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Mitochondrial DNA reveals monophyly of New Zealand's *Gobiomorphus* (Teleostei: Eleotridae) amongst a morphological complex

Mark I. Stevens[1,2] and Brendan J. Hicks[1]

[1]*Centre for Biodiversity and Ecology Research, Department of Biological Sciences, School of Science and Engineering, The University of Waikato, Private Bag 3105, Hamilton, New Zealand and* [2]*South Australian Museum, Adelaide, SA 5000 and School of Biological Sciences, Flinders University, Adelaide, SA 5001, Australia*

ABSTRACT

Goal: To examine the phylogenetic relationships within *Gobiomorphus* to explore Australasian biogeography, origins (single or multiple), and evolutionary loss of a marine life stage (loss of diadromy).

Methods: We analyse a fragment of the mitochondrial DNA (Cyt-b) using phylogenetic analyses, and use several meristics (spines, rays, and vertebrae) in a discriminant function analysis.

Organisms: All nine species of the genus *Gobiomorphus* (Eleotridae) found in New Zealand and Australia, with the two Australian *Philypnodon* species included as outgroup taxa.

Conclusions: Monophyly of the genus and all seven New Zealand species was strongly supported. The New Zealand *Gobiomorphus* are likely to be the result of a single introduction and to have been isolated from Australia since the Miocene or Pliocene (~18–28 Myr ago). These dates correspond well to the oldest fossil in New Zealand dated at 16–20 Myr. Diadromy was consistently at the root of the New Zealand group, and non-diadromy and morphological diversification among these species appears to have occurred relatively recently, with meristic comparisons supporting the molecular analyses. However, among the non-diadromous forms, species morphology contrasts with the molecular data suggesting a recent evolutionary history driven by ecological selection.

Keywords: amphidromy, *Gobiomorphus*, habitat fragmentation, island systems, mtDNA (Cyt-b), species radiation.

INTRODUCTION

The family Eleotridae (Teleostei: Perciformes: Gobioidei) comprises approximately 35 genera containing about 150 species (Nelson, 2006). They are present in a variety of habitats, from inland freshwaters and estuaries to coastal seas. Some species have diadromous life

Correspondence: M.I. Stevens, South Australian Museum, Adelaide, SA 5000 and School of Biological Sciences, Flinders University, Adelaide, SA 5001, Australia. e-mail: stevens.mark@saugov.sa.gov.au

strategies, utilizing both fresh- and saltwater as part of their reproductive cycles (McDowall, 1988). In New Zealand's eleotrids, known locally as 'bullies', this strategy involves amphidromy, a form of diadromy in which larvae migrate out to sea for a feeding phase before returning as juveniles to freshwater (Michel *et al.*, 2008). The presence of species near the coast and saltwater tolerance of some larvae has led to the suggestion that the Eleotridae are derived from marine origins and that the freshwater fauna is composed of secondary freshwater species.

Australia has approximately 14 freshwater eleotrid genera (Thacker and Hardman, 2005; Thacker and Unmack, 2005), but only one genus, *Gobiomorphus*, is found in New Zealand. McDowall (1975) suggested that New Zealand freshwater fish are derived from Australia via prevailing ocean currents, and that the Australian *G. coxii* is ancestral to, or has common ancestry with, the New Zealand *Gobiomorphus*. In New Zealand, *Gobiomorphus* currently comprises seven species, of which three are strictly diadromous (*G. hubbsi*, *G. gobioides*, and *G. huttoni*), three are non-diadromous (*G. breviceps*, *G. basalis*, and *G. alpinus*), and one species appears to be facultatively diadromous (*G. cotidianus*) (McDowall and Stevens, 2007; Michel *et al.*, 2008), with the potential of a further 'cryptic' species (see Smith *et al.*, 2005). The only other species (*G. coxii* and *G. australis*) are found along the south-eastern coast of Australia (Thacker and Hardman, 2005).

McDowall (2004) views amphidromy in island faunas as evidence that a marine juvenile phase provides a dispersal mechanism for freshwater taxa possessing this life-history trait rather than implying marine ancestry. In further support of this contention, Thacker and Hardman (2005) found that basal taxa of gobioid fishes are usually found in fresh or brackish water and the genus *Rhyacichthys* (basal within the Gobioidei) is exclusively freshwater-dwelling. Consequently, it has been proposed that gobioids speciated in freshwater from marine ancestors, and returned to marine habitats one or more times (e.g. Thacker and Hardman, 2005). Indeed, most of the basal gobioids examined by Thacker and Hardman (2005) exhibited at least partial saltwater tolerance, suggesting that diadromy may be the ancestral character. Waters and Wallis (2001) examined loss of diadromy in the *Galaxias vulgaris* complex (~10 non-migratory lineages) and found that three species had independently derived non-migratory life histories, indicating that non-migratory species were of a more recent origin, at least in *Galaxias*. In addition, Waters *et al.* (2006) have revealed high rates of speciation in *Galaxias divergens* associated with non-diadromous life histories among different isolated catchments, while Allibone *et al.* (1996) and Waters *et al.* (2000a, 2000b) found that speciation within *Galaxias* species has been associated with the loss of diadromy. This suggests that migratory ability is an ancestral characteristic in galaxiid fishes.

