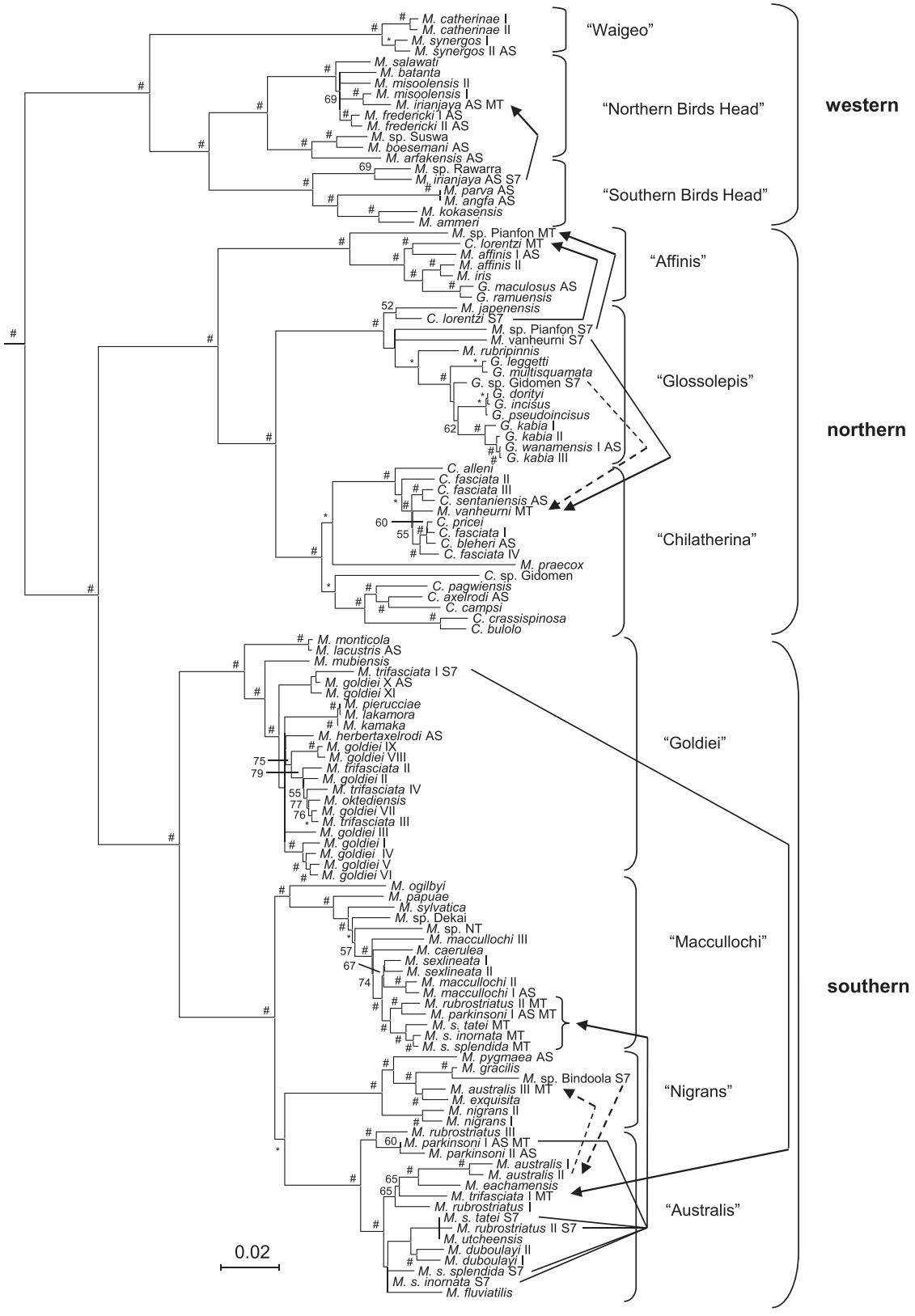

Fig. 2. (*continued*)

groups that extensively overlap in their geographic ranges. One group consists of mostly *Chilatherina* species plus *M. praecox* (which we refer to as the "Chilatherina" group). The second group has a mix of *Glossolepis* and *Melanotaenia* species plus *C. lorentzi* (which we refer to as the "Glossolepis" group). A third small group (which we refer to as the "Affinis" group) contains two *Melanotaenia* species, two *Glossolepis* species, plus two introgressed mitochondrial types that were acquired by two species from the "Glossolepis" group via introgression in the past. Southern New Guinea, plus Australia, contains four deeply divergent

