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Phylogenetics and the evolution of terrestriality in mudskippers (Gobiidae: Oxudercinae)

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ABSTRACT

The initial vertebrate conquest of land by stegocephalians (Sarcopterygia) allowed access to new resources and exploitation of untapped niches precipitating a major phylogenetic diversification. However, a paucity of fossils has left considerable uncertainties about phylogenetic relationships and the eco-morphological stages in this key transition in Earth history. Among extant actinopterygians, three genera of mudskippers (Gobiidae: Oxudercinae), *Boleophthalmus*, *Periophthalmus* and *Periophthalmodon* are the most terrestrialized, with vertebral, appendicular, locomotory, respiratory, and epithelial specializations enabling overland excursions up to 14 h. Unlike early stegocephalians, the ecologies and morphologies of the 45 species of oxudercines are well known, making them viable analogs for the initial vertebrate conquest of land. Nevertheless, they have received little phylogenetic attention. We compiled the largest molecular dataset to date, with 29 oxudercine species, and 5 nuclear and mitochondrial loci. Phylogenetic and comparative analyses revealed strong support for two independent terrestrial transitions, and a complex suit of ecomorphological forms in estuarine environments. Furthermore, neither Oxudercinae nor their presumed sister-group the eel gobies (Amblyopinae, a group of elongated gobies) were monophyletic with respect to each other, requiring a merging of these two subfamilies and revealing an expansion of phenotypic variation within the “mudskipper” clade. We did not find support for the expected linear model of ecomorphological and locomotory transition from fully aquatic, to mudswimming, to pectoral-aided mudswimming, to lobe-finned terrestrial locomotion proposed by earlier morphological studies. This high degree of convergent or parallel transitions to terrestriality, and apparent divergent directions of estuarine adaptation, promises even greater potential for this clade to illuminate the conquest of land. Future work should focus on these less-studied species with “transitional” and other mud-habitat specializations to fully resolve the dynamics of this diversification.

1. Introduction

Initial conquests of land by various plant and animal lineages (e.g. rhinopods, bryophytes, fungi, arthropods, molluscs, leeches and sarcopterygians) allowed access to new resources and exploitation of untapped ecological niche spaces precipitating major phylogenetic diversifications (Gordon and Olson, 1995). Such transitions required, among other things, capacities to: (1) resist gravity in a subaerial environment; (2) resist desiccation; (3) respire in air; (4) endure more drastic

diurnal temperature fluctuations than experienced in water; and among motile organisms, (5) means to propel themselves across subaerial substrates (Clack, 2002; Gordon and Olson, 1995; Graham, 1997).

The vertebrate transition to land is thought to have been first achieved by stegocephalians (Sarcopterygia: Tetrapodomorpha) between the Middle Devonian and early Carboniferous Periods (Eifelian stage ~395 million years; Clack, 2002; Shubin, 2008). Unfortunately, gleaned critical insights into their terrestrialization has been hampered by a sparse fossil record (e.g., Ahlberg and Milner, 1994; Ashley-Ross et al.,

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2013; Coates et al., 2002; Molnar et al., 2017) leaving considerable uncertainties about phylogenetic relationships, terrestrial capacities, and the phenotypic stages leading to the conquest of land. As such, extant actinopterygian and sarcopterygian fish showing some terrestrial capacities, for which much is known about their biology (incl. anatomy, physiology, ecology and locomotion) have been used as models for the first tetrapod water-land transition (e.g., Graham, 1997; Harris, 1959; Kawano and Blob, 2013; Ord and Cooke, 2016; Pierce et al., 2012; You et al., 2014). Nevertheless, most of these studies lack the strong phylogenetic context from well-resolved molecular global phylogenies that would enable the rich sampling across transformation series necessary to identify specific adaptations to terrestriality. As a result, it is unclear if these studies can make generalizations about how stegocephalians succeeded in their transition to the terrestrial realm.

Among extant actinopterygians, three genera of mudskippers (Oxudercinae), *Boleophthalmus*, *Periophthalmus* and *Periophthalmodon* are the most terrestrialized (Graham, 1997; Murdy, 1989). These estuarine gobies possess short rigid trunks that resist dorsoventral flexion of the vertebral column on land, robust ventrally-positioned lobefins for terrestrial propulsion, and extensive respiratory, epithelial and physiological modifications enabling extensive (i.e., up to 14 h) overland excursions (Graham, 1997; Harris, 1960). In contrast, other oxudercines show more eel-like body forms. These 15 or more non-terrestrialized species can further be classified as either: (1) fully aquatic and demersal (presumably the primitive character state within the subfamily); (2) semi-terrestrial airbreathers that when exposed at low tide can move sinuously through the mud; or (3) semi-terrestrial airbreathers capable of sinuous locomotion through mud and crutching short distances over drier substrates using semi-lobed pectoral fins (Graham, 1997; Murdy, 1989) (Fig. 1).

