

Ú@^|[*^}^cá&Áæ~, }ácá^•Á[~Ác@^ÁP^, ÁZ^æ|æ}áÁà|~^Áã~&\ÁÇ *Hymenolaimus malacorhynchos*)

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Abstract We investigate the phylogenetic affinities of the New Zealand blue duck (*Hymenolaimus malacorhynchos*), a riverine specialist of uncertain relationships, using 2613 bp of DNA sequence data from 3 mitochondrial genes. *Hymenolaimus* has variously been considered an aberrant *Anas* species, or an ancient taxa in the tribe Anatini. Presently, it is placed in a highly-derived clade (Tribe Merganettini) with the shelducks. Our findings show that *Hymenolaimus* forms a monophyletic clade, and does not fit within any of the other duck tribes around the world. Our study also confirms convergent evolution among duck species that inhabit fast flowing rivers.

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Keywords blue duck; Anseriformes; cytochrome *b* gene; control region gene; ND2 gene; mitochondrial DNA; phylogenetics

INTRODUCTION

A number of phylogenies for the Anatidae have been proposed (e.g., Delacour & Mayr 1945; Del Hoyo *et al.* 1992; Livezey 1986, 1997) and a consensus appears to be emerging (e.g., Donne-Goussé *et al.* 2002; Gonzalez *et al.* 2009; Bulgarella *et al.* 2010), yet uncertainty remains about the affinities of some monotypic genera. The endangered blue duck (*Hymenolaimus malacorhynchos*) of New Zealand, an endemic riverine specialist (Marchant & Higgins 1990; Robertson *et al.* 2007), is one such taxa of uncertain placement. *Hymenolaimus* inhabits

mountain rivers and shares ecological adaptations in common with other river ducks (e.g., torrent duck *Ź* “*Ź*” and Salavadori’s duck *Salvadorinia waigiensis*), which has contributed to speculation on the shared phylogenetic affinities of these species (see Kear 2005). Delacour and Mayr (1945), however, refuted any suggestion that blue ducks were similar to either *Ź* “*Ź*” or *Salvadorinia*.

Studies of morphology, behaviour and DNA have consistently placed *Hymenolaimus* in the Family Anatidae (Gill *et al.* 2010). However, the taxon has variously been suggested to be: (i) an aberrant *Anas* species in the dabbling ducks (Tribe Anatini: *sensu* Delacour & Mayr 1945); (ii) an ancient Anatini with no close relatives due to affinities with both the perching ducks (Tribe Cairinini) and

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the Tribe Anatini (Kear 1972; Kear 2005); or (iii) a member of a highly derived clade, along with *Tachyeres* (steamer-ducks) and *Zonotrichia* within the shelducks (Tribe Tadornini, subtribe Merganettae: Livezey 1986). The presently accepted taxonomy (e.g., Kear 2005) has *Hymenolaimus* placed in a clade with the shelducks (Tribe Merganettini: *sensu* Livezey 1997). To date, the only phylogenetic analysis of *Hymenolaimus* involved the species as an outgroup species in an investigation of South American duck genera (Bulgarella *et al.* 2010). *Hymenolaimus* was placed in a clade with an Australian (*Hymenolaimus jubata*) and an Old World/Neotropical (*Sarkidiornis melanotos*) taxon.

Here we use DNA sequences from 3 mitochondrial regions (cytochrome b gene, ND2 & control region sequence), plus a large number of taxa, to examine the phylogenetic affinities of the blue duck in a wider phylogeny of Anseriformes. Based on this new analysis and the available literature, we evaluate the historic placement of *Hymenolaimus*.

METHODS

Hymenolaimus samples were collected and genomic DNA was extracted as described in Robertson *et al.* (2007). Cytochrome b gene was amplified from 3 blue duck samples, one each from the Manganuiateao River (WG118), Clinton River (L36927) and the Cleddau River (L38360) (see Robertson *et al.* 2007) using PCR primers L14841 (Kocher *et al.* 1989) and H16064 (Sorenson *et al.* 1999). The complete cytochrome b gene was amplified in a 25 µl reaction volume containing 50 ng genomic DNA, 1.0 pmol of each primer, 200 µM each of dATP, dGTP, dTTP, and dCTP, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂ and 0.5 unit of Taq polymerase (Bioline USA, Inc, Randolph MA 02368-4800). The thermal cycling parameters were an initial 2 min denaturation at 94°C, followed by 35 cycles at 94°C/30 sec, 58°C/45 sec and 72°C/90 sec, and finally 72°C/5 mins. PCR reactions were purified with Millipore Montage PCR96 Multiscreen filter plates (Biolab, New Zealand). Sequence was generated using a Big Dye v. 3.1 sequencing kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions using the L14841 and H16064 primers.