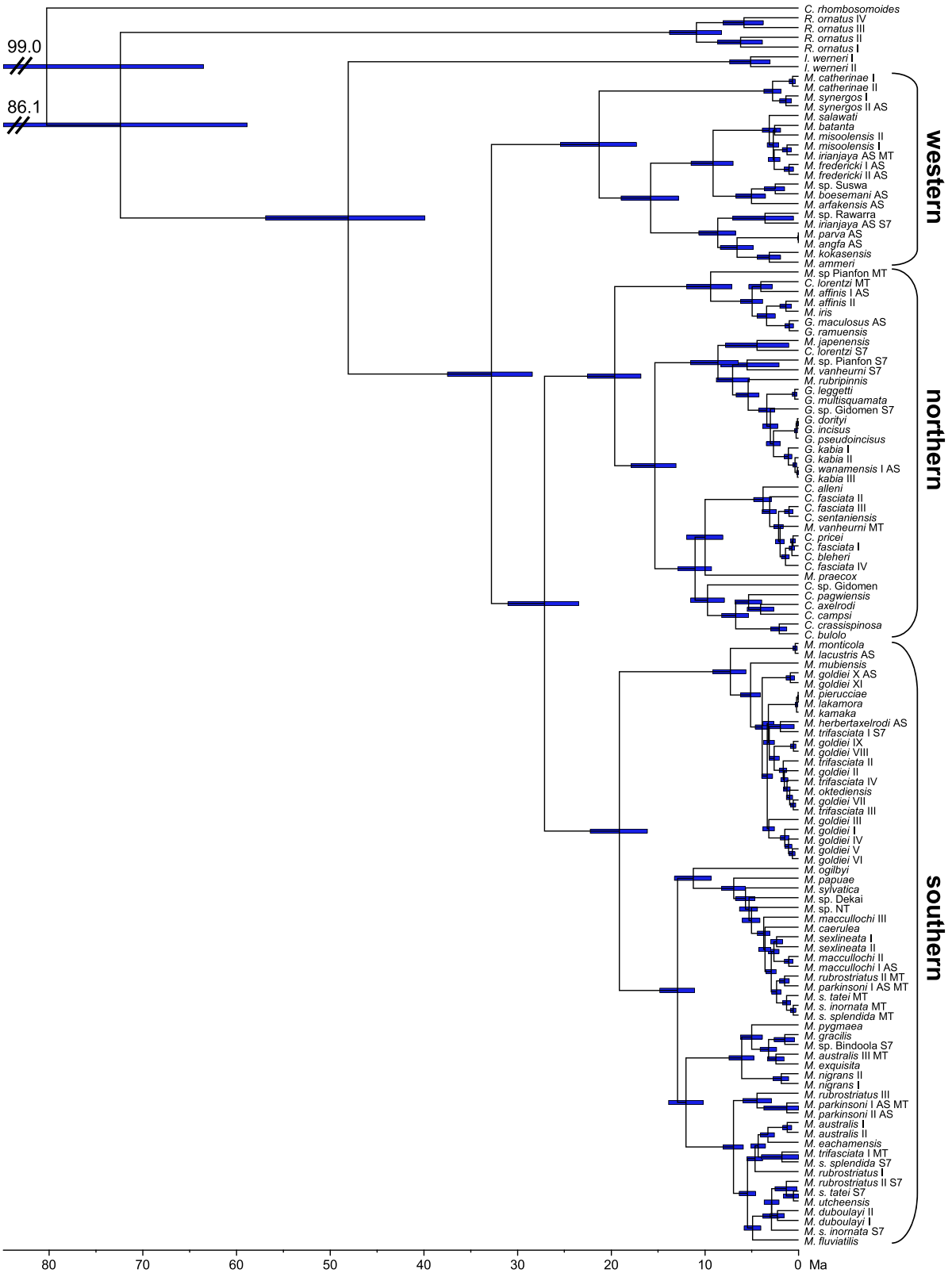

Fig. 3. Bayesian tree and molecular clock estimates for Melanotaeniidae based on analysis of combined mitochondrial DNA and the nuclear S7 gene. Horizontal bars represent the 95% highest posterior density ranges. A label ending in MT or S7 indicates that the OTU is based on only that portion of the genetic data with MT representing an introgressed mitochondrial genome. A label ending with AS indicates a specimen obtained from rainbowfish aquarium hobbyists. Locality details are provided in Table 1, OTU order and labels match those in Fig. 2 except when topology differs.

