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Gene Trees versus Species Trees: Reassessing Life-History Evolution in a Freshwater Fish Radiation

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Abstract.—Mechanisms of speciation are best understood in the context of phylogenetic relationships and as such have often been inferred from single gene trees, typically those derived from mitochondrial DNA (mtDNA) markers. Recent studies, however, have noted the potential for phylogenetic discordance between gene trees and underlying species trees (e.g., due to stochastic lineage sorting, introgression, or selection). Here, we employ a variety of nuclear DNA loci to reassess evolutionary relationships within a recent freshwater fish radiation to reappraise modes of speciation. New Zealand's freshwater-limited *Galaxias vulgaris* complex is thought to have evolved from *G. brevipinnis*, a widespread migratory species that retains a plesiomorphic marine juvenile phase. A well-resolved tree, based on four mtDNA regions, previously suggested that marine migratory ability has been lost on 3 independent occasions in the evolution of this species flock (assuming that loss of diadromy is irreversible). Here, we use pseudogene (*galaxiid Numt*: 1801 bp), intron (*S7*: 903 bp), and exon (*RAG-1*: 1427 bp) markers, together with mtDNA, to reevaluate this hypothesis of parallel evolution. Interestingly, partitioned Bayesian analysis of concatenated nuclear sequences (3141 bp) and concatenated nuclear and mtDNA (4770 bp) both recover phylogenies implying a single loss of diadromy, not three parallel losses as previously inferred from mtDNA alone. This phylogenetic result is reinforced by a multilocus analysis performed using Bayesian estimation of species trees (BEST) software that estimates the posterior distribution of species trees under a coalescent model. We discuss factors that might explain the apparently misleading phylogenetic inferences generated by mtDNA. [Coalescence; Galaxiidae; incongruence; introgression; life history; mitochondrial DNA; nuclear copy; parallel evolution.]

The accurate reconstruction of phylogenetic relationships is a fundamental goal of evolutionary biologic research (Avise 2004; Felsenstein 2004). To this end, systematists have routinely employed DNA sequence data for estimation of gene trees. Even today, however, many (if not most) such studies infer evolutionary relationships on the basis of single loci, such as mitochondrial DNA (mtDNA) (e.g., Arnason et al. 2002; Ishiguro et al. 2003; Phillips and Penny 2003; Douglas et al. 2006; Pereira and Baker 2006). It could be argued that the abundance of single-locus phylogenetic studies reflects pragmatism (e.g., ease of analysis)—rather than scientific merit—as theoreticians have long recognized the potential for discordance among gene trees and among gene trees and their underlying species tree (Pamilo and Nei 1988; Wu 1991; Doyle 1992; Maddison 1997; Ballard and Rand 2005). That is, in simple terms, even if a particular gene tree is well supported, it may misrepresent the associated species phylogeny for a variety of reasons, including lineage sorting, horizontal gene transfer, selection, or unrecognized paralogy. The phylogenetic pitfalls posed by incomplete lineage sorting and hybridization, in particular, may be especially problematic for studies attempting to estimate branching order of recent and rapid radiations (e.g., Albertson et al. 1999; Takahashi et al. 2001; Kai et al. 2002; Ballard and Whitlock 2004; Pollard et al. 2006; Peters et al. 2007). Although the small effective population size of mtDNA markers relative to nuclear loci makes the former less prone to incomplete lineage sorting (Moore 1995), recent multilocus studies suggest that phylogenetic reliance on any single locus remains hazardous (Buckley et al. 2006; Maddison and Knowles 2006; Carstens and Knowles 2007; Edwards et al. 2007; Chen et al. 2009; Edwards 2009; Nadachowska and Babik 2009; Spinks and Shaffer 2009). Phylogenetic studies should, therefore, ideally aim to incorporate data from multiple loci and sample multiple individuals per taxon (Degnan and Rosenberg 2006; Maddison and Knowles 2006).

Phylogenetic analysis is a key method for inferring character state transformations associated with organismal evolution. Life-history transitions, for instance, have long been considered a significant factor underlying the diversification of a variety of taxonomic groups (e.g., Strathman 1985; McDowall 1987, 1990; Ronce 2007). To investigate the evolutionary importance of such phenomena, several recent studies have used single-locus phylogenetics to track life-history transitions putatively associated with cladogenesis (e.g., Duda and Palumbi 1999). Parallel transitions in larval history mode are inferred to have promoted cladogenesis in asteroids (Hart et al. 1997), echinoids (Wray 1996), gastropods (Duda and Palumbi 1999), and teleosts (Waters and Wallis 2001a). More broadly, transitions from dispersive to nondispersive adult behaviour are thought to have driven speciation in beetles (Emerson and Wallis 1995), fishes (Taylor et al. 1996; Taylor 1999), and birds (e.g., Trewick 1996, 1997).

The cladogenetic and ecological importance of life-history transitions may vary in different biogeographic settings. Loss of saltwater tolerance, for instance, is thought to play a particularly important role in the

formation of freshwater biotic communities, particularly those of remote oceanic regions (McDowall 2003, 2004). Such communities may be primarily composed of species recently derived from marine and/or diadromous (migratory) taxa (Lee and Bell 1999; McDowall 2003). For instance, New Zealand's (NZ's) isolated landmass is arguably equivalent to an oceanic island (Pole 1994; Campbell and Landis 2001; Waters and Craw 2006; Trewick et al. 2007), and its freshwater fish fauna is accordingly characterized by diadromous taxa—migrating between freshwater and marine systems—and their freshwater-limited derivatives (McDowall 1990, 2000). Several galaxiid fish species, for instance, live and breed in freshwater but have a 6-month marine juvenile phase (amphidromous). As a case in point, the widespread *Galaxias brevipinnis*, an upstream migrator with strong climbing ability, has "spawned off" numerous landlocked (non-diadromous) lake populations. Similar losses of diadromy are thought to have initiated speciation, as exemplified by the *G. vulgaris* complex (Waters and Wallis 2001b)—a species flock of freshwater-limited taxa—presumably derived from a *G. brevipinnis*–like diadromous ancestor (McDowall 1990). Although losses of diadromy occur commonly in galaxiids, these life-history shifts are thought to be irreversible (McDowall 1990; Waters and Wallis 2001a).

Although originally considered a single widespread taxon (McDowall 1970, 1990), the *G. vulgaris* complex was subsequently found to be speciose on the basis of fixed differences at isozyme loci (*G. anomalus*, *G. depressiceps*, *G. vulgaris*, *G.* "sp D") (Allibone and Wallis 1993; Allibone et al. 1996; McDowall and Wallis 1996), morphological differentiation (*G. pullus*, *G. eldoni*, *G. gollumoides*) (McDowall and Wallis 1996; McDowall 1997; McDowall and Chadderton 1999), and reciprocal monophyly of mtDNA lineages (all the above taxa plus *G.* "southern," *G.* "teviot," *G.* "northern") (Waters and Wallis 2001b). On the basis of a well-resolved mtDNA tree (combined control region (CR), cytochrome *b* (cyt *b*), ATPase 6, ND5, ND6; Fig. 1), it was inferred that diadromy was lost on three independent occasions during the formation of this species flock (Waters and Wallis 2001a; Fig. 1).

Given the potential shortcomings of single-locus phylogenetics, here, we employ three nuclear loci, with several individuals per taxon, to reassess the evolutionary relationships of the 10 lineages/species comprising the *G. vulgaris* complex. Specifically, we use pseudogene, intron, and exon sequences to evaluate phylogenetic relationships and test the mtDNA-derived hypothesis of parallel losses of diadromy as initiators of speciation.