Control region sequences obtained by Robertson *et al.* (2007) were used in this study, in combination with sequences from GenBank for all 3 genes (Table A1). In particular, GenBank sequences from previous molecular phylogenetic studies of Anseriformes were used (Donne-Goussé *et al.* 2002; Gonzalez *et al.* 2009; Bulgarella *et al.* 2010). We were unable to source DNA from Salvadori's duck *Salvadoria waigiensis* to fully examine putative convergent evolution among

the riverine ducks. A multiple alignment of all sequences for each gene was achieved using default parameters in ClustalX (Thompson *et al.* 1997), and all variable sites and ambiguous sections were confirmed by visual inspection in BIOEDIT version 5.0.6 (Hall 1997).

Phylogenetic analysis

Maximum likelihood and Bayesian Probability methods were used to test the phylogenetic relationship of *Hymenolaimus* with other Anseriformes taxa. A partial fragment of the mtDNA control region was analysed separately from the concatenated ND2 (954 base pair, bp) and cytochrome b (1018bp) genes. For control regions sequence, domains II and III were aligned as per Donne-Goussé *et al.* (2002), with gaps excluded. Major gaps of 30 - 100bp were observed only in outgroup taxa and so were deleted as these groups were not the focus of the study. Monophyly of outgroups was maintained despite this exclusion. The remaining sequences were reviewed for substitution/saturation, with no asymptote observed for transitions or transversions, which was also observed when these data were published previously (*i.e.*, Donne-Goussé *et al.* 2002). Domain I was not used due to its hypervariable nature and the absence of sequence available for this region for many taxa. We did not combine the 3 genes for mixed analyses (*cf.* Donne-Goussé *et al.* 2002), as there are few taxa that have been sequenced for both the control region and cytochrome b genes, due to the different foci of previous studies. For both datasets, we determined the best fit evolutionary model using Modeltest v. 3.06 (Posada & Crandall 1998) following the Akaike Information Criterion (AIC: Sakamoto *et al.* 1986).

Maximum likelihood analysis was performed with 100 bootstrap replicates using PAUP*4.0b10 (Swofford 1998) for the control region data only. The genetic distance among taxa was also determined using maximum likelihood parameters. Bayesian analysis was conducted for 1,000,000 generations, sampled every 100 generations (25% burnin) in Mr Bayes (Ronquist & Huelsenbeck 2003), using likelihood parameters determined by Modeltest: 4by4 nucleotide model with substitution type 6 (GTR) for all sequence alignments and among-site rate variation estimation for invariable sites and the gamma distribution.

RESULTS

The 2 sequence alignments used in this study consisted of 641bp of control region fragment and 1972bp of ND2 and cytochrome b sequence. The control region dataset included 46 Anseriformes taxa and the ND2/cytochrome b dataset included 82 taxa from the Anatinae sub-family (Table 1). Although

Table 1. Scientific name, region of mtDNA and accession number of relevant sequences.