groups, all of which are currently placed in the genus *Melanotaenia* and all have extensive geographic overlapping distributions. Three are shared between Australia and southern New Guinea (which we refer to as the "Goldiei," "Maccullochi" and the "Australis" groups), the fourth is limited to northern Australia (the "Nigrans" group).

Table 2
Mean genetic divergences between Melanotaeniidae groups for mitochondrial DNA genes calculated using *p*-distances. The last column represents mean within group divergences. Groups are based on phylogenetic relationships shown in Fig. 2.

Group		1	2	3	4	5	6	7	8	9	10	11	12	13	within
"Waigeo"	1														0.018
"Nth Birds Head"	2	0.117													0.043
"Sth Birds Head"	3	0.125	0.101												0.053
"Affinis"	4	0.158	0.150	0.153											0.040
"Glossolepis"	5	0.157	0.147	0.151	0.119										0.030
"Chilatherina"	6	0.157	0.146	0.149	0.112	0.093									0.054
"Goldiei"	7	0.142	0.133	0.138	0.126	0.123	0.121								0.029
"Maccullochi"	8	0.147	0.143	0.147	0.133	0.133	0.132	0.089							0.035
"Nigrans"	9	0.153	0.145	0.150	0.137	0.137	0.136	0.094	0.080						0.036
"Australis"	10	0.153	0.149	0.152	0.137	0.138	0.136	0.096	0.077	0.086					0.042
Iriatherina	11	0.191	0.187	0.188	0.190	0.189	0.186	0.177	0.180	0.182	0.184				0.040
Rhadinocentrus	12	0.238	0.238	0.239	0.239	0.239	0.238	0.230	0.234	0.235	0.237	0.235			0.065
Cairnsichthys	13	0.258	0.254	0.249	0.254	0.249	0.250	0.247	0.246	0.250	0.252	0.255	0.271		n/c
Outgroups	14	0.266	0.264	0.264	0.262	0.264	0.262	0.257	0.257	0.260	0.261	0.259	0.272	0.238	0.173

4. Discussion

4.1. Biogeographic patterns

Melanotaeniidae are generally thought to have a close relationship to Bedotidae, which is otherwise restricted to Madagascar. There are two alternative explanations for the relationship between these families in Australia/New Guinea and Madagascar. The first is a single freshwater origin in Gondwana, followed by separation via continental drift, which started around 90–120 Ma (Sparks and Smith, 2004; Ali and Krause, 2011). The second explanation is for independent origins from a marine ancestor that is now extinct (Ivantsoff et al., 1997). Our molecular clock estimate for the separation of *Cairnsichthys* from the rest of the family (Fig. 3, mean 80.2 Ma, 95% HPD of 63.5–99.0 Ma) is consistent with the Gondwanan continental drift hypothesis for the separation of Melanotaeniidae from their sister group Bedotidae (Sparks and Smith, 2004; Ali and Krause, 2011), although the range of estimated ages suggests that this conclusion should be viewed with caution.