Materials and Methods

Data Collection

We genetically analyzed a total of 135 fish specimens, including 119 representatives of the nonmigratory *G. vulgaris* complex (Appendix 1). Many of these samples were previously included in detailed isozyme analyses (e.g., Allibone and Wallis 1993; Allibone et al. 1996; Wallis et al. 2001), and all have been identified on the basis of mtDNA. Here, partial sequences for mtDNA CR and cyt *b*, generated using primers P4 and S-Phe (CR) and cytb-Glu and cytb-Thr (cyt *b*) (see Waters and Wallis 2001b), were employed for lineage designation. Each nonmigratory taxon was represented by multiple samples from distinct drainage systems and/or regions (Fig. 2). In addition, we included samples of the migratory taxon *G. brevipinnis* from five NZ (10 specimens) and two Tasmanian (three specimens) sites. Samples of *G. prognathus* and *G. postvectis* were included as outgroups. All fish were collected using pole-nets and electrofishing. Protocols employed for total DNA extraction, polymerase chain reaction amplification, and sequencing are as described in Waters and Wallis (2001b). In the current study, we generated and analyzed data from three nuclear loci. First, we amplified and sequenced approximately 2 kb of a large mitochondrial pseudogene (*galaxiid Numt*; see Waters and Wallis 2001b), comprising "fossil" CR and cyt *b* regions. This region was amplified with Cytb-PSGf1 (5′ ttc cgc tcc ctt cta gga 3′) and CR-Nuc-R (5′ acc act tta agg gtt tta tc 3′). Second, we amplified exon sequences of the *RAG-1* locus using primers Rag1F1 and Rag1R1 (López et al. 2004). Third, we amplified intron sequence from the *S7* ribosomal protein gene using primers S7EX1F and S7EX3R (Chow and Hazama 1998). Sequences for each locus were aligned manually (by eye) in PAUP*4.0b10 (Swofford 2003).

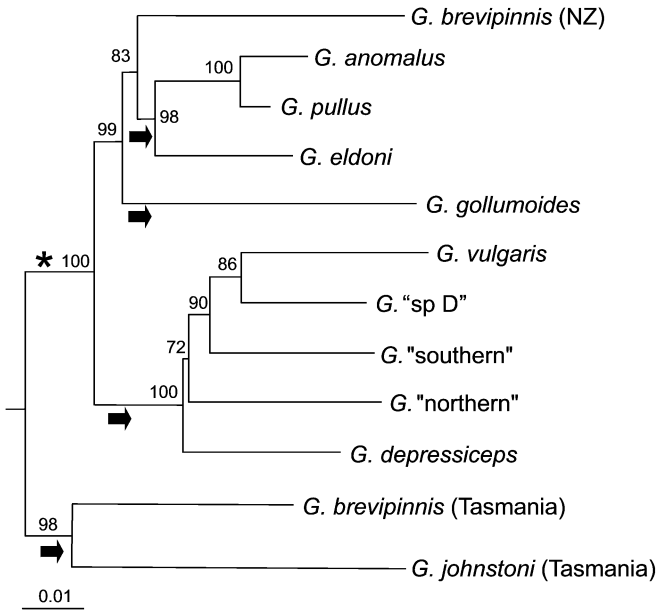

FIGURE 1. Mitochondrial DNA phylogeny of NZ's *Galaxias vulgaris* complex, redrawn from Waters and Wallis (2001b), with outgroups omitted. Numbers at nodes are bootstrap values based on ML analysis (GTR + G + I model). All taxa are freshwater limited, with the exception of *G. brevipinnis*, which retains the ancestral marine larval phase (diadromy; Tasmania, NZ). Inferred losses of diadromy—assumed to be irreversible (Waters and Wallis 2001b)—are indicated by arrows, and the monophyly of the NZ radiation is indicated by an asterisk.

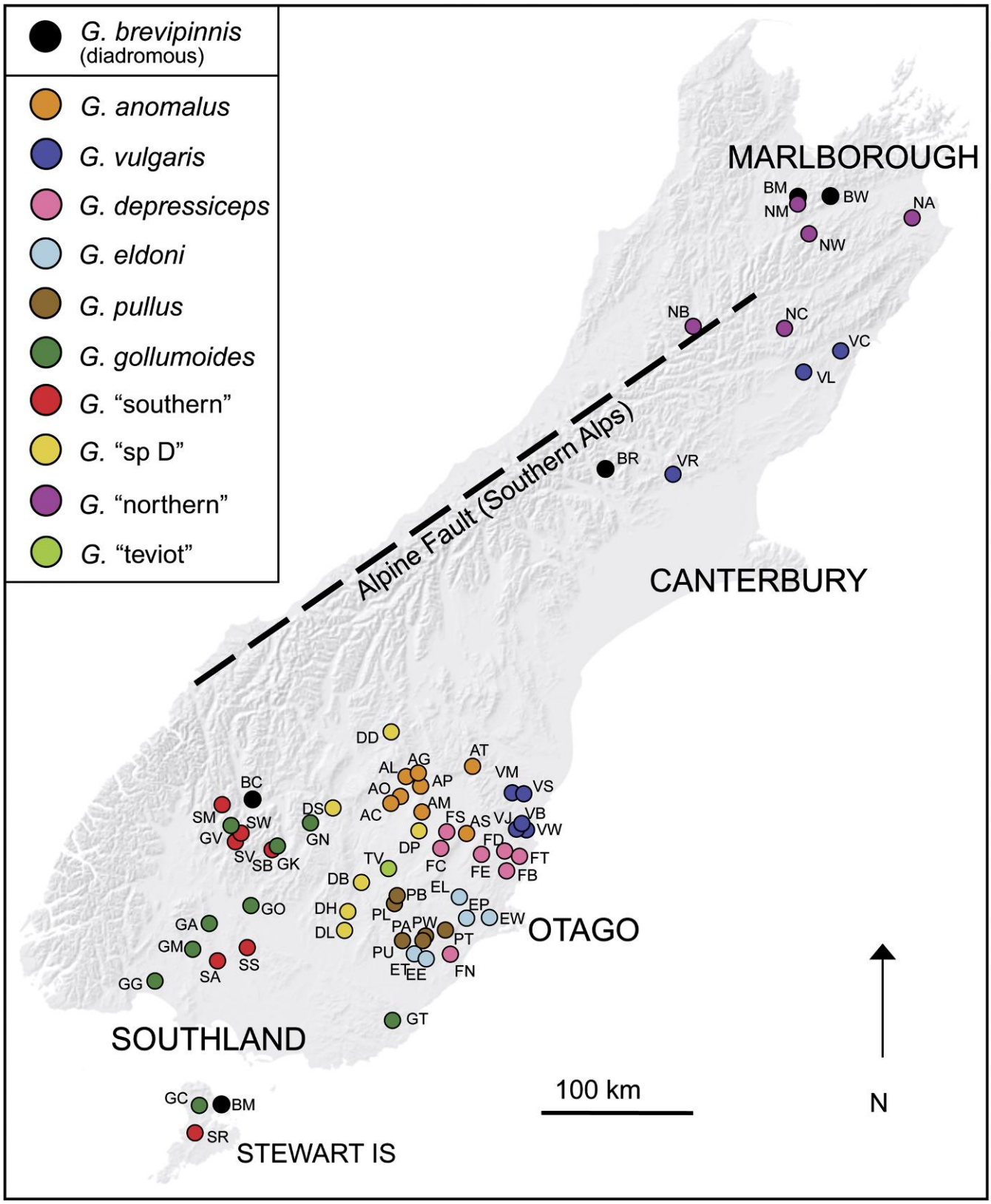

FIGURE 2. Map of South Island, NZ, showing sample sites for migratory *Galaxias brevipinnis* (black circles) and derived nonmigratory taxa (colored symbols). Identity of samples was confirmed on the basis of mtDNA data; codes are from Appendix 1.