Because of the highly terrestrialized nature of *Boleophthalmus*, *Periophthalmus* and *Periophthalmodon* that employ a form of “walking” (=

“crutching”; Harris, 1960) involving leg-like fins, these taxa have been most extensively studied as a model system for the initial tetrapod water-to-land transition, particularly with regard to locomotion (Graham, 1997; Harris, 1960; You et al., 2014). However, as with the other lineages of extant terrestrialized fish, sparse phylogenetic sampling among these taxa or their more aquatic relatives has hindered robust reconstruction of transitional stages. For instance, Harris (1960) in his study on the evolution of mudskipper locomotion assumed, as did previous researchers (Eggert, 1929; Harms, 1929) that all highly terrestrialized mudskippers are descended from a single terrestrial ancestor. His reconstruction of the likely anatomical and locomotory transition was limited in precision by his comparison to distantly related species, the demersal *Eleotris* (Eleotridae) and the pelagic *Gobius* (Gobiidae) (Agorreta et al., 2013; Thacker, 2003; Tornabene et al., 2013) to represent the primitive fully-aquatic character state.

Murdy (1989) conducted the first comprehensive phylogenetic analysis of Oxudercinae (Günther, 1861), consisting of 10 genera and based on morphological characters. He subsequently identified eelgobies (Ambylopininae; Günther, 1861) as their sister group (Murdy, 2012). His studies supported the long-accepted assumption that terrestriality evolved just once within the clade. Specifically, *Periophthalmus*, *Periophthalmodon*, and *Boleophthalmus* comprised a derived clade within Oxudercinae, with *Boleophthalmus* sister to *Periophthalmus* + *Periophthalmodon*. Semi-terrestrial eel-like relatives (e.g., *Scartelaos*, *Pseudapocryptes*) were recovered as closely related lineages. The mud-swimming and fully-demersal taxa (e.g. *Apocryptes* and *Parapocryptes*, respectively) represent successively more distant outgroups.

Molecular phylogenies that included multiple members of Oxudercinae (Thacker, 2003, Agorreta et al., 2013 and You et al., 2014) — with three, eight, and four oxudercine species, respectively — surprisingly did not place Murdy’s (1989) terrestrialized genera as a monophyletic group. Nor were either Oxudercinae or Ambylopininae monophyletic with respect to one another. An analysis of thousands of nuclear loci in gobioids that included six members of these two subfamilies likewise separated the most terrestrial genera and Oxudercinae was paraphyletic (Kuang et al., 2018). The most taxonomically comprehensive analyses to date are (Polgar et al., 2017) with 17 members of these subfamilies and three genes (coxI/cytb and RAG1) and McCraney et al. (2020). The latter is a supermatrix study of 827 gobioids that included 34 oxudercines and ambylopinines represented by nine genes. We chose a similar sampling approach to McCraney et al. (2020) except that we focused on a smaller number of genes with broader taxonomic coverage to reduce the non-overlapping data coverage, selected sequences from some different vouchers, and conducted new targeted sequencing to fill holes in the matrix. Both Polgar et al. (2017) and McCraney et al. (2020) corroborated polyphyly of the subfamilies and convergence of terrestrialization. In sum, mudskippers may have made evolutionary transitions on to land on at least two occasions, and the transformation sequence may have been complex. However, taxonomic sampling has been too sparse or resolution too limited to permit more detailed conclusions about the morphological transformations.

Here we present the more comprehensive molecular and phylogenetic analysis of Oxudercinae and Ambylopininae to test the veracity of the aforementioned morphological and molecular analyses and improve resolution of the region of the phylogeny where morphological transformations may have been concentrated. We then use these data to reconstruct the sequence of eco-morphological locomotory states and compare it to the model inferred from prior morphological studies of mudskippers (i.e. demersal to mudswimming to pectoral-aided mudswimming to terrestrial crutching). We provide a more nuanced understanding of this particular clade’s locomotory evolution on to land as an analogous model for how stegocephalians may have first conquered terrestrial niches.

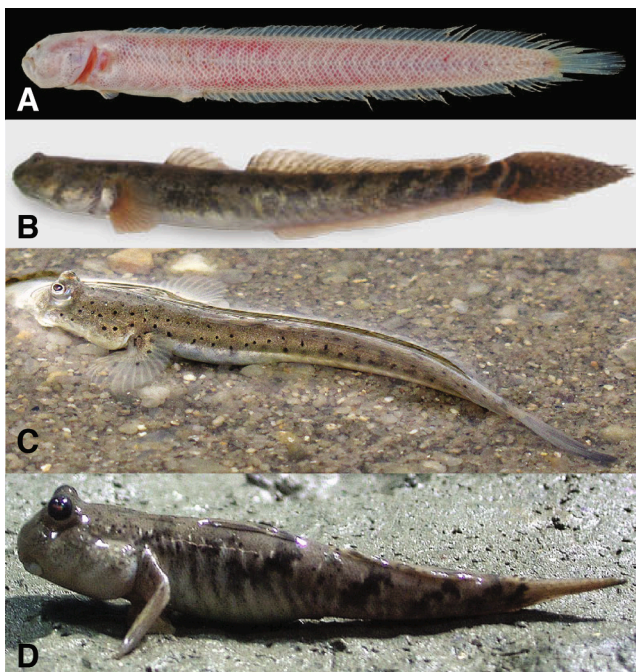


Fig. 1. Representative taxa of mudskippers and allies. (A) a demersal burrowing goby *Trypauchen vagina* (Dinh D. Tran, FiMSeA, <http://ffish.asia>, CC 2.0.); (B) a mud swimmer *Pseudapocryptes elongatus* (G. Polgar, <http://www.mudskipper.it/>, CC BY-NC-SA 3.0); (C) a pectoral-aided swimmer, the bearded mudskipper, *Scartelaos histophorus* (H.T. Cheng, iNaturalist.org. License: CC by Attribution-NonCommercial); (D) a terrestrial crutcher, Shutles mudskipper *Periophthalmus modestus* (Daiju Azuma, https://commons.wikimedia.org/wiki/File:Periophthalmus_modestus.jpg, CC by-sa/2.5).