Species	Sample code	Control Region	ND2	Cytb
<i>Aix galericulata</i>		AY112953*	-	-
<i>Aix sponsa</i>	2	-	EU585668~	AF059053^
<i>Alopochen aegyptiacus</i>	7	AY112964*	HM063564^	^
鸕鶿	17	-	AF059115`	AF059054^
<i>Anas acuta</i>	29	AY112939	-	AF059055`
<i>Anas americana</i>	30	-	AF059163`	AF059103^
<i>Anas aucklandica</i>	31	-	AF059117`	AF059059
<i>Anas bahamensis</i>	32	AY112940*	AF059120`	AF059058`
<i>Anas bernieri</i>	33	-	AF059121`	AF059060`
<i>Anas capensis</i>	34	-	AF059165`	AF059105`
<i>Anas castanea</i>	35	-	AF059125`	AF059065`
<i>Anas chlorotis</i>	36	-	AF059122`	AF059061`
<i>Anas c. carolinensis</i>	13	-	AF059123`	AF059063`
<i>Anas c. crecca</i>	14	AY112942*	EU585670~	AF059064`
<i>Anas c. cyanoptera</i>	37	-	AF059127`	AF059067`
<i>Anas clypeata</i>	12	AY112941*	AF059174`	AF059062`
<i>Anas diazi</i>	38	-	AF059129`	AF059069`
<i>Anas discors</i>	39	-	AF059128`	AF059068`
<i>Anas erythrorhyncha</i>	40	-	AF059130`	AF059070`
<i>Anas falcata</i>	41	-	AF059166`	AF059106`
绿翅鸭	42	-	AF059131`	AF059071`
<i>Anas f. oxyptera</i>	43	-	AF059132`	AF059072`
<i>Anas formosa</i>	44	-	AF059133`	AF059073`
<i>Anas fulvigula</i>	46	-	AF059134`	AF059074`
<i>Anas g. spinicauda</i>	45	-	AF059135`	AF059075`
<i>Anas g. gracilis</i>	47	-	AF059136`	AF059076`
绿翅鸭	48	-	AF059137`	AF059077`
<i>Anas laysanensis</i>	49	-	AF059138`	AF059078`
<i>Anas luzonica</i>	50	-	AF059139`	AF059079`
<i>Anas melleri</i>	51	-	AF059140`	AF059080`
<i>Anas penelope</i>	52	-		AF059107`
<i>Anas platalea</i>	53	-	AF059144`	AF059084`
<i>Anas platyrhynchos</i>	15	AY112938*	EU585672~	AF059081`
<i>Anas poecilorhyncha</i>	54	-	AF059143`	AF059083`
<i>Anas puna</i>	64	-	AF059145`	AF059085`
<i>Anas querquedula</i>	55	-	AF059146`	EU585610~
<i>Anas r. rhynchotis</i>	56	-	AF059147`	AF059087`
<i>Anas rubripes</i>	57	-	AF059148`	AF059088`
<i>Anas sibilatrix</i>	58	AY112943*	AF059168`	AF059108`
<i>Anas smithii</i>	59	-	AF059149`	AF059089`
<i>Anas sparsa</i>	60	-	AF059151`	AF059091`

Table 1. Continued.

<i>Anas strepera</i>	61	AY112944*	AF059169`	AF059109`
<i>Anas s. rogersi</i>	62	-	AF059152`	AF059092`
<i>Anas undulata</i>	63	-	AF059153`	AF059093`
<i>Anas versicolor</i>	65	-	AF059154`	AF059094`
<i>Anser albifrons</i>		AY112967*	-	-
<i>Anser anser</i>		AY112966*	-	-
<i>Anser caerulescens</i>		AY112968*	-	-
<i>Anser canagicus</i>		AY112969*	-	-
<i>Anser erythropus</i>		AY112970*	-	-
<i>Anser indicus</i>		AY112971*	-	-
<i>Anser rosii</i>		AY112972*	-	-
<i>Asarcornis scutulata</i>	9	-	AF059159	AF059099^
· ¼« ½» □ ± ¶	67	-	EU585684~	EU585621~
<i>Aythya americana</i>		AY112946*		AF090337^
<i>Aythya australis</i>	66	-	EU585685~	EU585622~
<i>Aythya ferina</i>	68	-	EU585686~	EU585623~
<i>Aythya fuligula</i>	69	-	EU585687~	EU585624~
<i>Aythya marila</i>	70	AY112947*	EU585688~	EU585625--
<i>Aythya nycora</i>	71	AY112948*	EU585689~	-EU585626~
<i>Branta bernicla</i>	1	AY112973*	EU585691~	EU585628~
<i>Branta canadensis</i>		AY112974*	-	-
<i>Branta leucopsis</i>		AY112975*	-	-
° µ±· □ µ ½²~¶		AY112976*	-	-
<i>Bucephala albeola</i>	72	-	EU585696~	EU585633~
<i>Bucephala clangula</i>	24	AY112959*	EU585697~	AF515261*
<i>Bucephala islandica</i>	74	-	EU585698~	EU585635~
<i>Cairina moschata</i>	75	AY112952*	AF059158`	AF059098`
“ □²±” □ “, ½²«µ¶	27	AY112960*	AF059157`	AF059097^
<i>Cereopsis novaehollandiae</i>		AY112977*	-	-
<i>Chauna torquata</i>		AY112982*	-	-
“ «”±²±” □ “, ½³ □	22	AY112951*	AF059160`	AF059100`
<i>Chloephaga picta</i>	4	AY112965*	AF515266*	AF515262*
<i>Clangula hyemalis</i>	77	AY112954*	EU585701~	-EU585638~
<i>Coscoroba coscoroba</i>		AY112979*	-	-
<i>Cyanochen cyanopterus</i>	11	-	AF059161`	AF059101^
<i>Cygnus atratus</i>		AY112978*	-	-
<i>Cygnus olor</i>		-	-	-
<i>Dendrocygna bicolor</i>		AY112980*	-	-
<i>Dendrocygna eytoni</i>		AY112981*	-	-
<i>Hymenolaimus malacorhynchos</i>	28	EF395946/955>	>	#
<i>Lophodytes cucullatus</i>	78	-	EU585650~	EU585713~
ž²³«²±” □ ¶³· ½, ¾²·S·¶	16	AY112945*	AF059162`	AF059102`