Here, we use the mitochondrial cytochrome *c* oxidase *b* gene to provide further understanding of the origins of New Zealand *Gobiomorphus*. In particular, we examine the relationships among all the New Zealand and Australian *Gobiomorphus* species. The present study aims to test the following specific hypotheses: (1) *Gobiomorphus* dispersed from Australia to New Zealand in a single event (rather than multiple events); and (2) diadromy in *Gobiomorphus* is an ancestral character and therefore diadromous species should occupy positions closer to the root among the New Zealand species (with respect to the Australian species and outgroups), while species that have lost the marine life stage (non-diadromous) will be derived.

METHODS

Collection of samples

Gobiomorphus and *Philypnodon* specimens were collected from localities in North Island, South Island, and Chatham Island of New Zealand, and from eastern Australia (see Online Appendix T1: www.evolutionary-ecology.com/data/2369T1.pdf) by electric fishing and baited traps. Specimens were identified to genus, and where possible to species, in the field. All specimens were identified to species in the laboratory using the relevant meristic information (McDowall, 1975; McDowall and Stevens, 2007).

DNA sampling, extraction, and analysis

Up to 10 individual fish from each location were anaesthetized in the field and 10–50 μl of blood was taken from the caudal vein using a syringe and fine-gauge needle (29G), primed with 5–10 μl of sterilized EDTA (100 mM) as an anticoagulant (Gleeson *et al.*, 1999). The blood samples were flash-frozen in liquid nitrogen and stored at −75°C until needed for molecular analyses. Total genomic DNA was extracted from the blood and EDTA solution using 300 μl of lysis buffer (5 ml 1 M Tris, 5 ml 10% SDS, 5 ml 0.5 M EDTA, 1 ml 5 M NaCl, 34 ml H_2O) and shaken for 30 min at 65°C; then 300 μl LiCl (5 M) were added and mixed for 30 min; then 750 μl of chloroform were added and mixed for 30 min. This mixture was then centrifuged (10 min at 10,000 rcf), after which the aqueous phase was removed to a new tube and DNA precipitated using an equal volume of isopropanol and left for 2 h; DNA was pelleted by centrifugation (15 min at 13,000 rcf) and washed with 70% ethanol. The DNA pellet was air-dried and re-suspended in 50 μl sterilized dH_2O.

PCR amplification (Saiki *et al.*, 1988) of a 450 base pair fragment of the mitochondrial (mt) cytochrome *c* oxidase b (Cyt-b) gene was amplified using the universal primers Cyb2 (modified) (5′-CCC TCA GAA TGA TAT TTG TCC T-3′) (Kocher *et al.*, 1989) and tgludg (5′-TGA CTT GAA RAA CCA YCG TTG-3′) (Palumbi *et al.*, 1991). Amplification (via PCR) was carried out using a 25-μl reaction volume consisting of 0.75 μl of DNA (not quantified), $1 \times$ PCR buffer (Roche), 1.5 mM $MgCl_2$, 0.2 $mmol \cdot l^{-1}$ of each dNTP (Boehringer Mannheim), 10 pmol of each primer, and 0.5 units of *Taq* DNA polymerase (Roche) on a Eppendorf Mastercycler gradient thermocycler.

The thermal cycling conditions included an initial 2-min denaturation step at 94°C, followed by 35 cycles at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 30 s, with a final cycle of 5 min extension at 72°C. All PCR products were purified by either a QIAquick PCR purification kit (Qiagen) with an additional 750 μl guanidine hydrochloride (35%) wash to remove residual primers and unincorporated dNTPs, or using SAPEXO (Amersham, Inc.). Sequencing was performed using the forward and reverse primers on an ABI 377XL automated sequencer (Applied Biosystems) at the University of Waikato DNA Sequencing Unit or on an ABI 3730 capillary sequencer (Applied Biosystems) at the Allan Wilson Centre (Massey University) Genome Service.

Sequences were individually verified using the GenBank BLAST algorithm, and were aligned using SEQUENCHER (Gene Codes version 4.7) sequence editor. In addition, all sequences were aligned with the complete cytochrome *b* sequence for *Gobiomorphus hubbsi* (accession no. AB021239, AY722227) and several other *Gobiomorphus* sequences obtained from GenBank (see Online Appendix T1: www.evolutionary-ecology.com/data/2369T1.pdf). These data were analysed using PAUP* 4.0b10 (Swofford, 2002), PhyML version

2.4.4 (Guindon and Gascuel, 2003), MrBayes version 3 (Ronquist and Huelsenbeck, 2003), and BEAST version 1.4.7 (Rambaut and Drummond, 2007). All sequences obtained here have been submitted to GenBank (see Online Appendix T1: www.evolutionary-ecology.com/data/2369T1.pdf).