2. Materials and methods

2.1. Taxon and character sampling

We analyzed 125 sequences from 39 species. Twenty-three of the sequences were new, acquired from six species obtained by the authors (GME, ASYA; Table 1, Supplemental Material). Collections by GME were made under Permit QFA 1994 PM110043011 to Dr. M. Sheave, James

Cook University, and in accordance with IACUC 83–99 (Brown University) and ACUC 1645 (FSU). Collections by ASYA were conducted in Kuwait Bay, Kuwait. The remainder were downloaded from GenBank, originating from 20 published studies. We included all available species of Oxudercinae and Amblylopininae as well as a stratified sampling of gobioid outgroups, using the eleotrid *Dormitator* as the basal outgroup. All oxudercine genera were represented (with the exception of *Zappa*) as well as three of the 14 known amblylopinine genera. In addition, because

Table 1

Sampling and voucher information for gobioid representatives used in this study. Locomotory mode corresponds to that used in Fig. 3. Museum Abbreviations: FLMNH – Florida Museum of Natural History (University of Florida), Gainesville, USA; GME – collection of Gregory Erickson. Italicized collector and voucher numbers indicate newly acquired sequences. Locomotory mode state definitions: 0 = fully aquatic, no terrestrial ability (either demersal or restricted to tidal exposed burrows with water); 1 = mud swimmers (generally eel like, no substantial use of pectoral fins); 2 = pectoral aided mud-swimmers (generally eel like with either reported sculling or semi-lobed fins but unable to fully lift body), 3 = terrestrially crutchers (short stout bodies fully developed lobe fins).

Taxon	Cyt b	ND1	Gpr85	RAG1	Rhodopsin	Zic1	Locomotory mode
Outgroup							
<i>Dormitator maculatus</i>		<i>MT83200</i>	KF416157	<i>MT843224</i>	KU765128	KF415947	0
		<i>GME 1</i>		<i>GME 1</i>			
<i>Awaous banana</i>	KF415507		KF416118			KF415907	0
<i>Awaous flavus</i>	KF415508		KF416119	KF415703		KF415908	0
<i>Awaous guamensis</i>	JN387711	AF391410			HQ639147		2
<i>Gillichthys mirabilis</i>	KF415563	AF391412	KF416182	KF415764		KF415969	1
<i>Gobioides broussonnetii</i>			KF416189			KF415977	0
<i>Redigobius bikolanus</i>	KF415651		KF416279			KF416063	0
<i>Sicyopterus lagocephalus</i>	KF415661	AF391461	KF416287			KF416073	1
Ingroup							
<i>Apocryptes bato</i>	KF415502		KF416114	KF415698		KF415902	2
<i>Apocryptodon</i> sp.	KF415503		KF416115	KF415699		KF415903	0
<i>Boleophthalmus boddarti</i>	KF874277	KF874277		JX003116			3
<i>Boleophthalmus dussumieri</i>	<i>MT800981 Boleokw 001</i>	<i>MT832008 Boleokw 001</i>		<i>MT843223 Boleokw 001</i>	<i>MT800989 Boleokw 001</i>		3
<i>Boleophthalmus pectinirostris</i>	KF415515		KF416126	KF415710		KF415915	3
<i>Boleophthalmus</i> sp.				JX003093			3
<i>Odontamblyopus lacepedii</i>				JX003119	KF235517		0
<i>Odontamblyopus rebecca</i>				JX003120			0
<i>Odontamblyopus</i> sp.	KF415617		KF416243	KF415824		KF416028	0
<i>Oxuderces dentatus</i>	KF415622	NC016194	KF416249	KF415828		KF416034	1
<i>Parapocryptes serperaster</i>	KF415629		KF416256	KF415834		KF416041	0
<i>Periophthalmadon freycineti</i>	<i>MT800982</i>	<i>MT832006</i>		<i>MT843225</i>	<i>MT800990</i>		3
<i>Periophthalmadon schlosseri</i>	<i>GME 4</i> JACM01085727	<i>GME 4</i>	JACM01052059	<i>GME 4</i> JACM01010566	<i>GME 4</i>	JACM01015942	3
<i>Periophthalmus argentilineatus</i>	AB021251			KF304810			3
<i>Periophthalmus barbarus</i>	<i>MT800983</i>	AF391411	KF416261	<i>MT843226</i>	<i>MT800991</i>	KF416045	3
<i>Periophthalmus c.f. minutus</i>	<i>GME 5</i> LK391944	LK391944		<i>GME 5</i>	<i>GME 5</i>		3
<i>Periophthalmus kalolo</i> Kenya				KF304816			3
<i>Periophthalmus kalolo</i> Palau				KF235483	KF235559		3
<i>Periophthalmus kalolo</i> Sulawesi				KF304820			3
<i>Periophthalmus magnuspinnatus</i>	KT357639	KT284931					3
<i>Periophthalmus malaccensis</i>				KF304821			3
<i>Periophthalmus minutus</i>	<i>MT800988 FLMNH 185058</i>	<i>MT832007 FLMNH 185058</i>	KP638476	<i>MT843228 FLMNH 185058</i>	<i>MT800993 FLMNH 185058</i>		3
<i>Periophthalmus modestus</i>	DQ901366			JX003122			3
<i>Periophthalmus waltoni</i>	KX998291			MK570137	MK570143		
<i>Pseudapocryptes elongatus</i>	EU380957	AF391466					1
<i>Pseudapocryptes</i> sp.	KF415643		KF416270	KF415847		KF416055	1
<i>Scartelaos histophorus</i>	KF415656	AF391418		KF415860		KF416068	2
<i>Scartelaos tenuis</i>	<i>MT800984 sckw001-2015</i>	<i>MT832009 sckw001-2015</i>		<i>MT843227 sckw001-2015</i>	<i>MT800992 sckw001-2015</i>		2
<i>Taenioides limicola</i>	AB021253						0
<i>Taenioides</i> sp.	KF415671		KF416298	KF415874	KF235513	KF416084	0
<i>Trypauchen</i> sp.	KF415676		KF416306	KF415882		KF416092	0