Cairnsichthys, *Rhadinocentrus* and *Iriatherina* represent the three earliest lineages to branch within Melanotaeniidae as per the findings of Allen (1980a) and Sparks and Smith (2004). These genera were estimated to have origins that pre-date ~40 Ma (Fig. 3). All three genera have restricted distributions relative to other lineages within the family. The monotypic genus *Cairnsichthys* has a very limited distribution restricted to just a few drainages in the Wet Tropics of northeastern Queensland (Allen and Cross, 1982; Thuesen et al., 2008). Suitable habitat for this species in Australia is almost certainly limited to the Wet Tropics region due to the combination of high rainfall and larger topographic gradients combined with a tropical climate. Its distribution may be further limited by competition from *M. splendida*, which is common in lowland areas as the two species only have narrow zones of sympatry (Pusey et al., 2004), or by more abundant populations of predators in larger lowland streams (Thuesen et al., 2008). The genus *Rhadinocentrus* is more widespread than *Cairnsichthys*, but still limited to fairly specific habitat types that are geographically limited to drainages near the central coast of eastern Australia. Recent evidence (Page et al., 2004) suggests that the genus consists of four species, which, based on our divergence estimates diversified between 3.8 and 13.9 Ma (Fig. 3). *Iriatherina* has a broader although disjunct distribution, being limited to drainages in northern Cape York, Arnhem Land and southern central New Guinea (Allen and Cross, 1982; Allen et al., 2002). They primarily inhabit shallow floodplain wetlands. Australian populations show a large divergence consistent with the presence of two species (Figs. 2 and 3). Within the family, *Iriatherina* has the most divergent morphological appearance, with a relatively small size (<4 cm standard length) and extremely long ornate fin filaments (Allen and Cross, 1982).

All of the remaining diversity of the family is contained within three lineages that we refer to as western, northern and southern. The ancestor to these lineages diverged sometime between 39.8 and 57.1 Ma, while the three individual lineages were estimated to have diverged from each other between 23.6 and 37.3 Ma (Fig. 3). These three lineages geographically correspond to the major aquatic biotic provinces of New Guinea (Allen, 1991; Abell et al., 2008). The northern and southern lineages are separated by the Central Highland Mountains that extend east–west through New Guinea. The western lineage is restricted to the Birds Head region, which has a narrow connection to the rest of New Guinea with very rugged limestone terrain, often with little coordinated surface drainage (Polhemus and Allen, 2007; Bailly et al., 2009). These three regions have essentially no obligate freshwater fishes in common except for five species. Two species are shared between western and southern New Guinea, while four species are found on both sides of the Central Highlands (Allen, 1991). Three of these exceptions include species that likely consist of multiple cryptic taxa (the plotosid catfish *Neosilurus brevidorsalis*; the eleotrid, *Oxyeleotris fimbriata*; and the goby, *Glossogobius bulmeri*), which if recognised, will reduce faunal similarity. The other two exceptions are due the presence of the rainbowfish *C. campsi* and the plotosid *N. gjellerupi* in the upper reaches of the Purari River, which is on the southern side of the central highlands presumably due to faunal exchange between their headwaters (Allen, 1991). In addition, we now document the close relationship of *M. iris* to *M. affinis* (Fig. 2). *Melanotaenia iris*, a member of the northern lineage is only known from the upper reaches of the Strickland River, a major tributary to the southern flowing Fly River. However, this portion of the Strickland River rises on the northern side of the Central Highlands before flowing to the south, thus the presence of *M. iris* could possibly be a result of a faunal transfer from a northern drainage. Despite these few possible exceptions, the biogeographic pattern in rainbowfishes closely matches those of the overall freshwater fish fauna. Further phylogenetic work is required to determine if other groups show similar monophyletic patterns within each of the three major geographic regions.

4.2. Divergence times and geology

Our molecular clock results provide much older estimates of divergence than the present geological setting, even when allowing for faster rates of evolution than we applied. For instance, the current formation of the Central Highlands, the integration of the Birds