We obtained sequences of each locus for multiple representatives of each nonmigratory taxon, with the exception of *Galaxias* "teviot," for which only one sequence (*RAG-1*) was obtained. All DNA sequences are available on GenBank (accession numbers GU269411–GU269540). Amplification results varied among loci, with high success rates for *galaxiid Numt* (122 specimens, 1801 bp alignment) and *RAG-1* sequences (117 specimens, 1427 bp alignment) but reduced success for *S7* (92 specimens, 903 bp alignment). Many specimens of *G. depressiceps*, in particular, failed to amplify for both the *galaxiid Numt* and *S7* fragments. In addition, a number of specimens sequenced successfully for the CR region of *galaxiid Numt* (sequence from primer CR-Nuc-R) but not for the cyt *b* region (sequence from primer Cytb-PSGf1). To minimize problems associated with missing data, phylogenetic analyses of concatenated nuclear loci (3141 bp alignment) were performed on a reduced suite of 82 specimens for which complete (or near-complete) sequences of *RAG-1*, *S7*, and the 3′ (CR) portion of the *galaxiid Numt* were obtained. For each analysis, we first collapsed the data set, removing redundant sequences prior to phylogenetic reconstruction.

Phylogeny Reconstruction

Phylogenetic relationships among DNA sequences were reconstructed via maximum-likelihood (ML) and Bayesian methods. Data matrices and associated trees are available from TreeBASE (study accession S2545; matrices M4865-9) (see also online Figures S1 to S5, available from http://www.sysbio.oxfordjournals.org/). ML analyses were performed using PAUP*4.0b10 (Swofford 2003), and employed 10 heuristic searches with random sequence addition, under a single substitution model selected from a set of 56 hierarchically nested candidates using the Akaike Information Criterion implemented in ModelTest 3.7 (Posada and Crandall 1998). Selected evolutionary models for the three nuclear data sets were as follows: *galaxiid Numt*: F81 + G (nst = 1; rates = gamma); *S7*: HKY (nst = 2; rates = equal); *RAG-1*: TrN + I (nst = 6, rates = inv). Bayesian phylogenetic analyses of individual loci, and of concatenated loci, were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Although we note that there can be pitfalls associated with the analysis of concatenated phylogenetic data (Kubatko and Degnan 2007), we include this approach here as a crude means of exploring phylogenetic signal across multiple loci, in addition to preferable multilocus methodologies that accommodate the separate phylogenetic histories of distinct loci (see below). Concatenated Bayesian analyses were conducted with nucleotide substitution models (see above) and parameters unlinked among data partitions. Markov chain Monte Carlo (MCMC) searches were performed with four chains of 10,000,000 generations, and trees were sampled every 100 generations. Three of the chains were heated according to "Temp = 0.1" to improve mixing. The first 10,000 trees were discarded as "burn in," based on stationarity of LnL and other parameters. Replicate Bayesian runs were performed for each data set, with convergence of split frequencies between runs confirmed using AWTY (Nylander et al. 2008).

We used multilocus sequence data (*RAG-1*, *S7*, *galaxiid Numt*, and mtDNA) to estimate the underyling species phylogeny using Bayesian estimation of species trees (BEST) version 2.2 (Liu and Pearl 2007; Liu 2008), a program based on a modified version of MrBayes 3.1.2. This Bayesian hierarchical method employs coalescent theory, incorporating multiple alleles per species, across multiple loci (Liu et al. 2008), to generate a posterior distribution of species trees. This methodology assumes that lineage sorting, rather than horizontal gene transfer, explains incongruence among loci (Liu and Pearl 2007). These species-tree estimates were carried out following the exclusion of samples that showed evidence of mtDNA introgression between geographically adjacent taxa (PW2; DH1–4; *G. anomalus;* see below). BEST analyses were conducted with nucleotide substitution models unlinked among data partitions (see above). *Galaxias postvectis* and *G. prognathus* were included as outgroups. Initial BEST MCMC analyses using two chains yielded high standard deviations in split frequencies between runs. Subsequent BEST MCMC analyses were performed using six chains, run for 20,000,000 generations, with trees sampled every 1000 generations. Of the six chains, one was cold, and five were heated with a low temperature setting (0.1) to improve precision of split frequencies (Beiko et al. 2006). mtDNA sequences were specified as haploid to account for differences in population size between nuclear versus cytoplasmic markers. Priors for mutation rate were selected to account for the substantial variation in evolutionary rates previously inferred for nuclear versus mtDNA loci in galaxiid fishes (see Waters and Wallis 2001b) (GeneMuPr = uniform (0.5, 1.5)). Analyses were conducted using unlinked priors for species topology, branch lengths, and mutation rate. A conjugate prior distribution for population size (thetapr = invgamma [3, 0.003]) was implemented to reduce computational time. Replicate BEST runs were conducted to assess convergence of species-tree topologies and other parameters. The resultant posterior distribution of species trees was summarized using the "sumt" command, generating a majority rule consensus of trees across both replicate runs. BEST analyses were repeated multiple times under alternative prior settings to assess reproducibility of species-tree estimates.

RESULTS

Galaxiid Numt *Analysis*

The 1801 bp mitochondrial pseudogene (*galaxiid Numt*) alignment included 125 variable nucleotide positions (6.9%) (ignoring indels). Sequencing of 119 ingroup samples yielded 45 unique sequences, with three