deep genetic divergences associated with large geographic distances suggested possible non-monophyly, three representatives of *Periophthalmus kalolo* were also analyzed. Six genes were sampled: mitochondrial cytochrome *b* (*cytb*; 1141 bp [base pairs]), and ND1 (975 bp) and from the nuclear genome, portions of RAG1 (1149 bp), Rhodopsin (856 bp), *Zic1* (849 bp), and *Gpr85* (990 bp) for a combined alignment length of 6296 bp. The average coverage per taxon was 3793 bp (=40% missing data).

All available sequences for these genes were first downloaded from GenBank, and alignments were pruned of redundant population samples. These pruned alignments were the basis for preliminary single-gene analyses to test species identifications by corroboration among studies. Multiple identification conflicts were diagnosed and sequences were retained for the final alignment if they were most in agreement with identifications from other studies. Taxonomic conflict was limited to *cytb* and RAG1, the two genes sequenced by multiple labs (the other three genes were largely sequenced by just one lab each). When one individual was retained for each locus, we gave preference to using the same voucher animal as retained for other loci. Phylogenies estimated from these larger single-gene alignments for *cytb* and RAG1 are available from Supplemental Material, where retained OTUs are indicated in bold and problematic sequences are highlighted. In the most extreme case of conflict, for *cytb*, a subset of the sequences were virtually identical, yet vouchers they were derived from were variously identified as either *Periophthalmus magnuspinatus*, *P. modestus*, or *Scartalaos histophorus*. Other sequences purportedly from these three same species were distantly related. Species identifications were further evaluated by comparing the voucher localities to known geographic distributions (IUCN, 2015; Polgar, 2014) and by cross-referencing voucher information from multiple studies. Voucher and accession information are summarized in Table 1 and Appendix S1, Supplemental Material.

2.2. DNA sequencing and alignment

All tissues were preserved in 70–100% ethanol or were frozen prior to DNA extraction. Total genomic DNA was extracted using standard phenol–chloroform extraction (Maniatis et al., 1982) or alcohol precipitation (Warwick et al., 2015). PCR reactions were conducted in 25 µl volumes, containing a mix of 1X GoTaq Reaction Buffer (Promega), 0.08 mM dNTPs, 0.2 µM each primer, (Table 2), 1 U GoTaq (Promega) polymerase, and 7.5–25 ng template DNA. Some reactions also included 1.5 µl MgCl₂ and 0.5 µl BSA.

Amplification conditions for *cyt b*, ND1, RAG1, and Rhodopsin included an initial denaturation at 94 °C for 2 m, 40 cycles of 94 °C for 30 s, 53–55 °C for 45 s, 72 °C for 60–90 s, and a final extension at 72 °C for 4 m. PCR products were visualized on an agarose gel with ethidium bromide. Successful amplifications were then prepared for sequencing using enzymatic digestion with ExoSAP-IT (USP, Cleveland, USA). Complementary strands were sequenced on an ABI Prism 3100 Genetic Analyzer using big-dye terminator chemistry (Applied Biosystems).

The sequences were edited and initially aligned by eye using Sequencher 3.1.1. (Gene Codes Corp.). All protein-coding sequences were translated into amino acids and easily aligned by eye using Mesquite v3.08 (Maddison and Maddison, 2017). These served to refine the alignments and test for possible pseudogenes. The new sequences were deposited on GenBank with accession numbers MT800981–MT800993, MT832005–MT832009, MT843223–MT843228 (Table 1).

2.3. Phylogenetic analyses

All Maximum Likelihood (ML) analyses were conducted using PAUP 4.0 v167 (Swofford, 2002) for individual genes and RAxML (Stamatakis, 2014) on the Cipres gateway (Miller et al., 2015) for the concatenated dataset. For Bayesian Inference (BI) we used MrBayes v 3.0 (Huelsenbeck and Ronquist, 2003) on the Cipres gateway for the concatenated dataset. Concatenated analyses were partitioned by codon position and

Table 2

Primers used in this study.