We used χ^2-tests, as implemented in PAUP*, to determine whether the assumption of equal base frequencies among sequences was violated on all sites and parsimony-informative sites only. Modeltest version 3.7 (Posada and Crandall, 1998) was used to determine the appropriate model parameters for the maximum likelihood (ML) heuristic search and the program PhyML was used to obtain 1000 bootstrap replicates (using unique haplotypes). The ML model selected was GTR + I + Γ (–lnL = 2250.3982; substitution model: A-C = 2.7439, A-G = 19.3443, A-T = 0.9505, C-G = 1.9543, C-T = 10.8831, G-T = 1.00; I = 0.5527; Γ = 1.183; with base frequencies set to A = 0.2881, C = 0.3238, G = 0.1401, T = 0.2480; all other options in PAUP* remained as default). Distance matrices of pair-wise nucleotide sequence divergence were calculated using the ML settings, as well as uncorrected distances. Bayesian phylogenetic analyses [MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003)] were used to perform a partitioned-likelihood Bayesian search in which a separate substitution model was applied to each codon position [obtained using MrModeltest version 2.2 (Nylander, 2004)] (1st codon: 2 state model (K80 + I), ti/tv = 2.9792, I = 0.7554; 2nd codon: 1 state model (F81), base frequencies set to A = 0.2012, C = 0.2318, G = 0.1709, T = 0.3961; 3rd codon: 6 state model (GTR + Γ), substitution model: A-C = 0.5829, A-G = 19.7503, A-T = 0.1449, C-G = 0.9422, C-T = 3.9393, G-T = 1.00, Γ = 1.4544; base frequencies set to A = 0.3300, C = 0.4148, G = 0.0574, T = 0.1978). Four incrementally heated Metropolis-coupled Markov chain Monte Carlo (MCMCMC) were each run for 10,000,000 generations, sampling trees and parameters every 100 generations, with the first 100,000 generations discarded as burn-in determined from plotting log-likelihood values against generation time in TRACER 1.4 (Rambaut and Drummond, 2007). The consensus (majority-rule) tree was obtained from 180,000 trees sampled after the initial burn-in period, obtained with the *sumt* command using MrBayes, and visualized using TreeView ver. 1.6.6 (Page, 1996).

Divergence time estimates were calculated using both strict and relaxed molecular clocks in a Bayesian framework using BEAST version 1.4.7, which employs a Markov chain Monte Carlo (MCMC) to co-estimate topology, substitution rates, and node ages. The data were partitioned by codon (1st, 2nd, and 3rd). Based on the best-fit substitution models identified for the MrBayes analyses, we chose the GTR + Γ [six gamma categories, using the SRD06 Model in BEAUti version 1.4.7 (Rambaut and Drummond, 2007)] with unlinked model parameters across partitions with a Yule branching rate prior with rate variation across branches assumed to be uncorrelated and log-normally distributed (Drummond *et al.*, 2006). Four MCMC chains were each run for 10,000,000 generations (with the first 100,000 generations discarded as burn-in) and the chain was sampled every 1000 generations. TRACER version 1.4 (Rambaut and Drummond, 2007) was used to determine that the independent chains had converged on the stationary distribution and adequately sampled the probability distribution, and FigTree version 1.0 (Rambaut and Drummond, 2007) was used to visualize the consensus tree.

Strict molecular clock estimates of divergence times used previously reported evolutionary rates of 0.5–1.7%/Myr (Thacker and Hardman, 2005; Burridge *et al.*, 2006; Wang *et al.*, 2007). The substitution rate (for *Gobiomorphus*) was modelled using log-normal (mean zero offset = 16, standard deviation = 0.5) and normal (mean = 16, standard deviation = 0.5) distributions. For the relaxed molecular clock analysis, substitution rates were calibrated using the single known

fossil record (McDowall *et al.*, 2006) that gives a minimal age for the group of 16 Myr. The age of this fossil was modelled with a translated log-normal distribution and a normal distribution using the same parameters as above (to reflect a minimum age). This yields a skew consistent with the bias in the fossil record by placing a hard minimum bound (zero probability) of dates much younger than the oldest known fossil to allow for errors in dating, and a soft maximum bound (indefinite, but increasingly unlikely) for dates much older than the fossil. All analyses used a log-normal prior at the root to reflect a date greater than 20 Myr (mean zero offset = 20, standard deviation = 2.0). The fit of the different models given the data was compared using Bayes Factors (see Kass and Raftery, 1995; Suchard *et al.*, 2001) as implemented in BEAST version 1.5a (Rambaut and Drummond, 2007; see also Suchard *et al.*, 2001). Performing these analyses without data indicated that the effective and the original priors were similar and that the posterior and prior distributions were different, suggesting that the data were informative (*sensu* Drummond *et al.*, 2006).

Meristic analysis

Spines and rays were counted in the first and second dorsal fins, the anal fin, and the right pectoral fin (McDowall, 2000). An anterior spine in the second dorsal and anal spines was present in all fish, and was therefore omitted from the analyses. Differences between mean counts were tested with analysis of variance (ANOVA), and a Bonferroni adjustment of *P*-values was used to test for pair-wise differences among means. Discriminant function analysis was used to examine the ability of these meristics to define species groups. All statistical analyses were performed with SYSTAT 11.

RESULTS

MtDNA variability

All sequences were checked for open reading frames [using MacClade version 4 (Maddison and Maddison, 2000)] to check for the presence of nuclear copies or other unintended sequence types and were confirmed as being from the target DNA using the GenBank BLASTn search. In addition, all new sequences obtained in this study aligned with the relevant sequences (and species) obtained from GenBank (see Online Appendix T1: www.evolutionary-ecology.com/data/2369T1.pdf). A 366-bp fragment (122 codons) of the mtDNA (Cyt-b) was used in these analyses with no insertions or deletions detected. The nucleotide composition of all sequences was biased for C and T (A = 0.227, C = 0.295, G = 0.187, T = 0.291). Among the 161 eleotrid sequences (71 unique), there were 234 constant sites, 17 variable sites that were parsimony uninformative, and 115 parsimony informative nucleotide substitutions. We were unable to reject the hypothesis of homogeneity across base frequencies among sequences for all positions ($\chi^2_{210} = 56.77$, $P = 1.00$), for the parsimony-informative positions ($\chi^2_{210} = 206.23$, $P = 0.56$) or for third codon positions ($\chi^2_{210} = 177.81$, $P = 0.95$). The number of nucleotide substitutions between each haplotype ranged from 1 (0.2% sequence divergence) to 77 (22.4% sequence divergence) (see Online Appendix T2: www.evolutionary-ecology.com/data/2369T2.pdf).