Head region with the rest of New Guinea, and the present proximate position of Waigeo and Batanta islands relative to the Birds Head are all relatively recent events within the last 1–14 million years (Hall, 2002; Hill and Hall, 2002; Polhemus, 2007). New Guinea has an extremely complicated and poorly understood geological history which makes comparisons with biogeography difficult. The Australian continent (of which New Guinea forms the northern portion) has been interacting with multiple plates to the north, which have been moving in different directions throughout the Tertiary. In brief, much of northern New Guinea represents a series of accreted terrains from plates moving west across the northern margin. Accretion has resulted in folding and subduction with subsequent volcanism and uplift around 10 million years after accretion (Hill and Hall, 2002). The current Central Highlands were primarily formed over the last 12–14 million years (Hill and Hall, 2002). However, there were probably also earlier periods of uplift as well, perhaps dating to Eocene (Davies et al., 1996) as well as a possible extension of the Australian Eastern Highlands through central and northwestern New Guinea around 70 million years ago (Hill and Hall, 2002). Clearly, the recent uplift of the Central Highlands is too young to explain the divergence time estimates for the separation of the northern and southern lineages (Fig. 3, mean 27.0 Ma, 95% HPD of 23.8–30.8 Ma). To force the molecular clock estimates to be consistent with the geological estimates of 12–14 million years requires a pairwise rate of evolution of ~2%, which is beyond current estimates in the literature for fishes (0.68% and 1.66% pairwise divergence per million years, Burridge et al., 2008). However, the current mountain chain is clearly responsible for their isolation today, as well as for a considerable time back into the past. It is also important to note that uplift patterns in New Guinea were likely not uniform in time or space. Terrains were thought to have accreted earlier in the west and later in the east, with uplift following these events up to around 10 million years later (Hill and Hall, 2002). When the issue of the different directions of plate movement is combined with the sheer number of accreted terrains (at least 32, Pigram and Davies, 1987), it makes determining the uplift history and its timing extremely difficult, especially with our current limited geological knowledge of New Guinea (Hill and Hall, 2002; Polhemus, 2007). The key point relative to the evolution of the northern lineage is that any of these earlier uplift and accretion events could have helped to isolate the ancestors of this lineage, with the most recent uplift reinforcing their isolation.

The geological history of the Birds Head region is difficult to reconcile with the phylogenetic relationships recovered and our molecular clock estimates. The western lineage, which is restricted to the Birds Head and several offshore islands, was the first to diverge relative to the northern and southern lineages (Fig. 3, mean 32.7 Ma, 95% HPD of 28.4–37.3 Ma). Within the western lineage, the "Waigeo" group was the first to diverge relative to the Northern and Southern Birds Head groups (Fig. 3, mean 21.4 Ma, 95% HPD of 17.5–25.3 Ma). The current integration of the Birds Head with the rest of New Guinea was established around 10 Ma, while the current position of Batanta and Waigeo islands has only been relatively recent, perhaps within the last 1 million years (Hill and Hall, 2002; Polhemus, 2007; Bailly et al., 2009). Clearly the geological dates massively underestimate the molecular divergence and contradict the phylogenetic patterns relative to the early divergence of the "Waigeo" group (Fig. 2). Batanta and Waigeo have a Pacific plate origin that has followed a path to the north of New Guinea (Hill and Hall, 2002; Polhemus, 2007). It is possible, although speculative, that the Batanta and Waigeo arc fragments were temporarily attached to northern New Guinea, but were then sheared off again after being colonised by rainbowfishes. One problem with this explanation is that the islands did not come close to the Birds Head region until the last few million years. It seems almost inescapable that the rainbows living on Batanta and Waigeo islands did not evolve there, but colonised from somewhere else. But from where is unclear at this stage. Similar issues exist relative to the colonisation of the Birds Head region. Presumably rainbowfishes were present on the Birds Head prior to its current integration with New Guinea ~10 Ma. The Birds Head is hypothesised to have always been relatively close to the western end of New Guinea (Hill and Hall, 2002; Polhemus, 2007) and it seems to share aspects of its geological evolution which is consistent with them being in close proximity (Dow and Sukamto, 1984). We favour the possibility that there may have been some old contact that allowed colonisation, but was subsequently severed. One problem with that hypothesis is with all the plate movements and shearing in this region, then why are not rainbowfishes present on any landmasses west of the Birds Head that had potentially had contact with it too? Perhaps the land sheared off was too small, or became submerged. Alternatively, rainbowfishes simply could not persist, or perhaps were never present. Another possibility is that the Birds Head was colonised by a series of invasions after it was integrated with New Guinea. The first invasion made it all the way to Batanta and Waigeo, the second to the northern Birds Head (replacing the first invaders), the third only made it as far as the southern Birds Head. Their ancestors from New Guinea were then subsequently replaced by other rainbowfish lineages. Any potential hypotheses seem highly speculative though given the lack of other data to support or refute any of these explanations.