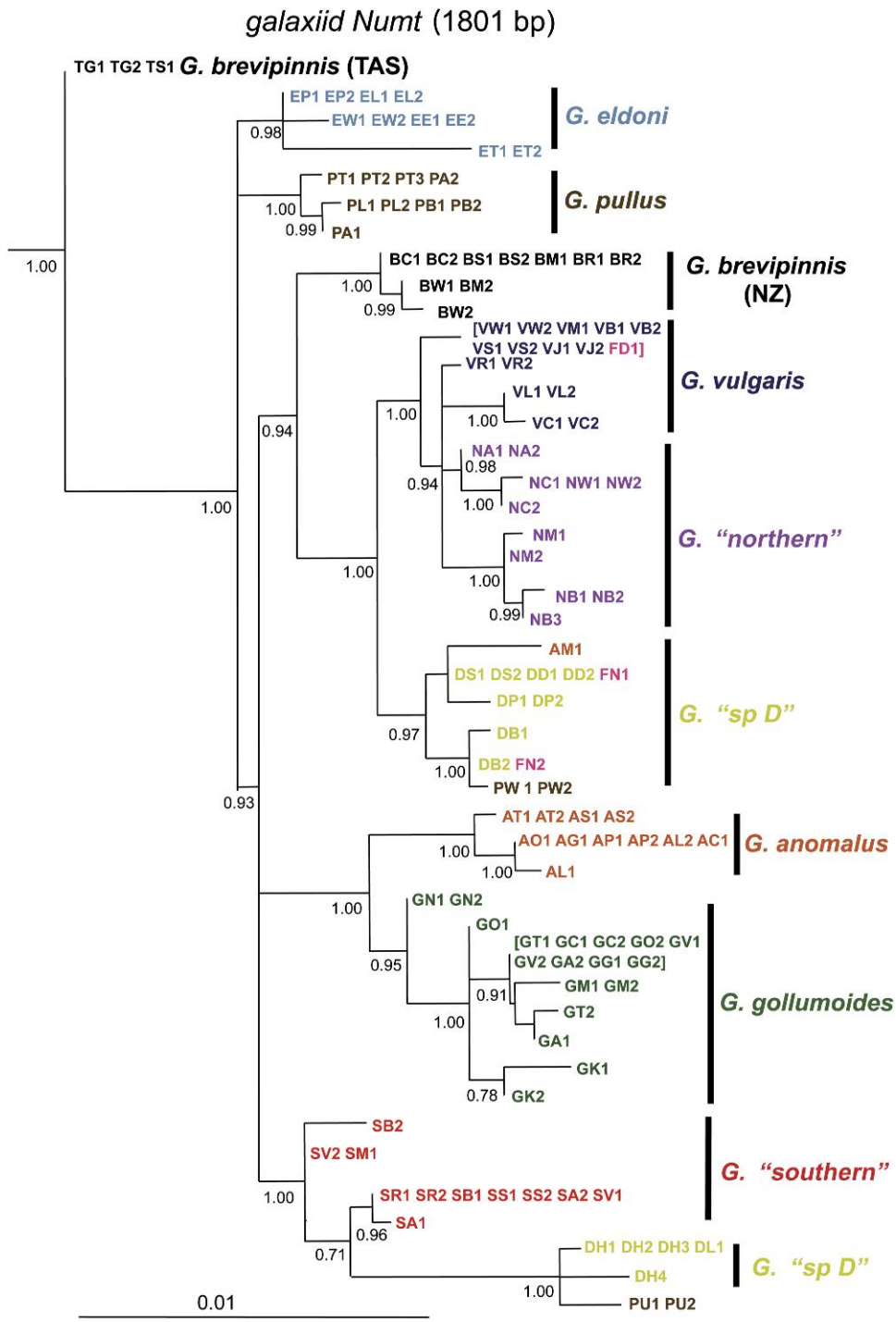

FIGURE 3. ML phylogeny of *galaxiid Numt* (pseudogene) sequences with Bayesian posterior probability values. Outgroup taxa *Galaxias postvectis* and *G. prognathus* are removed for diagrammatic purposes; color codes are from Fig. 2.

identical sequences shared across multiple taxa. Twenty indels were detected, ranging in length from 1 to 381 bp. A distinctive high-frequency deletion (106 bp) was detected in most *G.* "sp D" samples (DB1,2; DP1,2; DD1,2; DS1,2) along with 2 *G. depressiceps* (FN1, FN2) and 2 *G. pullus* samples (PW1, PW2).

ML and Bayesian analyses of *galaxiid Numt* data (Fig. 3) strongly supported the monophyly of several taxa: Tasmanian *G. brevipinnis*, NZ *G. brevipinnis*, *G. eldoni*, *G. pullus* (with the exception of samples PW1, PW2), *G. anomalus* (with the exception of sample AM1), and *G. gollumoides*. The analysis strongly supported

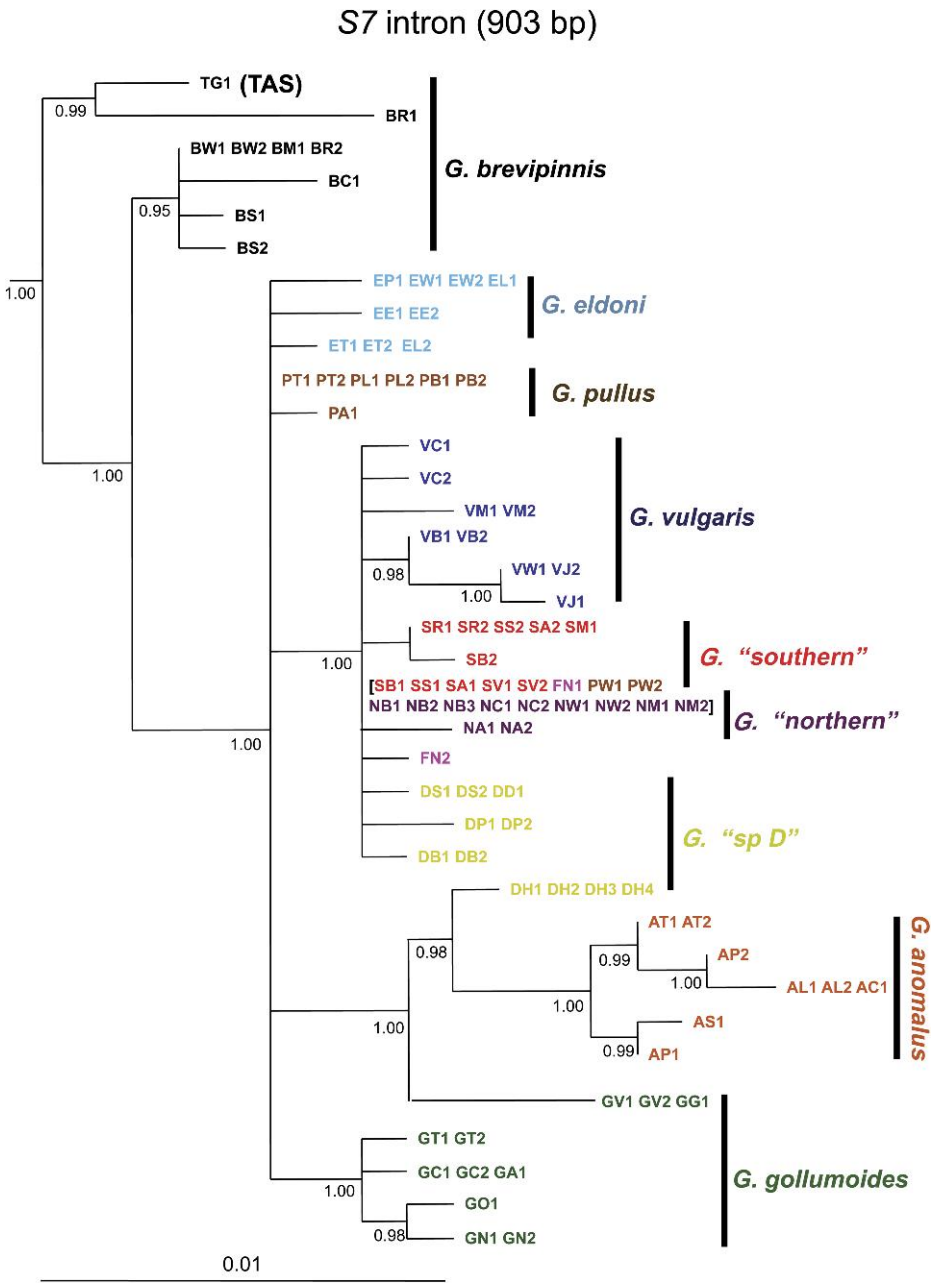

FIGURE 4. ML phylogeny of *S7* sequences with Bayesian posterior probability values. Outgroup taxa *Galaxias postvectis* and *G. prognathus* are removed for diagrammatic purposes; color codes are from Fig. 2.

the position of Tasmanian *G. brevipinnis* as sister to the NZ radiation (i.e., NZ *G. brevipinnis* plus nonmigratory taxa). We detected substantial support (0.94) for a sister relationship between NZ *G. brevipinnis* and a clade of "flathead" nonmigratory taxa (*G. vulgaris*-*G.* "northern"-*G.* "sp D"-*G. depressiceps*).

S7 Analysis

The *S7* intron alignment (903 bp) contained 85 variable sites (9.4%) (ignoring indels). Eighteen indels were detected, ranging from 1 to 9 bp in length. Sequencing of 90 ingroup samples yielded 36 unique sequences, with 1 shared across multiple mtDNA-defined lineages. Phylogenetic analysis of *S7* (Fig. 4) yielded strong support for the monophyly of the *G. vulgaris* complex (posterior probability 1.00), consistent with a single loss of diadromy. In addition, we detected strong support (1.00) for a clade of flathead nonmigratory taxa [*G. vulgaris*-*G.* "northern"-*G.* "sp D"-*G. depressiceps*]. Monophyly at *S7* was observed for only one species: *G. anomalus*.