Figure 1 shows *Philypnodon grandiceps* and *P. macrostomus* as outgroup taxa, with a well-supported (bootstrap and posterior probabilities) position for the ingroup and high support for most branches. The ingroup consisted of all nine *Gobiomorphus* species. The two species

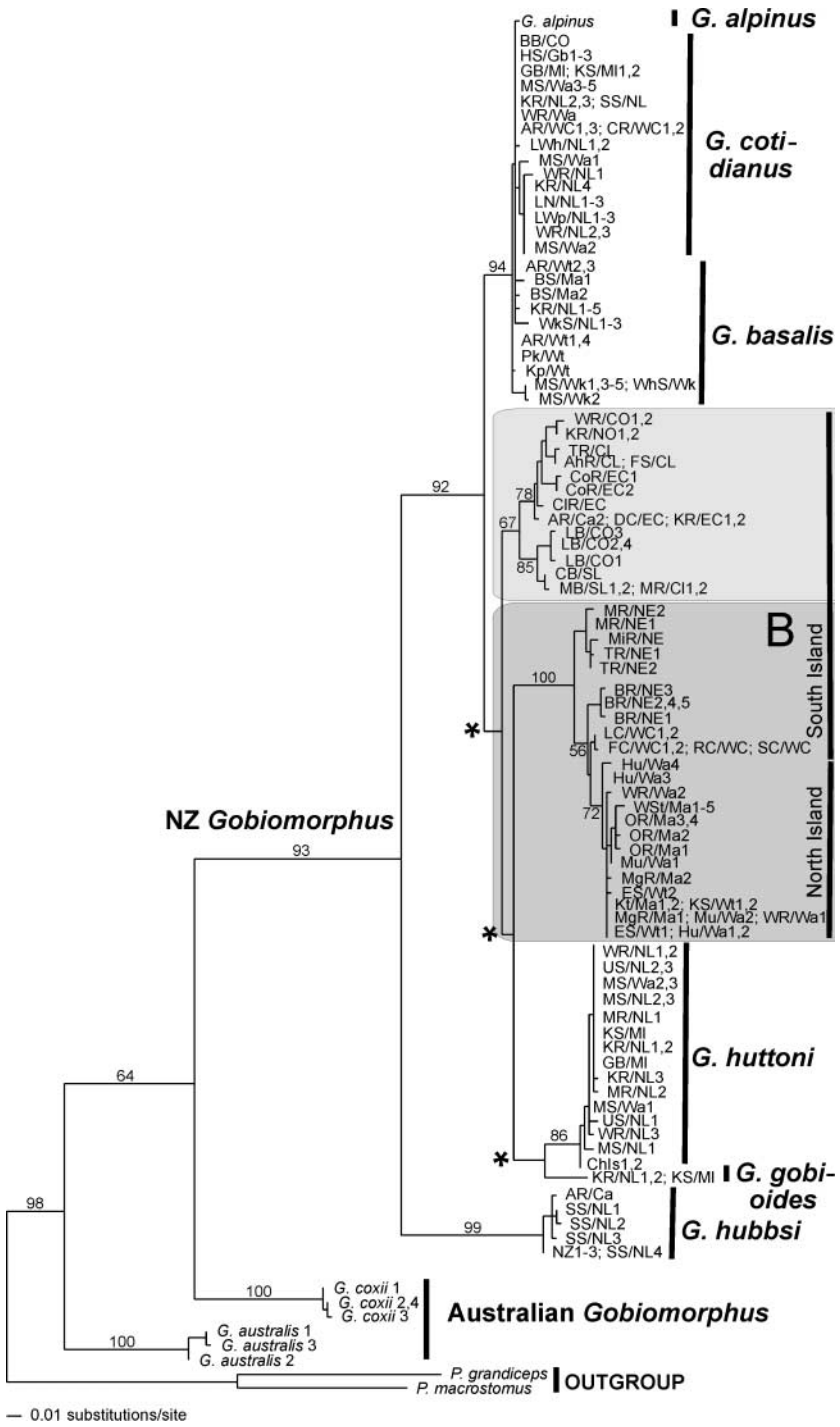
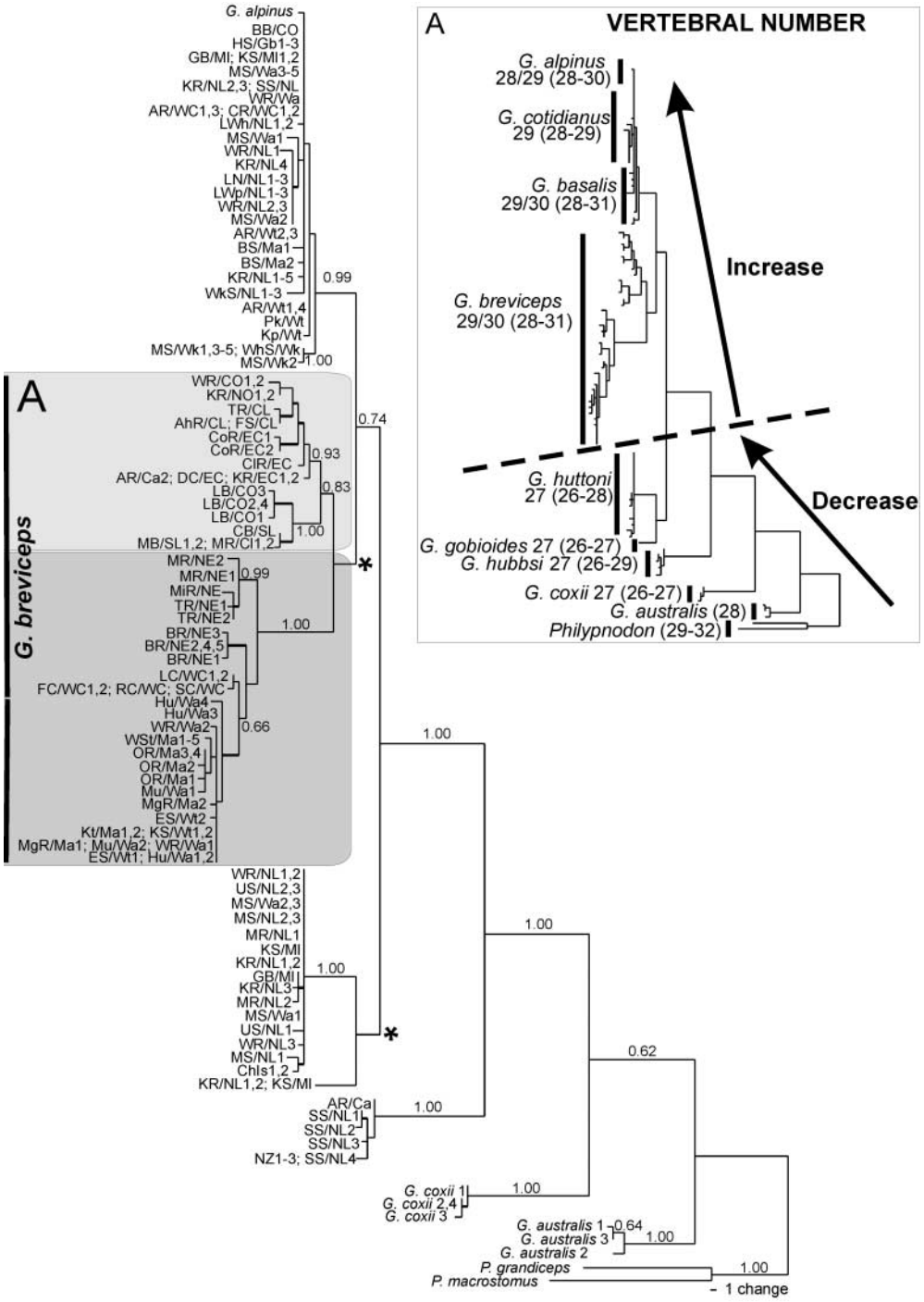