4.3. Introgression

It is clear that previous work on the family has underestimated the degree to which introgression has occurred. Given the limited breadth of our sampling strategy within species, there are likely to be additional examples of introgression not detected here, especially in cases where introgression is geographically limited to a small portion of a species' range. Broad sampling within each species distributional range will be necessary to more fully understand patterns of introgression. Still, we identified 13 populations representing 10 species that have likely experienced historical introgression with replacement of their original mtDNA type (Fig. 2). Six of those species were involved in old indeterminate introgression events (sensu Near et al., 2011) such that sufficient time has passed for these mitochondrial lineages to represent divergent genotypes that are as different today to their donor sister species as many rainbowfish sister species are to each other. The remaining four species are examples of proximal introgression. Nine of the ten introgression events are between different groups within the northern New Guinea lineage and the southern New Guinea/Australian lineage. In the northern lineage we identified four introgression events as follows. *Melanotaenia vanheurni* ("Glossolepis" group) was a recipient of mtDNA that is closely related to *C. fasciata* ("Chilatherina" group), a species that it is sympatric with today. *Glossolepis* sp. Gidomen ("Glossolepis" group) has a mitochondrial genome inherited from *C. fasciata* via *M. vanheurni*, which represents an unusual case of mitochondrial DNA passing from one species (*C. fasciata*) to another (*M. vanheurni*), and then to a third species (*Glossolepis* sp. Gidomen). Both *C. lorentzi* and *M.* sp. Pianfon ("Glossolepis" group) were recipients of mtDNA that is sister to the rest of the "Affinis" group. *Chilatherina lorentzi* is sympatric with *M. affinis* in parts of its range, but *M.* sp. Pianfon is not, although that region of New Guinea remains poorly sampled. In the southern New Guinea/Australian lineage we identified four introgression events. One major introgression occurred between the "Australis" and "Maccullochi" groups. Essentially, *M. splendida* and some New Guinea populations of *M. rubrostriatus* and *M. parkinsoni* were the recipients in an old introgression event with an ancestor to *M. maccullochi*/*M. sexlineata,* a group with which they are frequently sympatric (except *M. parkinsoni*). In

the "Goldiei" group, one small isolated population of *M. trifasciata* (South Alligator R., Leichhardt Springs) was the recipient of an indeterminate introgressed mtDNA type from the "Australis" group that is weakly supported as being related to *M. eachamensis*, *M. australis* and *M. rubrostriatus* from the Aru Islands. This result implies extinction of this lineage in parts of northern Australia as these lineages are not sympatric today. Today *M. trifasciata* (Leichhardt Springs) is allopatric (or close to) *M. splendida* populations, but these *M. splendida* populations now have "Maccullochi" group mtDNA due to introgression. Presumably the introgression event occurred prior to the *M. splendida* introgression, which is consistent with our molecular clock estimates (Fig. 3). We also found introgression between the "Australis" and "Nigrans" groups. *Melanotaenia* sp. Bindoolah is sympatric with, and was a recipient of *M. australis* mtDNA (data not shown), but it is otherwise closely related to *M. gracilis* based on the S7 data. One *M. australis* population (Mitchell R., Camp Ck) was the recipient of an mtDNA type related to *M. exquisita*, which is otherwise found several drainages distant to the east. In addition, Zhu et al. (1994) and McGuigan et al. (2000) also found evidence for introgression between *M. australis* and both *M. gracilis* and *M. nigrans* based on shared or similar haplotypes that were inconsistent with morphological identifications. Only one introgression event was identified within western lineage and that was *M. irianjaya* ("Southern Birds Head" group). Its nuclear DNA placed it as sister to *M.* sp. Rawarra, whereas mtDNA placed it close to *M. misoolensis* ("Northern Birds Head" group). We note however that this conclusion should be confirmed with analysis of wild fish as our *M. irianjaya* samples were based on an aquarium hobby strain. In summary, we found a striking degree of introgression in rainbowfishes which begs for further investigation to understand factors that might promote hybridisation and promote successful introgression.

4.4. Taxonomic implications

There are multiple taxonomic issues at both the generic and species levels. The largest problem is how to systematically deal with the generic nomenclature of *Melanotaenia*, *Chilatherina* and *Glossolepis*, as none were monophyletic. Both *Chilatherina* and *Glossolepis* are mostly monophyletic, but deeply nested within *Melanotaenia*. This could imply that all species should be placed into a single genus (*Melanotaenia*), or that *Melanotaenia* be separated into multiple new genera. Determining the appropriate generic nomenclature here is beyond our scope, but clearly, a reassessment of the characters defining these genera is required.