NAME	SOURCE	SEQUENCE	GENE
S410	modified from Thacker	YAATGRCCTTGAAAAACCCACCGTTGT	<i>cyt b</i>
S411	modified from Thacker	AACCACCGTTGTTATTCAACTA	<i>cyt b</i>
S412	modified from Thacker	CACCNTAYTTCTCHTYAAAGA	<i>cyt b</i>
S414	modified from Thacker	TTTGAAGCAGYAGGGAGGATTYTA	<i>cyt b</i>
S415	modified from Thacker	TTTAGAATGCTAGCTTTGGGAG	<i>cyt b</i>
GOBYL3543	Thacker (2003)	GCAATCCAGGTCAGTTTCTATC	ND1
S394	modified from Thacker	AACCYCATKWTCTACTCTATCAAA	ND1
RAG1-F1	López et al. (2004)	CTGAGCTGCAGTCAGTACCATAAGATGT	RAG1
RAG1-R1	López et al. (2004)	CTGAGTCCCTGTGAGCTTCCATRAAYTT	RAG1
RAG1-R3	López et al. (2004)	GTCTTGTGSAGGTAGTTGGT	RAG1
S416	modified from Chen et al.	CATATGAAATAYCCTCAGTACTACC	Rhodopsin
S418	modified from Chen et al.	TGCITGTTCATGMAGATGTAGA	Rhodopsin
Rh1073R/ S419	Chen et al. (2003)	CCRCAGCACARCCTGGTGATCATC	Rhodopsin

genome (six partitions). We selected the best-fit model of evolution using the Akaike Information Criterion in PAUP*. RAxML applied the GTR + Γ substitution model. BI analyses used the GTR + I + Γ substitution model with all parameters estimated for each partition separately. ML analyses used 200 bootstrap replicates. For our BI analysis we ran MCMC chains for 10 million generations, 2 parallel runs of 4 chains, and temp = 0.2. Convergence and stationarity were assessed using Tracer v.1.4 (Rambaut and Drummond, 2005) and split frequency standard deviations. In all analyses, stationarity was reached in the first 10%. We excluded the first 30% of the MCMC chains as burn-in. The results of BI analyses were summarized with TreeAnnotator v1.6.1 (Drummond and Rambaut, 2007). The data matrix and ML tree are deposited on Treebase with accession 26662.

Mudskippers and their allies lack a known fossil record that can provide internal fossil constraints (Murdy, 1989), and the record in Gobiidae in general is limited. Indeed, the only recent molecular study to date gobies used only the mitochondrial gene 16S and was restricted to fossil calibrations in distantly related families outside the suborder Gobioidae (Polgar et al., 2014). Consequently, we did not estimate a dated tree and await future nuclear gene studies with broader taxonomic sampling.

2.4. Character state reconstruction

We reconstructed ancestral character states to test the hypothesis based on morphological studies that the transformation series to terrestriality progressed from: (1) a primitive fully-aquatic demersal state to; (2) animals exposed during low tide that sinuously swim through custard mud (a medium that provides moderate aquatic support against gravity in a subaerial environment); (3) animals exposed at low tide that can sinuously swim through mud but aided by pectoral crutching of their semi-lobed fins for short bouts across solid substrates; and finally, (4) highly terrestrial forms that can crutch across hard substrates for long distances. Each species was assigned to one of these four locomotory character states based on a combination of personal observations (GME), accounts in Fishbase.org (Froese and Pauly, 2019), and the primary literature (Graham et al., 2007; Graham, 1997; Harris, 1959; Murdy, 1989; Pace, 2017; Polgar et al., 2017; Townsend and Tibbetts, 2005). Species were assigned to the states (0) fully aquatic, (1) mudswimming, (2) pectoral-aided mudswimming, and (3) terrestrial crutching,

respectively (see Table 1 for species specific state assignments).

Ancestral state reconstructions were conducted using Mesquite v. 3.11 (Maddison and Maddison, 2017) under both maximum parsimony (MP) and maximum likelihood criteria. For MP reconstructions, we treated the states as unordered. This conservative ordering does not assume a transitional series exists, and thus allows the phylogenetic pattern to suggest likely transitions, specifically the transitional model described above. ML reconstruction used the symmetrical Mk1 model. We also reconstructed ancestral states in addition to evaluating various models of character transformations using simulation mapping (SIMMAP) in the phytools package (Revel, 2012) in R. All reconstructions were conducted on the ML phylogram. Six models of character evolution were evaluated and compared using weighted AIC (Akaike, 1973): all rates equal and unordered (Equal Rates), ordered according to the traditional model with forward and reverse rates equal (Ordered1), ordered with unequal but symmetrical rates (Ordered2), ordered with equal rates among pairs but asymmetrical forward or reverse transformations (Ordered3), separate rates for each transformation pair but symmetrical between forward and reverse (SYM), and all rates different (ARD). The models were fitted using maximum likelihood with the *fitm* command and parameter values reported in Appendix S2 (Supplemental Material).

3. Results

3.1. Phylogenetic results

Each gene analyzed separately yielded a single ML tree (Supplemental Material). The congruent results included well-supported monophyly of the combined oxudercine and amblyopine taxa. Two major lineages arose from the base of this clade; the terrestrialized clade of *Periophthalmus* plus *Periophthalmodon* and the clade consisting of all

amblyopines and the remaining oxudercines. *Periophthalmodon* was the sister-group to *Periophthalmus*, and most relationships among the species of the latter genus were moderately to well-supported (65–100% bootstrap percentage). ML and BI analyses of the concatenated dataset were congruent for all nodes except for several short branches at the base of the non-*Periophthalmus* clade, involving the amblyopines and a subset of oxudercines (Fig. 2). There is decisive support for the paraphyly of Oxudercinae with respect to Amblyopinae. *Periophthalmus kalolo* appeared to be paraphyletic with the Palau population more closely related to *Periophthalmus modestus* than to other populations.