Table 1. Continued.

<i>Malacorhynchus membranaceus</i>	79	-	EU585714~	EU585651~
ž ął ął±" ą ą±ā, ą-ł±ł ą±ł	8	AY112950*	AF059164`	AF059104`
ž " ą±- ą±-ā ą±ł	25	-	AF515267*	AF515263*
ž " ą±- ą±-ā " ą±- ą±-ā	80	-	EU585715~	EU585652~
ž " ą±- ą±-ā ą ą ą	3	-	HM063566^	^
<i>Mergus albellus</i>	81	AY112957*	EU585716~	-EU585653~
<i>Mergus cucullatus</i>		AY112958*	-	-
<i>Mergus m. merganser</i>	82	-	EU585717~	EU585654~
<i>Mergus serrator</i>		AY112956*	-	-
<i>Neochen jubata</i>	5	-	HM063564^	^
! " ą±- ą±-ā ą ą	83	-	EU585719~	EU585656~
! " ą± ą±		AY112949*	-	-
# " ą±- ą±-ā " ą±- ą±-ā	10	-	AF059170`	AF059110^
<i>Sarkidironis melanotos</i>	23	-	EU585723~	AF059111^
<i>Somateria mollissima</i>	26	AY112955*	EU585724~	AF515264*
<i>Somateria spectabilis</i>	84	-	EU585725~	EU585662~
<i>Speculanus specularis</i>	18	-	AF059150`	AF059090^
<i>Tachyeres bachypterus</i>	20	-	HM063559^	^
<i>Tachyeres leucocephalus</i>	19	-	HM063560^	^
<i>Tachyeres pteneres</i>	21	-	AF059172`	AF059112^
<i>Tadorna cana</i>	85	-	EU585726~	EU585663~
<i>Tadorna ferruginea</i>	87	-	EU585727~	EU585664~
<i>Tadorna radjah</i>	86	-	EU585728~	EU585665~
<i>Tadorna tadorna</i>	6	AY112962*	AF059173`	AF059113`
<i>Tadorna tadornoides</i>	88	AY112963*	EU585729~	-EU585666~

Note: symbols represent the respective publications in which the sequences are published. ^ Bulgarella et al. 2010. * Donne-Goussé et al. 2002, ^ Johnson & Sorenson 1998, ~ Gonzalez et al. 2009, > Robertson et al. 2007, # present study. - shows where no sequence was available.

the 2 datasets were not directly comparable, due to the different taxa available for each of the genes, consistencies did exist in the relationships among major taxonomic units (Figs. 1 & 2). For example, the placement of the Tadornini, Aythyini, Anatini, and Mergini tribes were consistent for each dataset, despite bootstrap and Bayesian support for the short control region fragment being low for these clades (Fig. 1).

Hymenolaimus was placed firmly within the Anatinae sub-family for both datasets, but maximum likelihood and Bayesian analyses were unable to resolve many of the relationships within the Anatinae (Figs. 1 & 2). For both analyses, *Hymenolaimus* was not placed within any of the existing tribes, nor was it associated with any of the historical placements (e.g., in the Anatini or in the Merganettini with torrent ducks and steamer ducks; Fig. 2). The concatenated ND2/cytochrome b duck phylogeny placed the *Hymenolaimus* in

a clade with *S. melanotos*, that did not include *C. jubata*.