Fig. 1. Phylogenetic relationships among the seven New Zealand and two Australian *Gobiomorphus* species (with the two *Philypnodon* spp. used as outgroups) for cytochrome b gene using maximum likelihood (ML) (left) and Bayesian analyses (right). Individual codes correspond to those in Online Appendix T1 (www.evolutionary-ecology.com/data/2369T1.pdf). The grey boxes show inconsistent topology (A and B, for the two *G. breviceps* lineages) between the ML and Bayesian analyses caused by



the low support at the nodes indicated by an asterisk. ML bootstrap replicates (1000) and posterior probabilities (greater than 55% and 0.55, respectively) shown at the main nodes, or indicated by a 'bold' branch. Inset (A) maps on the Bayesian tree the change in vertebral number with species (see text and Table 1). High-resolution version: evolutionary-ecology.com/data/2369Fig.1.tif

endemic to Australia (*G. australis* and *G. coxii*) were closer to the root (*Philypnodon* spp.) of the tree with *G. coxii* as sister group to all seven endemic New Zealand species. Within the New Zealand species, *G. hubbsi* was sister to a large group containing the six remaining species in both analyses. Some internal branches with less than 50% support in the ML tree (but higher posterior probabilities for the Bayesian analyses) resulted in a conflicting position between the two divergent *G. breviceps* groups [previously identified by Smith *et al.* (2005)], which also produce an alternate position for *G. gobioides* and *G. huttoni* (Fig. 1). The topology seen in the Bayesian analyses is consistent with previous molecular data and also based on morphological relationships (McDowall, 1975). This large group was split into defined subgroups: (1) *G. gobioides* and *G. huttoni*; (2) northern South Island and all North Island *G. breviceps* (this group 'B' was not well resolved and had alternative positions between the ML and Bayesian analyses in Fig. 1), and south-eastern South Island *G. breviceps* (group 'A') (see also Smith *et al.*, 2005); and (3) a complex of *G. basalis*, *G. alpinus*, and *G. cotidianus*.