Although relationships at the base of the amblyopine/*Boleophthalmus* clade were poorly resolved, there was mixed support for the non-monophyly of Amblyopinae. Three species of the amblyopine *Odontamblyopus* were most closely related to *Pseudapocryptes* and this generic-pair was then sister to the oxudercine *Boleophthalmus-Parapocryptes* clade. Bootstrap support was weak for all nodes that separated *Odontamblyopus* from the core amblyopines, *Taenioidea* and *Trypauchen*, but the posterior probability was moderately high for the latter clade.

Given the low support values, there is uncertainty regarding the relationships among the less terrestrialized mudskipper genera *Parapocryptes*, *Pseudoapocryptes*, and *Oxuderces* relative to the more terrestrialized *Boleophthalmus* and *Scartelaos*. Monophyly among the sampled species is strongly supported for the genera *Boleophthalmus*, *Pseudoapocryptes*, *Odontamblyopus*, and *Scartelaos*, as well as for *Periophthalmus* and *Periophthalmodon*.

3.2. Locomotory evolution

ML, SIMMAP, and MP reconstructions of locomotory grades were all unequivocal that the most derived terrestrial mode evolved twice — once in the *Periophthalmus/Periophthalmodon* clade and once in the lineage leading to *Boleophthalmus* (Fig. 3 for ML and SIMMAP, Fig. S3 for