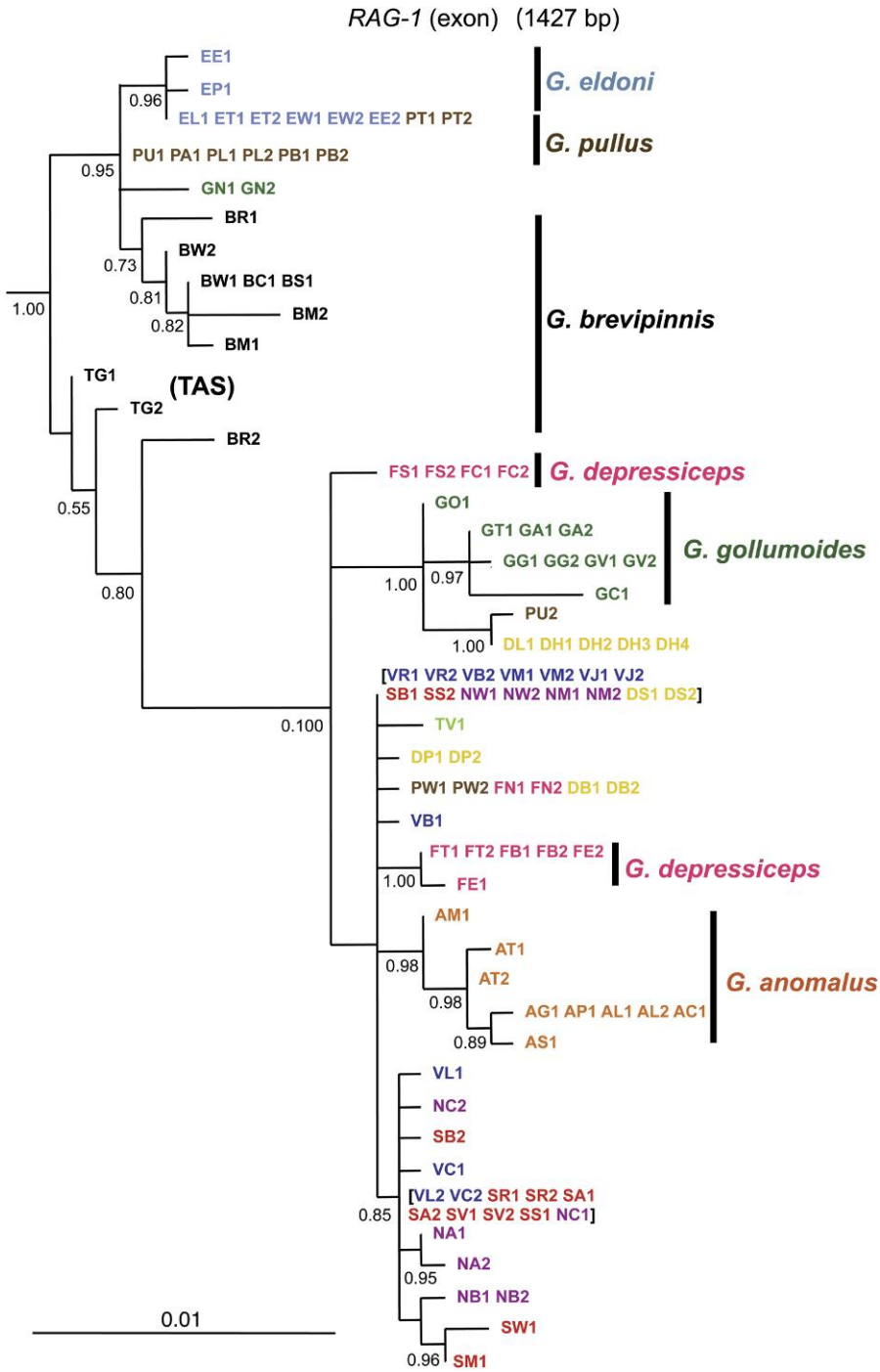

FIGURE 5. ML phylogeny of *RAG-1* sequences with Bayesian posterior probability values. Outgroup taxa *Galaxias postvectis* and *G. prognathus* are removed for diagrammatic purposes; color codes are from Fig. 2.

RAG-1 *Analysis*

The *RAG-1* exon sequences yielded a 1427 bp alignment, with no indels. Variation was detected at 90 (6.3%) of the nucleotide positions, typically transitions at third codon positions. Sequencing of 112 ingroup specimens yielded 42 unique sequences (Fig. 5), with four alleles shared across multiple taxa. Phylogenetic analysis of *RAG-1* yielded support (0.95) for a clade comprising samples of NZ *G. brevipinnis* and two nonmigratory taxa (*G. eldoni*, *G. pullus*) in an unresolved polytomy (Fig. 5). All other nonmigratory taxa formed a distinct monophyletic group (1.00; Fig. 5). Only one

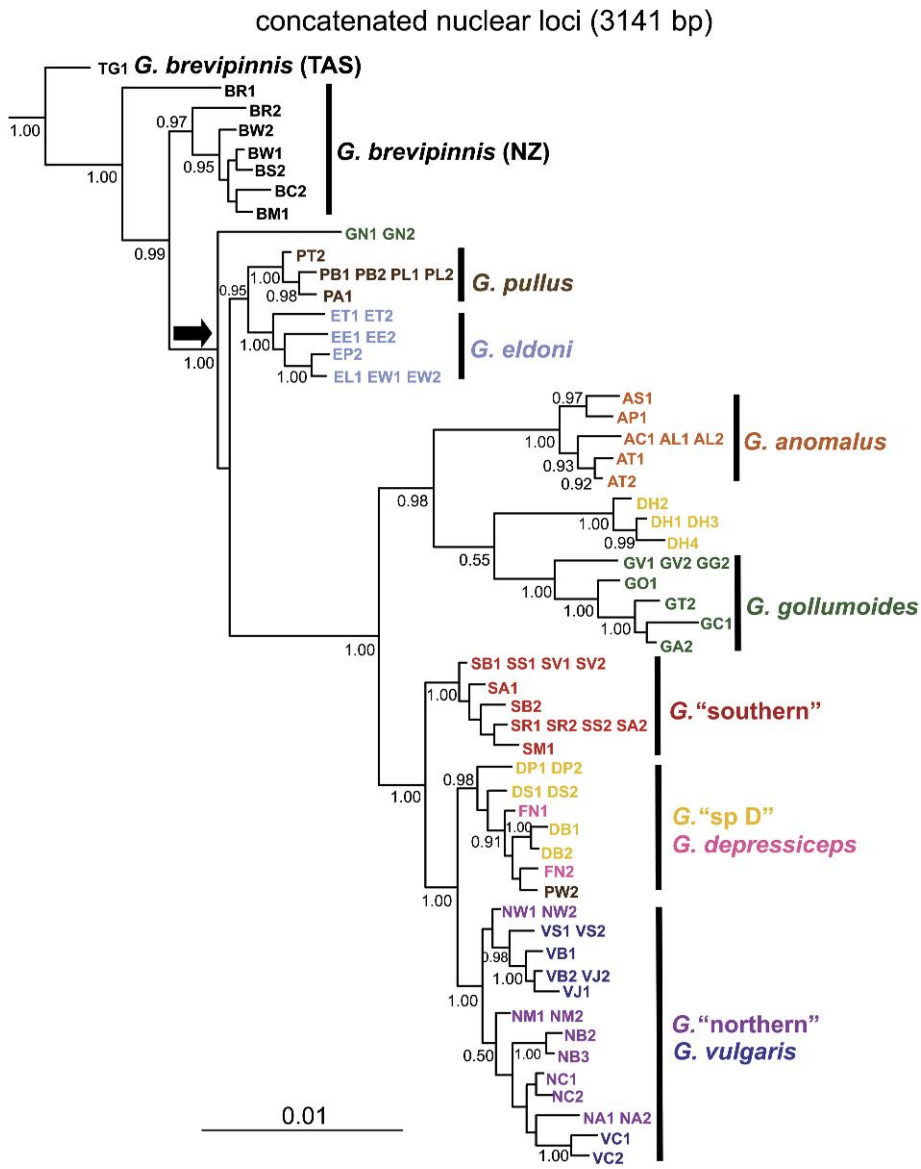

FIGURE 6. Combined nuclear DNA phylogeny based on partitioned Bayesian analysis of combined *galaxiid Numt*, *S7*, and *RAG-1* data sets. Outgroup taxa *Galaxias postvectis* and *G. prognathus* are removed for diagrammatic purposes; color codes are from Fig. 2. An arrow represents the inferred loss of diadromy under the assumption that this character change is irreversible.