We examined the age of the New Zealand *Gobiomorphus* using strict and relaxed molecular clocks. Calculating divergence dates based on a strict molecular clock with previously reported evolutionary rates of 0.5–1.7%/Myr provides a date of 18–28 Myr for the New Zealand lineage. We examined this further using MCMC relaxed clock analyses and the oldest known fossil record (McDowall *et al.*, 2006). Although the relaxed clock had a slightly more positive (with Bayes Factor always less than 4.4) marginal likelihood (-1615.2369 ± 0.1543 and $\pm 1614.3407 \pm 0.1376$ for the log-normal and normal model, respectively, with Bayes Factor < 2) than the strict clock (-1618.6494 ± 0.1387 and -1618.4270 ± 0.1267 for the log-normal and normal model, respectively, with Bayes Factor < 1), the increase in fit between the log-normal and normal models was not important (see Kass and Raftery, 1995). Based on these analyses, we present the relaxed clock with the fossil calibration treated as a log-normal distribution (Fig. 2) as described above. The dates obtained with this analysis are similar, albeit more conservative than those calculated using the strict clock, and estimate the divergence of the New Zealand lineage of *Gobiomorphus* in New Zealand (from an ancestral lineage indistinguishable from Australian or New Zealand species) between 16 and 28 Myr ago.

Meristic analysis

Gobiomorphus cotidianus and *G. basalis* were distinguished from the other species by their greater number of spines in their anterior (first) dorsal fin (mean 6.9 and 7.3 respectively, versus means of 5.9–6.1 for other species, $P < 0.001$) (Table 1). *Gobiomorphus basalis* had a lower mean number of rays in their second dorsal and anal fins than other species except for *G. alpinus*; *G. basalis* and *G. breviceps* were distinguished by their low number of pectoral fin rays. The means of pectoral fin rays minus first dorsal fin spines accentuated the difference between *G. basalis* and *G. breviceps* (9.7 and 8.3 respectively; Bonferroni-adjusted ANOVA, $P < 0.001$) and the other species (11.7–13.3) (Table 1). *Gobiomorphus alpinus* had a mean value of 11.7 for this metric, and low means for counts of first dorsal fin spines, second dorsal fin rays, and anal fin rays compared with the other species, as clearly defined by the larger data set analysed by McDowall and Stevens (2007).

Because *G. hubbsi* and *G. huttoni* are readily identified by their coloration (McDowall, 2000), we included the five New Zealand species that are less readily identifiable in the discriminant function analysis. The resolving power of a multivariate combination of these four meristics clearly distinguished between these species (Table 2). The classification matrix from the

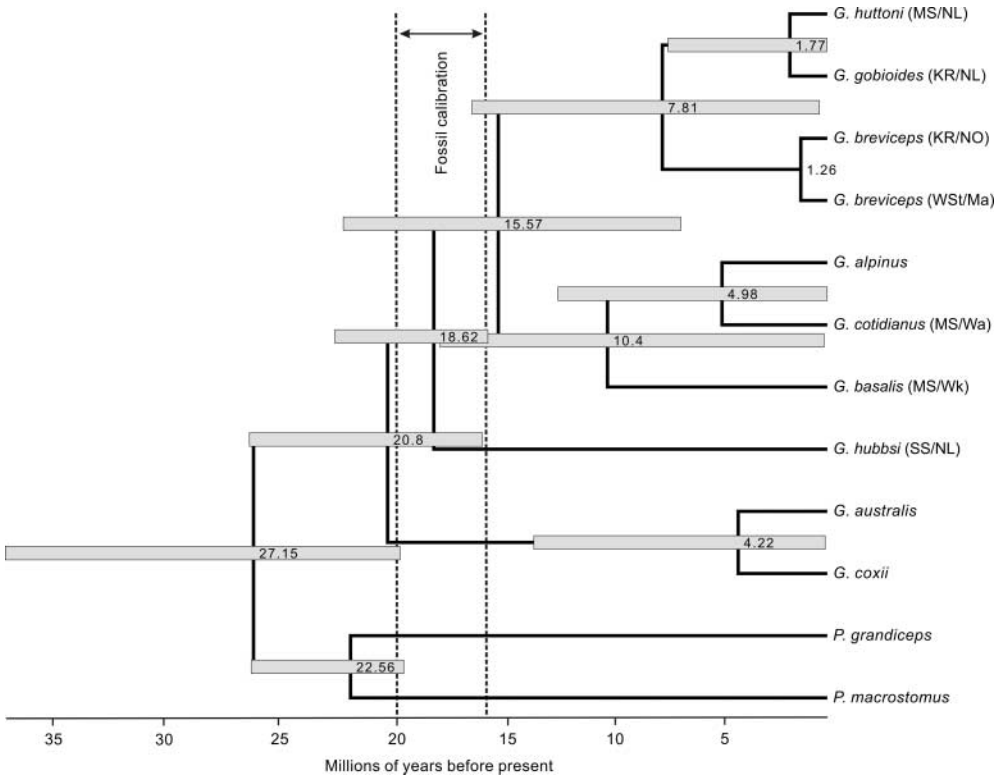


Fig. 2. Consensus of the posterior distribution of the divergence times corresponding to the mean posterior estimate of their age in millions of years (Myr) as calculated in BEAST (using log-normal priors; see Methods). The shaded bars represent the 95% highest posterior density (HPD) interval for the divergence time estimates (shown at each node). The oldest *Gobiomorphus* fossil date is indicated by the dashed lines (16 to 20 Myr). Taxa included are representative of the seven New Zealand and two Australian *Gobiomorphus* species (with the two *Philypnodon* spp. used as outgroups) (see Fig. 1). Individual locality codes correspond to those in Online Appendix T1 (www.evolutionary-ecology.com/data/2369T1.pdf). High-resolution version: evolutionary-ecology.com/data/2369Fig.2.tif

discriminant function was highly effective for discriminating *G. gobioides* and *G. breviceps*, but only 74–84% correct for *G. cotidianus*, *G. basalis*, and *G. alpinus* (Table 2). This shows that meristic determinations were partially successful in distinguishing among these morphologically similar species.