The genetic distances between *Hymenolaimus* and all other Anatinae taxa supports its distinct placement. Genetic distances ranged from 7% - 13% for the control region (Table 2) and 8% - 12% for the cytochrome b gene (Table 3). Genetic differentiation within *Hymenolaimus* was negligible in comparison, at 0 - 2% for the control region and 0% for the cytochrome b gene.

DISCUSSION

Although the precise placement of the New Zealand blue duck is unclear, it appears to be a unique entity within the Anatinae sub-family. Our results are consistent with the major Anseriformes clades of Donne-Goussé et al. (2002) and mostly consistent with the Anatinae clades identified by Bulgarella et al. (2010) in their phylogenetic study of South

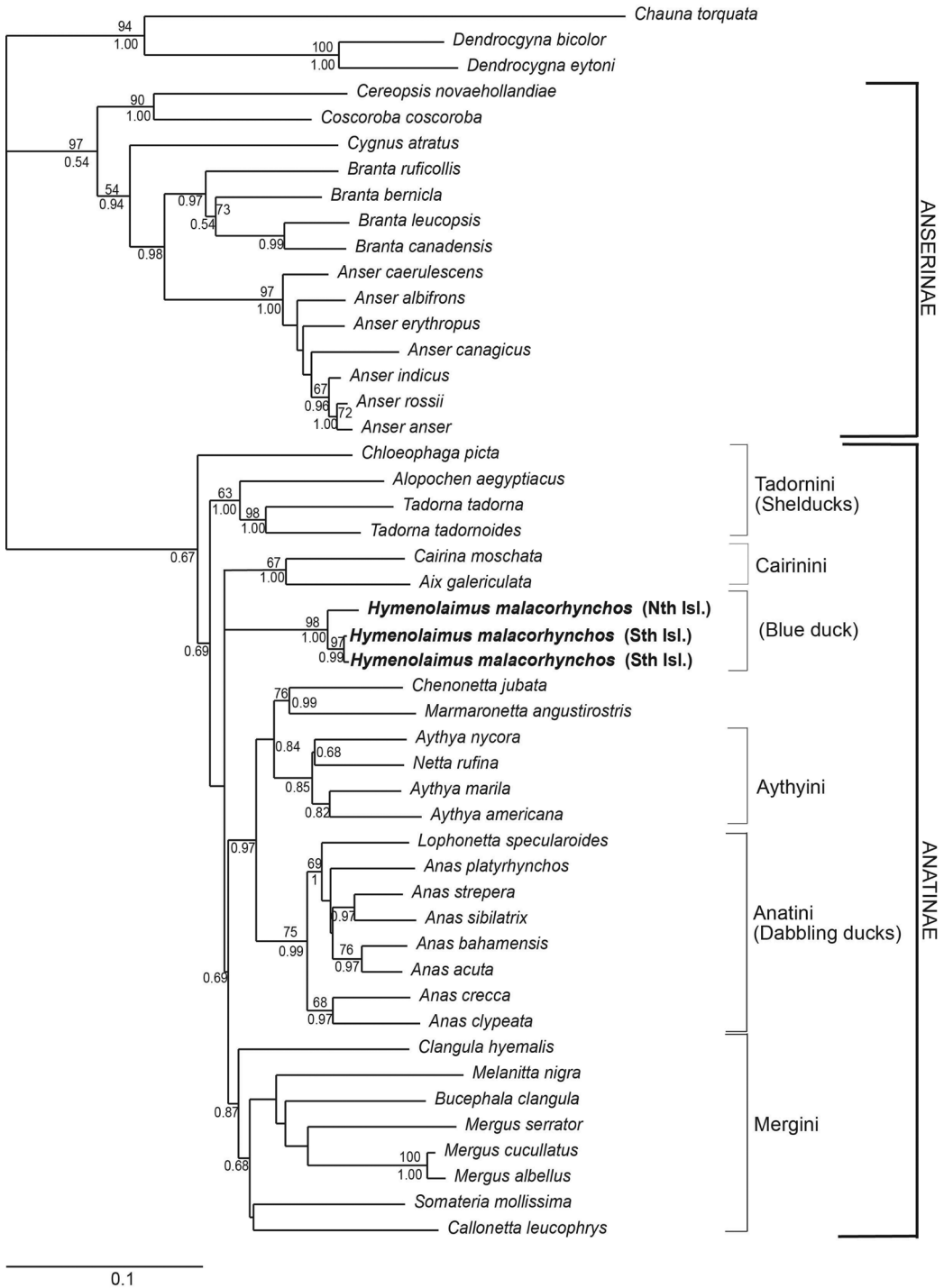