Our results demonstrate that considerable undescribed diversity exists within Melanotaeniidae, with at least 15–20 species included here likely representing undescribed, or unrecognised species. Some of this results from new species being found from previously unsampled areas, others represent existing species that appear to consist of multiple taxa. We found that species with the greatest cryptic diversity were typically the most widespread ones. Both *M. goldiei* (found across all of southern New Guinea) and *M. trifasciata* (found across northern Australia) have four distinct lineages present. *Melanotaenia maccullochi* has three non-monophyletic lineages within its patchy distribution from northeastern Australia, southern New Guinea and an isolated population near Darwin (Northern Territory). The most widespread species in the Birds Head region, *M. irianjaya*, consists of distinct forms such as *M.* sp. Suswa and *M.* sp. Rawarra. Both *M. affinis* and *C. fasciata* are widespread species across northern New Guinea and have distinct forms on their western range limits. In addition to splitting existing species, new species continue to be regularly discovered. Given the vast areas of New Guinea that remain either unsampled, or at best, poorly sampled, this trend will likely continue.

It is also clear that a number of currently recognised species have minimal genetic divergences between them (e.g., *G. leggetti* and *G. multisquamata*), or they are phylogenetically nested within other species (e.g., *C. bleheri* and *G. wanamensis*, Fig. 2), perhaps suggesting some of these taxa should be collapsed. Major differences in colour patterns have traditionally played a large role in species identification, especially between closely related taxa. These differences in colouration are usually associated with differences in meristic counts (modal values, sometimes with non-overlapping ranges), as well as variation in shape. Thus, most of the species with minimal genetic divergences were distinguished on the basis of differences in colouration, meristic counts and shape, e.g., *M. monticola* and *M. lacustris* (Allen, 1980b); *M. kamaka*, *M. lakamora* and *M. pierucciae* (Allen and Renyaan, 1996); *C. bleheri* and *C. fasciata* (Allen, 1985); etc. It would be worthwhile to revisit some of these species' distinguishing characteristics to confirm differences on the basis of broader sampling and/or more individuals should collections become available. The lack of genetic divergences could simply be a result of recent evolution in these traits, which would not have had long enough to leave genetic signatures based on the markers used in our study. Alternatively, introgression between closely related species may have occurred, thus reducing their genetic distinctiveness. In each of these cases, an assessment of taxonomic integrity based on multiple lines evidence (sensu Johnson et al., 2004) would help clarify species validity.

5. Conclusions

The family Melanotaeniidae represents an old and diverse monophyletic lineage that is endemic to Australia and New Guinea. We found that the most speciose genera, *Melanotaenia*, *Chilatherina* or *Glossolepis* were not monophyletic, but instead formed three different monophyletic lineages that are endemic to three discrete geographic regions: western New Guinea, northern New Guinea, and southern New Guinea plus Australia. Each of these geographic regions contain three or four additional monophyletic groups that are separated by large genetic divergences and are frequently sympatric (except in western New Guinea). Some of our molecular clock estimates conflict with present geological interpretations of the region relative to western and northern New Guinea. If these estimates are moderately accurate, our results either challenge current geological hypotheses, or suggest that speciation occurred prior to these geological events followed by dispersal to their present distributions. The most difficult result to reconcile with geology was the age of the lineage present on Waigeo and Batanta islands. Historical introgression was common between some lineages and in some species appears to have resulted in complete mitochondrial genome replacement (e.g., *C. lorentzi*, *M. vanheurni* and *M.* sp. Pianfon) over vast geographic regions. For instance, the entire Australian distribution of *M. splendida* has an introgressed mitochondrial genome from the "Maccullochi" group. Introgression has also been important in that it has conserved several unique mitochondrial genome types that have persisted over several million years since their presumed hybridisation event. Finally, considerable taxonomic work remains, with around 15–20 undescribed species identified within Melanotaeniidae. Our work suggests that this number will likely continue to increase as new areas of New Guinea are sampled and as more studies focused on within-species geographic variation are conducted.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.12.019.

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