DISCUSSION

New Zealand origins of *Gobiomorphus*

The monophyly of *Gobiomorphus* was previously implied by Thacker and Hardman (2005) for two Australian and two New Zealand species, and was strongly reinforced here with all nine *Gobiomorphus* species from Australia and New Zealand. In addition, we have demonstrated that one of our outgroup taxa *Philypnodon macrostomus*, recently described

as a separate species to *P. grandiceps* on morphological characters (Hoese and Reader, 2006), is well supported by our molecular data (see also Thacker *et al.*, 2008). Within *Gobiomorphus*, we found that the Australian species *G. coxii* and *G. australis* were sister to a monophyletic group of all seven New Zealand *Gobiomorphus* (Fig. 1). These are likely to have been present in New Zealand and isolated from the Australian group for around 16–28 Myr. These dates are based on evolutionary rates of 0.5–1.7%/Myr (Thacker and Hardman, 2005; Burridge *et al.*, 2006; Wang *et al.*, 2007) as well as using relaxed clock methods (Drummond *et al.*, 2006) (Fig. 2). The oldest fossil record indicates the presence of *Gobiomorphus* in New Zealand since the early Miocene epoch (16–20 Myr ago) when New Zealand and Australia were already separated by more than 1200 km, with no evidence of *Gobiomorphus* in New Zealand before the Oligocene (McDowall *et al.*, 2006). Based on the fossil and molecular data, we suggest that the current hypothesis supports a single *Gobiomorphus* dispersal to New Zealand from Australia via oceanic dispersal across the Tasman Sea after the break-up of Gondwana [~70 Myr ago (Cooper and Millener, 1993)].

Other fish species in New Zealand have been postulated as having achieved their current distributions by means of oceanic dispersal. Molecular studies support the importance of marine dispersal in *Galaxias maculatus* (Waters and Burridge, 1999; Waters *et al.*, 2000a), as the sister relationship of Australian and New Zealand haplotypes conflicts with plate tectonics being responsible for their observed distributions (Waters *et al.*, 2000b), as we found here for *Gobiomorphus*. This is further supported by the high dispersal ability of juveniles, which have been found as far as 700 km from continental land (McDowall, 1975), and by the low mtDNA divergence identified here for the Chatham Islands *G. huttoni* population (compared with the North and South Islands), which is consistent with other molecular studies and the proposed recent emergence of the Chatham Islands above sea-level (see Stevens and Hogg, 2004; McGaughran *et al.*, 2006 and references therein). Additional evidence for oceanic dispersal comes from the recent dispersal of the Australian anguilliid (*Anguilla reinhardtii*) to northern New Zealand that was previously only known from eastern Australia, New Caledonia, Norfolk Island, and Lord Howe Island (McDowall *et al.*, 1998). Monophyly of New Zealand *Gobiomorphus* suggests that colonization from Australia occurred as a single event followed by a series of *in situ* radiations.

New Zealand radiation and evolution of non-diadromy

Gobiomorphus hubbsi is a diadromous species and forms a sister group to all other New Zealand *Gobiomorphus* species, indicating that diadromy may be an ancestral characteristic, at least for the New Zealand radiation of *Gobiomorphus* [oceanic dispersal would be likely with a marine life-cycle phase, similar to *Neochanna* (Waters and McDowall, 2005)]. The loss of diadromous behaviour has been postulated as an important mechanism in speciation (result of a decrease in gene flow, where genes are no longer transferred from one population to another), especially within *Galaxias* species (Allibone *et al.*, 1996; Burridge *et al.*, 2006). *Galaxias vulgaris* has diverged into 10 lineages, of which three species have independently derived non-migratory life histories (Waters and Wallis, 2001). The loss of diadromy also appears to have contributed to the radiation of New Zealand *Gobiomorphus* (e.g. divergence within *G. breviceps*), as well as recently derived species (e.g. *G. basalis* and *G. alpinus*) (see also McDowall and Stevens, 2007), and appears to be congruent with meristic characters (Tables 1, 2).

The New Zealand radiation of *Gobiomorphus* may have involved the diversification of diadromous species, now represented by *G. hubbsi*, *G. huttoni*, and *G. gobioides*. Around this

Table 1. Meristics for all *Gobiomorphus* spp. used in the phylogenetic tree, other than specimens from Smith *et al.* (2003, 2005)