Fig. 1. A 50% majority-rule phylogenetic tree of the partial mitochondrial control region gene. Bootstrap (above branches) and Bayesian probability (below branches) support are shown. *Hymenolaimus* sequences are highlighted and the historically derived tribes and common names for the major tribes are shown. The scale bar represents the branch lengths as the number of substitutions per site.

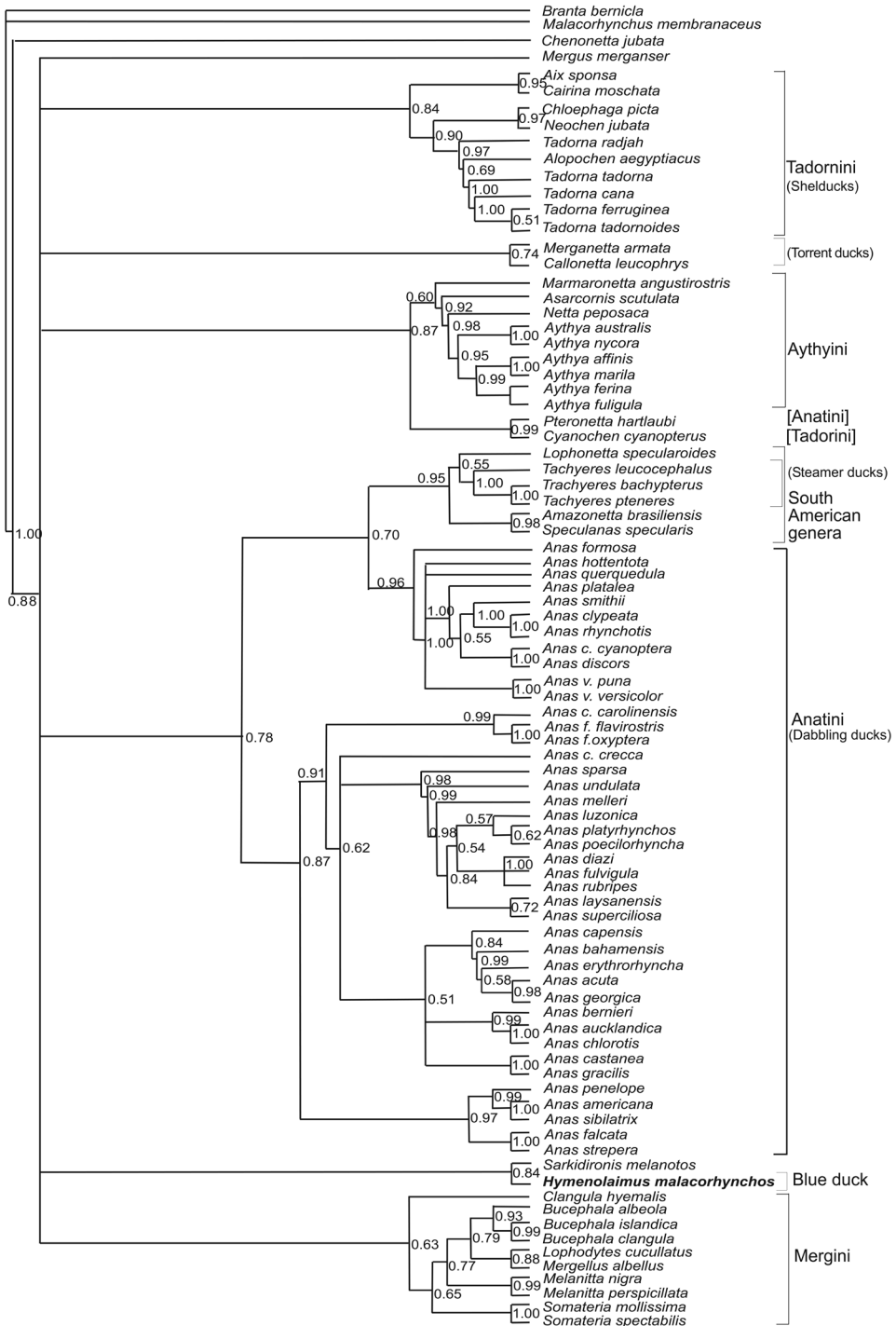


Fig. 2. A 50% majority-rule phylogenetic tree of the concatenated partial mitochondrial cytochrome *b* and ND2 genes. Bayesian probability (below branches) support is shown. *Hymenolaimus* sequences are highlighted and the historically derived tribes and common names for the major tribes are shown. The scale bar represents the branch lengths as the number of substitutions per site.