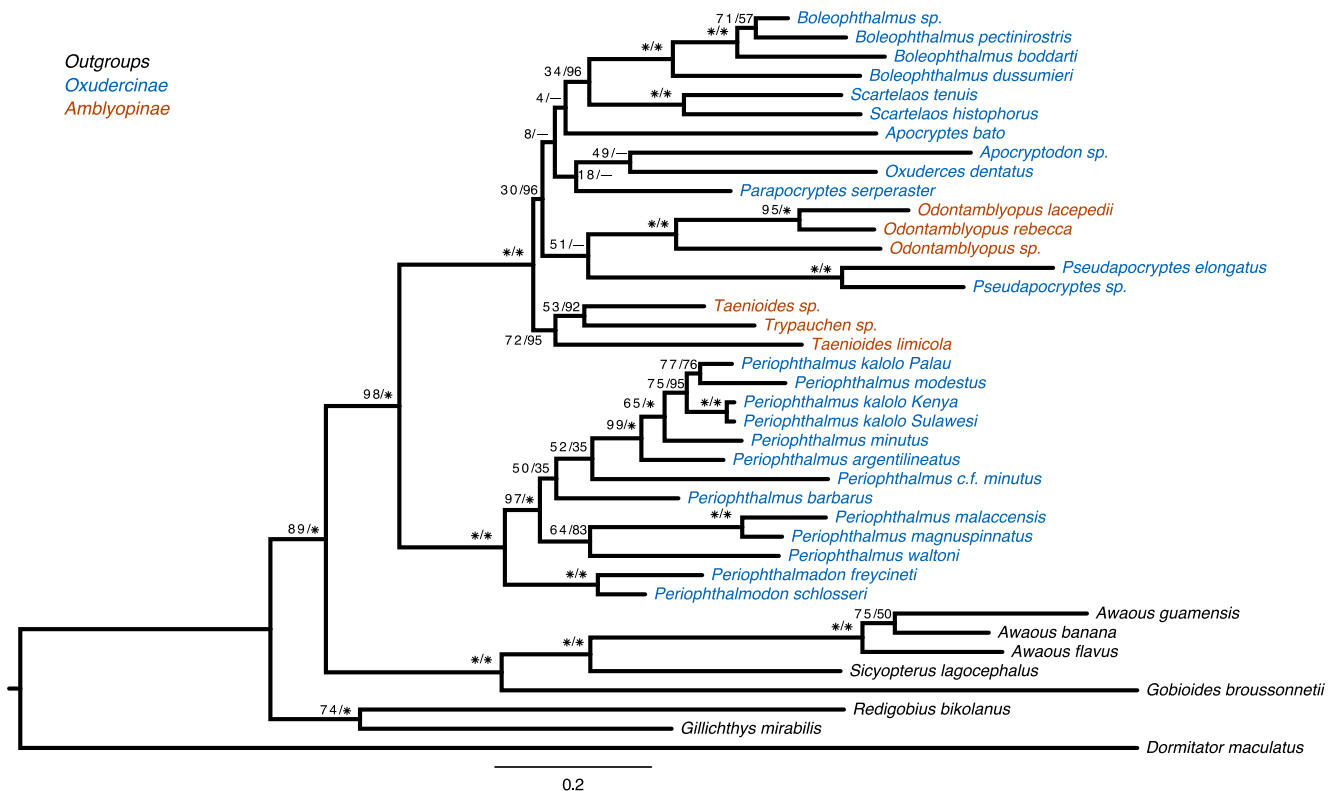


Fig. 2. Maximum-likelihood phylogram of concatenated dataset for six genes (cyt b, ND1, Gpr85, RAG1, Rhodopsin, Zic1). Numbers above branches indicate ML bootstrap and Bayesian posterior probabilities, respectively; “**” indicates 100%/1.00, dashes indicate absent from maximum clade credibility tree. Taxon colors indicate recognized subfamilies: blue, Oxudercinae (mudskippers); orange, Amblyopinae (eel gobies); black, outgroups. Labeled nodes correspond to discussion in the text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MP). Furthermore, the functionally intermediate condition of pectoral-aided mud swimming seen in *Scartelaos* and *Apocryptes* either evolved twice in parallel or was an intermediate grade leading to *Boleophthalmus*. The base of the *Boleophthalmus*/*Apocryptes* clade is poorly resolved phylogenetically and the clustering of pectoral-aided mud swimming leads to an equivocal reconstruction under both ML and SIMMAP. The best fit model of character transformation was Equal Rates, receiving 73% of the AIC weight, with the three ordered models summing to 17% (Table 3, Appendix S2). There is no clear evidence to support the previously proposed transitional series wherein evolutionary precursors passed from fully aquatic to mud-swimming to pectoral-aided mud-swimming and finally to terrestriality. Rather than an intermediate grade of terrestrialization, mud-swimming appears to be an alternative specialization that itself evolved more than once.

4. Discussion

4.1. Implications for understanding the initial vertebrate transition to land

Initial transitions to land by various higher order plant and animal clades led to major phylogenetic diversifications (Gordon and Olson, 1995). Consequently, there has been considerable interest in the suites of anatomical and physiological traits, be they exaptations or adaptations, that made them possible. Unfortunately, these transitions occurred hundreds of millions of years ago, leaving only their fossils from which to directly deduce salient character suites. In the case of the presumptive initial vertebrate conquest of land by stegocephalians, the fossil record is poor, consisting primarily of partial skeletons and tracks

Table 3

Comparisons of models of character state transformation in SIMMAP. AIC, Akaike Information Criterion; wAIC, AIC weights. Models are: Equal Rates, all rates equal and unordered; Ordered1, ordered according to the traditional model with forward and reverse rates equal; Ordered2, ordered with unequal rates among pairs of states but symmetrical forward and reverse; Ordered3, ordered with equal rates for each transformation pair but different (asymmetrical) forward or reverse transformations; SYM, unordered with separate rates for each transformation pair but symmetrical between forward and reverse; ARD, all rates different, unordered and asymmetrical. Estimated rate matrices reported in Appendix S2. The Equal Rates model is favored.

Model	AIC	wAIC
Equal Rates	69.743	0.7290
Ordered1	75.441	0.0422
Ordered2	73.574	0.1073
Ordered3	76.691	0.0226
SYM	73.780	0.0969
ARD	81.661	0.0019

(Ahlberg and Milner, 1994; Molnar et al., 2017; Shubin, 2008). As such their phylogenetic relationships are poorly understood and most aspects of their ecology, physiology, behavior and anatomy are not known. Fortunately, extant clades of fish that include terrestrial members can serve as analogs to generalize about how vertebrates make water-to-land transitions and infer the capacity of fossil taxa to have been terrestrial denizens. Oxudercine gobies, among the most terrestrialized extant fish, are a commonly cited model for the vertebrate transition to land (e.g. Graham, 1997; Harris, 1959; Kawano and Blob, 2013; Ord and Cooke, 2016; Pierce et al, 2012; You et al. 2014). Early studies of mudskippers