Common name	Species	*N*	First dorsal fin spines		Second dorsal fin rays		Anal fin rays		Pectoral fin rays		Pectoral rays minus first dorsal fin rays		Vertebrae	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bluegill	*G. hubbsi*	28	6.1	0.26	9.4	0.50	9.3	0.48	19.3	0.98	13.3	1.04	27.0	0.36
Giant	*G. gobioides*	94	6.0	0.00	9.9	0.31	9.8	0.47	18.6	0.67	12.6	0.67	27.0	0.22
Redfin	*G. huttoni*	30	6.1	0.31	9.8	0.43	9.4	0.50	18.2	0.57	12.1	0.68	27.0	0.22
Upland	*G. breviceps*	65	6.1	0.49	9.4	0.56	8.2	0.61	14.6	0.75	8.3	1.40	29.4	0.56
Common	*G. cotidianus*	88	6.9	0.55	9.9	0.71	10.0	0.68	18.7	0.93	11.8	1.09	29.0	0.14
Cran's	*G. basalis*	42	7.3	0.56	9.1	0.71	8.7	1.09	17.0	0.81	9.7	0.87	29.4	0.53
Tarndale	*G. alpinus*	39	5.9	0.55	8.7	0.52	8.7	0.51	17.4	0.75	11.7	1.37	28.7	0.62

Note: Counts of second dorsal fin rays exclude the anterior spine, which was present in all specimens. Additional data calculated from McDowall (1975), McDowall and Stevens (2007), and McDowall (unpublished).

Table 2. Classification matrix of five New Zealand bullies (*Gobiomorphus* spp.) from a discrimination function analysis based on the number of first dorsal fin spines, second dorsal fin rays, anal fin rays, and pectoral fin rays

	G. gobioides	*G. breviceps*	*G. cotidianus*	*G. basalis*	*G. alpinus*	Percent correct
G. gobioides	91	0	0	0	3	97
G. breviceps	0	62	0	0	3	95
G. cotidianus	8	0	65	15	0	74
G. basalis	1	1	6	34	0	81
G. alpinus	3	0	0	3	32	84
Total	103	63	71	52	38	87

Note: Additional data provided by McDowall (unpublished).

time (or later), diadromy became facultative (such as found in *G. cotidianus*) and may have initiated further diversification of non-diadromous species. *Gobiomorphus breviceps* is clearly separated into two groups based on morphological and phylogenetic evidence (Fig. 1, Table 1). The southern group is made up of South Island populations from the eastern coast down through to Southland (group A), and includes the type locality for *G. breviceps* [Kowai River (Smith *et al.*, 2005)]. The northern *G. breviceps* group B formed by populations from North Island as well as some populations from the north-east South Island clearly form a monophyletic group (see Fig. 1). The disjunction between the two groups was suggested by Smith *et al.* (2005) to be due to Pleistocene glacial periods (1,810,000 to 11,550 years ago) where sea levels were lower than present and northern South Island was periodically connected to the lower North Island. The molecular clock calibrations used here (strict and relaxed) agree with these dates, as the two *G. breviceps* groups are likely to have been isolated for less than 2 Myr.

A sister lineage to *G. breviceps* includes a three-species complex between *G. cotidianus*, *G. basalis*, and *G. alpinus* (see Fig. 1), which was examined using morphological, ecological, and genetic characters by McDowall and Stevens (2007). These three species did show meristic differentiation (Tables 1, 2), but had the lowest between-species distances (see Online Appendix T2: www.evolutionary-ecology.com/data/2369T2.pdf), and were generally not differentiated using mtDNA, indicating a recent/incipient radiation event [for a detailed morphological and molecular discussion of these three species, see Smith *et al.* (2003) and McDowall and Stevens (2007)]. Further support for the recent speciation between *G. cotidianus*, *G. basalis*, and *G. alpinus* comes from work on *G. cotidianus* that resolved genetic distributions that correlated to meristic and reproductive traits using AFLP data (van den Heuvel *et al.*, 2007; Michel *et al.*, 2008), a technique that may be highly useful in delineating the three-species complex formed by *G. cotidianus*, *G. basalis*, and *G. alpinus* that is clearly not resolved based on mtDNA alone (McDowall and Stevens, 2007).

Understanding speciation of freshwater fish species on island systems

The loss of diadromy appears to have played an important role in the speciation of New Zealand *Gobiomorphus*. Surprisingly, there are comparable levels of sequence divergence between two morphological diadromous species (*G. huttoni* and *G. gobioides*) and within

each non-diadromous species [see Smith *et al.* (2003) for *G. alpinus*]. Furthermore, incipient speciation in *Gobiomorphus* appears in non-diadromous species (*G. alpinus*, *G. cotidianus*, and *G. basalis*), which may be diverging rapidly through directional selection accelerating morphological differentiation in these species (McDowall and Stevens, 2007).

Interestingly, all strictly diadromous species have 27 vertebrae, whereas non-diadromous species have 29 (although *G. alpinus* has 28–29 vertebrae) and *G. cotidianus* (facultatively non-diadromous) also has 29 vertebrae (see inset Fig. 1 and Table 1), indicating an increase in vertebral number that, to our knowledge, has never been documented previously in derived, non-migratory forms (see also McDowall, 2008). For *Galaxias* and common smelt (*Retropinna retropinna*), there appear to be correlations (e.g. with latitude) associated with vertebral number, in which diadromous individuals tend to have more vertebrae than non-diadromous fish (McDowall, 2003, 2008). However, comparative information is currently lacking across the Eleotridae and the developmental mechanisms controlling vertebral number in advanced perciforms have yet to be thoroughly examined (see McDowall, 2008).

Collectively, these data reveal congruent phylogenetic relationships but morphological contradictions within the genus *Gobiomorphus*. With this in mind, the challenge now is to further explore these patterns using high-resolution markers (e.g. AFLPs), candidate genes, and quantitative trait loci, to increase our understanding of the drivers underlying speciation of freshwater fish species complexes on island systems.

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