American ducks. Both datasets used in our study show that historic placements of *Hymenolaimus* based on morphology and behaviour (Delacour & Mayr 1945; Kear 1972; Kear 2005; Livezey 1986; 1997) were incorrect. *Hymenolaimus* is not a member of dabbling (Anatini) or perching ducks (Cairinini), and the taxon does not group as a highly derived clade of shelducks (Merganettini), as it displays no phylogenetic affinities with steamer-ducks or the torrent duck. *Hymenolaimus* does not exhibit a close affinity to any other particular genus or tribe.

One major difference with our study was that Bulgarella *et al.* (2010) placed *Hymenolaimus* in a clade with *S. melanotos* and *C. jubata*. Bulgarella *et al.*'s placement of *Hymenolaimus* was a strongly supported grouping based on 3 mtDNA gene regions and 5 nuclear loci. In our study, we sampled 1972bp of cytochrome b and ND2 sequence (*cf.* 500bp in Bulgarella *et al.* 2010), which may account for the change in the relationship between *Hymenolaimus* and *C. jubata*. The additional sites in the cytochrome b fragment obtained in specimens used in our study have potentially increased resolution. The placement of *S. melanotos* and *C. jubata* is not consistent across studies, with some authors finding this relationship (Sorenson *et al.* 1999; Gonzalez *et al.* 2009; Bulgarella *et al.* 2010; present study for cytochrome b), yet others have not (Gonzalez *et al.* 2009; Johnson & Sorenson 1999). " $\llcorner\pm^2\pm$ " was placed basally in the Anatini by Gonzalez *et al.* (2009).

Many of the deeper phylogenetic clades within Anatinae were lost in our data, which is consistent with low support for these clades shown by Donne-Goussé *et al.* (2002) and Bulgarella *et al.* (2010). Most phylogenetic analyses involving Anseriformes have not been well resolved despite the large amount of nuclear and mitochondrial genetic data that has now been obtained by various studies (*e.g.*, Donne-Goussé *et al.* 2002; Bulgarella *et al.* 2010). Indeed, low resolution in phylogenetic analyses of avian orders is apparently common and most likely due to their rapid and ancient diversification (*e.g.*, Sorenson *et al.* 2003; Bulgarella *et al.* 2010).

Our results on the phylogenetic affinities of *Hymenolaimus* within the Anatinae provide evidence for convergent evolution among the duck species that inhabit fast flowing rivers (Williams & McKinney 1996). Three species, including *Hymenolaimus*, inhabit such rivers (torrent duck *M. armata* & Salavadori's duck *S. waigiuiensis*; Kear 2005). While it is clear that the torrent duck is not closely related to *Hymenolaimus*, it does share morphological (Livezey 1986) and behavioural (Kear 1972) similarities. However, *Hymenolaimus* also shares characters in common with some anatinine ducks (*e.g.*, syrinx structure, head-bobbing, patterning of ducklings & duckling calls), cairininine ducks (*e.g.*, patterning of ducklings),

tadorninine ducks (preflight signals) and aythyinine ducks (preflight signals; Kear 1972). In contrast, the knob-billed duck *S. melanotos* is described as a pond duck and shares no phenotypic characteristics that would suggest monophyletic affinities with *Hymenolaimus*, despite its apparent close genetic relationship. Livezey (1986) also noted widespread homoplasy among 120 morphological characters in his thorough phylogenetic analysis of the Anseriformes, which has undoubtedly contributed to the challenge of resolving the taxonomy of the group. Resolving the remaining phylogenetic uncertainty in the Anseriformes, and indeed the phylogenetic placement of *Hymenolaimus*, is going to require more comprehensive sampling among the " $\llcorner\pm^2\pm$ " taxa and more powerful application of genomics.

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