nonmigratory species (*G. anomalus*) exhibited monophyly for *RAG-1*

Multilocus Phylogenetic Analyses

A concatenated Bayesian nuclear DNA phylogeny (Fig. 6) strongly supported the combined monophyly of NZ's *G. vulgaris* complex (posterior probability 1.00), consistent with a single loss of diadromy during the evolution this freshwater radiation (Fig. 6). Combined nuclear DNA analyses also supported the reciprocal monophyly of several nonmigratory taxa (Fig. 6): *G. eldoni*, *G. anomalus*, *G.* "southern." Several additional taxa (e.g., *G. pullus* and *G. gollumoides*) were supported as monophyletic for the majority of samples. However, the multilocus nuclear analyses (Figs. 3–6) also revealed three cases of consistent incongruence between mtDNA and nuclear data. Based on geographic sampling of populations, we suggest that these cases of discordance likely reflect introgression. First, *G. pullus* was monophyletic with the exception of some Waitahuna River samples (PW1–2) that consistently grouped with geographically proximate flathead samples such as *G.* "sp D" (DB1–2). Second, Pomahaka River fish (DL1, DH1–4) classified as *G.* sp D based on mtDNA were genetically distinct from other *G.* "sp D" at all three nuclear loci. Third, despite their surprisingly close sister relationship for mtDNA (Waters and Wallis 2001a, 2001b), *G. anomalus* and *G. pullus* were not closely related at

any nuclear locus. It should be noted that these morphologically dissimilar species co-occur in the Clutha River system, implying historical opportunity for introgression. Indeed, we infer that an ancient mitochondrial replacement event (e.g., Irwin et al. 2009) likely occurred for *G. anomalus*. Ideally, such inferences of gene flow across species boundaries should be based on quantitative rather than qualitative analyses. Although we can provide no quantitative estimates of gene flow between these taxa, we have previously shown strong evidence for localized hybridization between some sympatric galaxiid species pairs (Allibone et al. 1996; Esa et al. 2000). Importantly, the failure to identify such cases of hybridization would compromise the BEST-derived estimates of species phylogeny (Liu and Pearl 2007) as these coalescent analyses cannot accommodate introgression. As a result, subsequent phylogenetic analyses based on all loci were conducted with the exclusion of putatively introgressed mtDNA alleles.

The combined monophyly of NZ's *G. vulgaris* complex (posterior probability 1.00) remained well supported by concatenated multilocus data when mtDNA sequence data were also included (Fig. 7, posterior probability 1.00). In addition, the estimated species phylogeny based on BEST analysis (Fig. 8) provided further support for the monophyly of this freshwater radiation (posterior probability 1.00). Interestingly, BEST analysis provided strong support for the combined monophyly of NZ and Tasmanian *G. brevipinnis* (posterior probability 0.99; Fig. 8), a relationship consistent with existing taxonomy but inconsistent with other multilocus phylogenetic analyses (Figs. 6 and 7).

Discussion

Phylogenetic Placement of G. brevipinnis

The phylogenetic placement of migratory NZ *G. brevipinnis* as sister to all nonmigratory members of the *G. vulgaris* complex, consistently recovered from multilocus analyses (Figs. 6–8), implies a single loss of diadromy in the evolution of NZ's *G. vulgaris* complex. Although we note that concatenated phylogenetic analyses can yield spurious conclusions (Kubatko and Degnan 2007), it is important to note that the monophyly of the *G. vulgaris* complex is strongly supported by a species-tree analysis that accommodates the distinct histories of different loci (Fig. 8). This result contradicts the previous hypothesis of parallel evolution (i.e., multiple independent losses of diadromy) during the evolution of the *G. vulgaris* species flock based on an interior phylogenetic position for *G. brevipinnis* mtDNA (Fig. 1). Explaining these conflicting phylogenetic placements of *G. brevipinnis* is key to interpreting the evolution of this freshwater fish radiation.

On the basis of ecological, morphological, and taxonomic relationships (McDowall 1970, 1990), a single loss of diadromy (i.e., a monophyletic origin; Fig. 7) is the simplest evolutionary scenario for the *G. vulgaris* complex. With the exception of the previously published mtDNA analysis (Fig. 1), it is now clear that combined genetic data overwhelmingly support a single loss of diadromy. There are several factors that could potentially explain the apparently misleading results previously obtained from mtDNA. First, this incongruence could reflect stochasticity associated with the coalescent process (Beiko et al. 2006; Maddison and Knowles 2006; Carstens and Knowles 2007; Edwards et al. 2007; Koblmüller et al. 2007; Liu and Pearl 2007), although retention of ancestral polymorphism would be unlikely to bias estimates of phylogenetic relationships of a relatively deep mtDNA lineage (e.g., *G. brevipinnis*)—especially given the small effective population size for this marker and the lengthy timescale involved. Alternatively, the incongruence could stem from introgressive hybridization among taxa (e.g., Buckley et al. 2006; Peters et al. 2007; Chen et al. 2009; Nadachowska and Babik 2009; Spinks and Shaffer 2009). For instance, ancient mitochondrial introgression between *G. brevipinnis* and a nonmigratory lineage (e.g., *G. eldoni*) could explain the internal phylogenetic placement of NZ *G. brevipinnis* mtDNA (Fig. 1). We note that *G. brevipinnis* (including both landlocked and diadromous populations) has often been collected in close proximity to populations of the nonmigratory *G. vulgaris* complex (McDowall 1990), implying physical opportunity for introgression (although these diadromous and non-diadromous galaxiid taxa have distinct spawning periods; McDowall 1990). Certainly, there is ample evidence for hybridization between nonmigratory lineages (Allibone et al. 1996; Esa et al. 2000). Third, it is possible that selective forces have undermined the phylogenetic accuracy of mtDNA in this case. Regardless of the underlying mechanism, based on the combined evidence of morphological and genetic data, it seems clear that multilocus data (Figs. 6–8), rather than mtDNA, most likely reflect the true species phylogeny.

Phylogenetic Relationships Among Nonmigratory Lineages

Concordance among gene trees is likely to reflect a common history: that is, their link to an underlying species phylogeny (Liu and Pearl 2007). Consistent with this view, the current study does provide some evidence of broad congruence between mtDNA and nuclear gene trees. For instance, a number of taxa characterized by reciprocally monophyletic mtDNA lineages (Waters and Wallis 2001a, 2001b) are also reliably diagnosable for nuclear DNA (e.g., *G. eldoni*, *G. anomalus*, *G.* "southern;" Fig. 6). In addition, some deeper phylogenetic relationships are also consistent across these nuclear and cytoplasmic markers (e.g., the strong support for [*G. vulgaris*-*G. depressiceps*-*G.* "sp D"-*G.* "southern"-*G.* "northern"]; Figs. 1, 6–8). On the other hand, we report three cases of nuclear versus cytoplasmic incongruence, consistent across all three nuclear loci. Importantly, each of these cases of consistent phylogenetic discordance between mtDNA and nuclear loci involves taxa that are

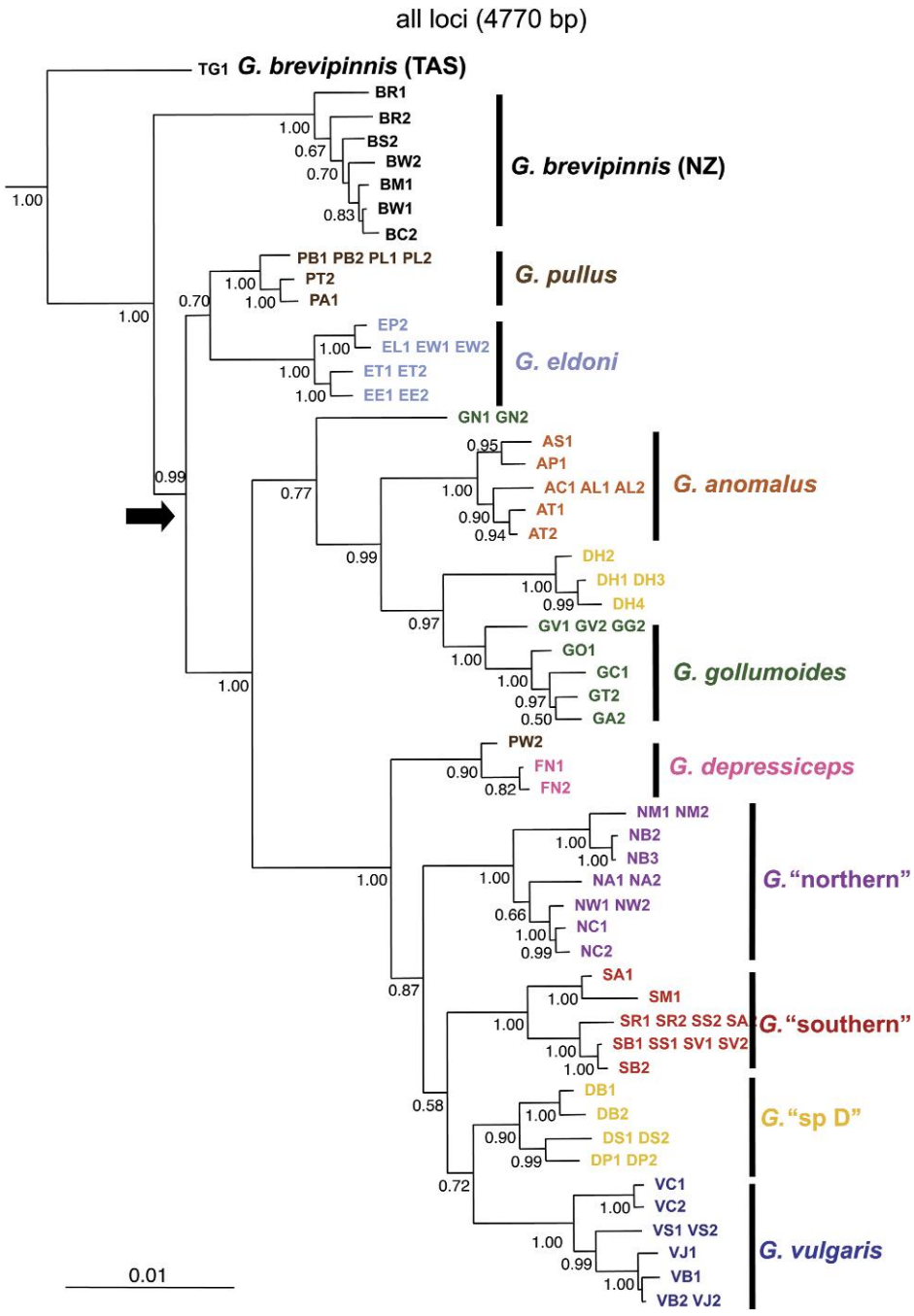

FIGURE 7. Combined DNA phylogeny based on partitioned Bayesian analysis of combined *galaxiid Numt*, *S7*, and *RAG-1* and mtDNA data sets. Outgroup taxa *Galaxias postvectis* and *G. prognathus* are removed for diagrammatic purposes; color codes are from Fig. 2. An arrow represents the inferred loss of diadromy under the assumption that this character change is irreversible.

geographically proximate, and all three cases therefore seem likely to reflect mtDNA introgression.

In contrast to the mtDNA findings of Waters and Wallis (2001a, 2001b), multilocus nuclear DNA analyses strongly suggest that *G. anomalus* is sister to *G. gollumoides* (and unrelated to *G. pullus*; Fig. 6). It is pertinent to note here that Southland populations of *G. gollumoides* were also previously considered to be *G. anomalus* on the basis of their distinctive "roundhead" morphology and mid-dorsal discontinuity in coloration (McDowall and Wallis 1996). Therefore, the nuclear DNA affinity of *G. anomalus* and *G. gollumoides* suggests that their morphological similarity is indeed a synapomorphic feature rather than a case of morphological convergence (Waters and Wallis 2001a, 2001b). We conclude that the mtDNA similarity of *G. anomalus* and *G. pullus* is best explained by historical introgression of *G. pullus* mtDNA into *G. anomalus*.

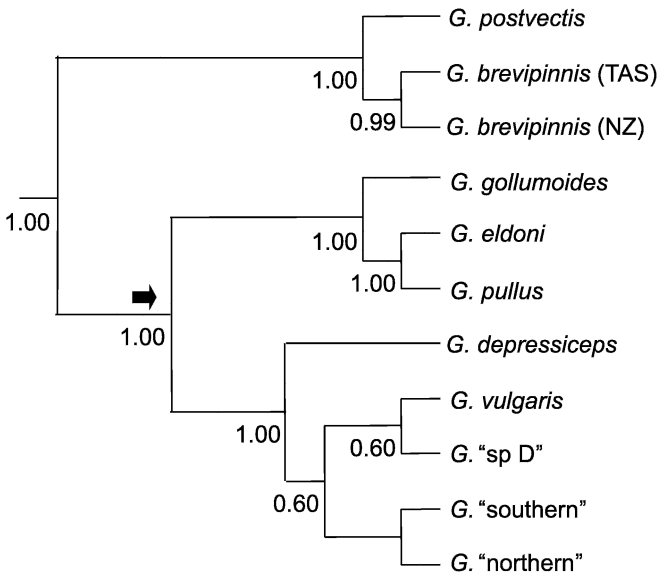

FIGURE 8. Majority rule consensus of the posterior distribution of *Galaxias* species trees estimated across all three nuclear loci and mtDNA using BEST. Outgroup taxon *Galaxias prognathus* is removed for diagrammatic purposes. Numbers at nodes are posterior probabilities, and an arrow represents the inferred loss of diadromy under the assumption that this character change is irreversible.

The finding that Pomahaka River fish (DL1, DH1–4)—classified as *G.* "sp D" on the basis of mtDNA—cluster with distinct taxa at all three nuclear loci implies that they are introgressed for mtDNA. These Pomahaka River fish also stand apart from other *G.* sp D (typically flathead morphotypes) in terms of their unusual roundhead morphology. We infer an ancient mtDNA introgression event involving geographically proximate flathead (*G.* "sp D") and roundhead lineages (e.g., lower Clutha River *G. gollumoides*).

In contrast to the above likely cases of mtDNA introgression involving geographically adjacent taxa, the general absence of reciprocal monophyly for *G. vulgaris*, *G.* "northern," *G.* "sp D," and *G. depressiceps* may simply reflect incomplete lineage sorting. This inference is based on the consistently shallow genealogies (i.e., lack of distinct clades) associated with these taxa at all nuclear loci (Figs. 3–6). Although it might be tempting to argue—on the basis of nuclear polyphyly—that these lineages are therefore unworthy of taxonomic recognition, we note that *G. vulgaris*, *G.* "sp D," and *G. depressiceps* are diagnosable by both morphology and fixed differences at multiple isozyme loci (McDowall and Wallis 1996). In addition, although only two of the taxa have been formally described, all four are associated with reciprocally monophyletic mtDNA lineages (Waters and Wallis 2001b). Indeed, polyphyly at nuclear loci is to be expected for recently evolved species (e.g., Palumbi et al. 2001; Hudson and Turelli 2003). More broadly, it should be noted that incomplete lineage sorting is hardly a rare biologic phenomenon, even for relatively rapid-coalescing mtDNA: A recent meta-analysis suggests that roughly one-quarter of animal species, for instance, may be polyphyletic for mtDNA (Funk and Omland 2003).

Evolutionary Importance of Landlocking

The combined evidence of three nuclear loci, analyzed together with mtDNA, implies that diadromy has been lost only once in the evolution of NZ's *G. vulgaris* complex. Although this finding overturns that of our previous study (Waters and Wallis 2001a), it is consistent with a priori expectations based on morphological data (McDowall 1970) and is certainly the most straightforward hypothesis in terms of life-history evolution. The finding is also more consistent with the absence of the *G. vulgaris* complex from NZ's North Island. The outcome of the current study therefore serves as a warning to biologists who derive evolutionary conclusions from phylogenetic results at any single genetic locus. Moreover, we reiterate recent calls for multilocus coalescent approaches to become routine in studies seeking to resolve the branching order of recent radiations (Maddison and Knowles 2006; Carstens and Knowles 2007; Liu and Pearl 2007; Edwards 2009).

Transitions from diadromous to freshwater-limited life history have long been thought to promote freshwater fish speciation (McDowall 1970, 1990; Taylor et al. 1996; Taylor 1999). Although the current nuclear phylogenetic study suggests that parallel life-history evolution has not driven the evolution of NZ's *G. vulgaris* complex, this is not to say that loss of diadromy is unimportant in the evolution of galaxiid fishes. Indeed, *G. brevipinnis* has produced parallel freshwater radiations in NZ (*G. vulgaris* complex), Tasmania (*G. johnstoni*, *G. pedderensis*, *G. fontanus*; McDowall and Frankenberg 1981; Waters et al. 2000; Waters and Wallis 2001a), and New Caledonia (*Nesogalaxias neocaledonicus*; Waters et al. 2000). In addition, the diadromous Australian *G. truttaceus* has given rise to non-diadromous *G. tanycephalus* and *G. auratus* in Tasmania (Ovenden and White 1990; Ovenden et al. 1993); whereas diadromous *G. maculatus* has produced parallel radiations of non-diadromous taxa both in NZ (*G. gracilis* complex; McDowall 1990) and in Australia (*G. occidentalis*, *G. rostratus*; McDowall and Frankenberg 1981). Overall, therefore, it is clear that parallel losses of diadromy underly speciation of galaxiid fishes in a broader context.

Fish Numts

A recent study by Venkatesh et al. (2006) highlighted the scarcity of mitochondrial pseudogenes (Numts: see Bensasson et al. 2001) in fish genomes, suggesting "it is likely that teleost fishes as a group are impervious to the transfer of mtDNA to the nuclear DNA." Furthermore, Venkatesh et al. (2006) concluded that putative Numts reported for the fugu genome (Antunes and Ramos 2005) were simply artefacts of genome misassembly. These authors proceeded to question the presence of Numts in *Danio* and *Tetraodon*. If Venkatesh et al.

(2006) are correct about the scarcity of Numts in fish genomes, then the presence of a substantial mitochondrial pseudogene in *Galaxias* seems noteworthy. Up until 2004, the only reported fish Numts were from *Galaxias* (ATPase 6 pseudogene: Waters and Wallis 2001b; CR pseudogene: Waters and Wallis 2001a). Here, however, we show that the CR Numt is a relatively extensive region of fossil mtDNA-like sequence (including cyt *b*: *galaxiid Numt*), and we thus speculate that the entire galaxiid mitochondrial genome may have been transposed into the nucleus of an ancestral *Galaxias* species. The simple model of sequence evolution (F81: nst = 1) favored for the *galaxiid Numt* is consistent with the suggestion that such pseudogenes are essentially free of selective constraint ("dead on arrival," Graur et al. 1989; Bensasson et al. 2001).

Supplementary Material

Supplementary material can be found at: http://www.sysbio.oxfordjournals.org/.

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APPENDIX 1. Details of ingroup samples included in genetic analyses, including number (n) of specimens sequenced per locality

Species	Code	*n*	Location	Drainage
Galaxias brevipinnis (Tas)	TG	2	Great Lake	Tamar R
	TS	1	Snug Falls	Snug R
G. brevipinnis (NZ)	BW	2	Lake Chalice	Wairau R
	BC	2	12 Mile St	Clutha R
	BS	2	Maori R	Stewart Is
	BM	2	Motueka Gorge	Motueka R
	BR	2	Ryton R	Rakaia R
G. vulgaris	VR	2	Rubicon R	Waimakariri R
	VL	2	Lottery R	Waiau R
	VC	2	Charwell R	Conway R
	VW	2	Main channel	Waianakarua R
	VM	1	Maerewhenua R	Waitaki R
	VB	2	Middle Branch	Waianakarua R
	VS	2	Sth Maerewhenua R	Waitaki R
	VJ	2	Jimmy Ck	Waianakarua R
G. anomalus	AO	1	Ophir	Clutha R
	AT	2	Timber Ck	Taieri R
	AS	2	Scrub Burn	Taieri R
	AM	1	Maori Ck	Clutha R
	AG	1	Long Gully Ck	Clutha R
	AP	2	Spains Ck	Clutha R
	AL	2	Lauder Ck	Clutha R
	AC	1	Chatto Ck	Clutha R
G. eldoni	EL	2	Lee St	Taieri R
	EP	2	Post Office Ck	Taieri R
	EW	2	Whare Ck	Taieri R
	ET	2	West Branch	Tokomairiro R
	EE	2	East Branch	Tokomairiro R
G."sp D"	DL	1	Pomahaka R	Clutha R
	DS	2	Shepherds Ck	Clutha R
	DP	2	Poolburn	Clutha R
	DH	4	Heriot Burn	Clutha R
	DB	2	Benger Burn	Clutha R
	DD	2	Dip Ck	Clutha R
G."southern"	SR	2	Rakeahua	Stewart Is
	SB	2	Bushy Ck	Mataura R
	SS	2	Stag Stream	Oreti R
	SA	2	tributary	Aparima R
	SV	2	South Von R	Clutha R
	SW	2	White Burn	Clutha R
	SM	1	Mararoa R	Waiau R
G. depressiceps	FN	2	Narrowdale St	Taieri R
	FS	2	Sow Burn	Taieri R
	FC	2	Cambridge Ck	Taieri R
	FE	2	Emerald St	Taieri R
	FB	2	Back Ck	Waikouaiti R
	FT	2	tributary	Shag R
	FD	1	Deepdell St	Shag R
G."teviot"	TV	1	Teviot R	Clutha R
G. gollumoides	GT	2	Tarwood St	Catlins R
	GC	2	Freshwater R	Stewart Is
	GK	2	Allen Ck trib	Mataura R
	GO	2	Ellis Rd	Oreti R
	GV	2	North Von R	Clutha R
	GN	2	Nevis R	Clutha R
	GA	2	tributary	Aparima R
	GG	2	Grove Burn	Waiau R
	GM	2	Morley R	Waiau R
G. pullus	PT	3	Waipori R	Taieri R
	PW	2	Waitahuna R	Clutha R
	PL	2	Little Beaumont St	Clutha R
	PB	2	Little Beaumont St	Clutha R
	PA	2	Waitahuna R	Clutha R
	PU	2	Tuapeka R	Clutha R
G."northern"	NA	2	Blairich St	Awatere
	NB	3	Maruia R	Buller R
	NC	2	Acheron R	Clarence R
	NW	2	Leatham R	Wairau R
	NM	2	Motueka Gorge	Motueka R