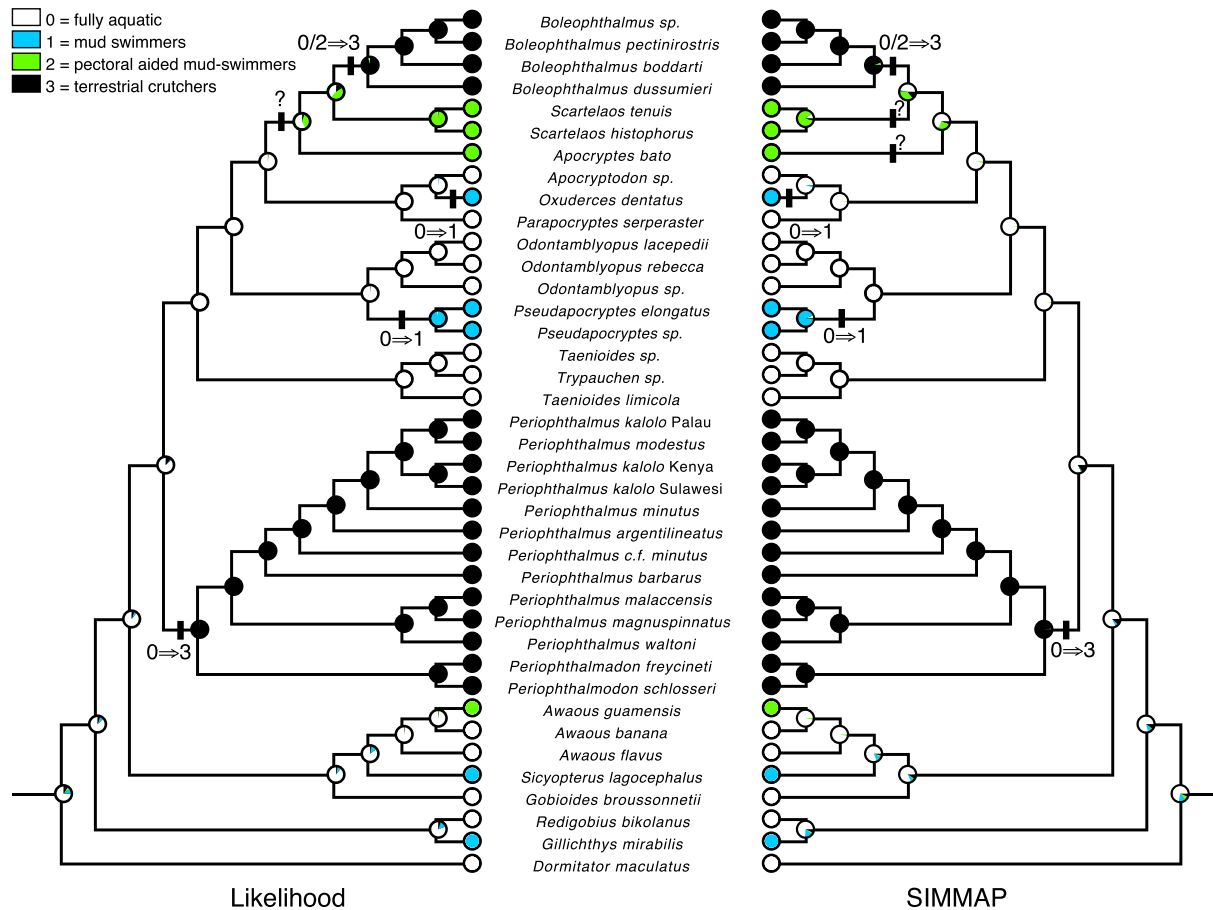


Fig. 3. Ancestral state reconstructions of locomotory mode. Maximum likelihood estimates on left conducted under Mk1 (symmetrical) model, SIMMAP reconstruction on right. Pie diagrams indicate proportional likelihoods or posterior frequencies of each state at each node. Hatch marks indicate transitions in mode within the ingroup for branches where there was a decisive change in the most-likely state (from >50% for one state to >75% for another).

in this regard assumed that they evolved terrestrial capacities in single cladogenic event (e.g. Graham, 1997; Harris, 1959; Murdy, 1989). However, molecular analyses suggested two terrestrial colonization events occurred within the clade (Agorreta et al., 2013; McCraney et al., 2020; Polgar et al., 2017; Thacker 2003; You et al., 2014). Our analysis, incorporating more genes and taxa than previously analyzed strongly corroborates those results. As such, previous and future analyses on mudskipper ecology, physiology, morphology and locomotion can now be analyzed in a more comprehensive, phylogenetic context (at low taxonomic levels) to identify the evolutionary modifications and stages by which they took to land. This more nuanced understanding will provide for better modelling of who, how, and when vertebrates first made their way onto land.

4.2. Phylogeny and taxonomic implications

Our results corroborate and consolidate those of previous studies (Agorreta et al., 2013; McCraney et al., 2020; Thacker, 2003) indicating that Oxudercinae and Amblyopinae form a clade and that neither is monophyletic with respect the other. As discussed in detail in Thacker (2003), the molecular results conflict strongly with the morphological phylogeny by Murdy (1989) for Oxudercinae and his later systematic treatment of the Amblyopinae (Murdy, 2012). For example, Murdy placed all three terrestrial genera *Boleophthalmus*, *Periophthalmus*, and *Periophthalmodon* in a clade exclusive of other oxudercines. The larger sequencing studies of Agorreta et al. (2013), McCraney et al. (2020) and this study demonstrate that *Boleophthalmus* is distantly related to the other two. Murdy's (1989) phylogeny showed a basal split between the clade of *Oxuderces*, *Apocryptodon*, and *Parapocryptes* as sister to all other oxudercines, with *Apocryptes* as the earliest branch of the latter. Our genetic data do not provide support for these relationships. Within the amblyopines, there is no support for the primary grouping by Murdy (2012) (following Birdsong et al., 1988) into the Trypauchen (for which we have only *Trypauchen*) and Taenioides groups. *Trypauchen* nests within *Odontamblyopus*. Two small areas of agreement between Murdy's (1989) phylogeny and ours are the sister-group pairs of *Periophthalmus*/*Periophthalmodon* and *Oxuderces*/*Apocryptodon*. Remaining areas of contention are concentrated among the genera of the amblyopine to the *Boleophthalmus* clade where our taxonomic sampling is poorest and most nodes are poorly supported in the molecular trees. The terrestrialized genera are relatively well sampled, thus future sampling efforts should concentrate on these other genera, especially among traditional amblyopines (eel gobies).

This study and McCraney et al. (2020) are in agreement on major results as expected given the large amount of shared data. The phylogeny in McCraney et al. (2020) exhibits a 10-lineage polytomy at the base of the amblyopine/*Boleophthalmus* clade where this study yielded many low support values. Stronger but limited conflict appeared within the well-sampled *Periophthalmus* clade. We suspect that these topological differences might be due to differences in screening of and possible misidentified vouchers among published sequences, and that sequencing multiple genes from the same vouchers to avoid chimeric data is needed to clarify this region of the tree.

Regarding the subfamilies, there is sufficient evidence from multiple genes representing both nuclear and mitochondrial genomes to recommend a taxonomic revision that merges these two taxa. The oldest available family-group name for this clade is Amblyopinae (Amplypolina Gunther 1861, p 133) making Oxudercinae (Oxudercidae Gunther 1861, p. 166) a junior synonym (although we note that the type *Amblyopus* has not yet been sequenced to definitively corroborate its membership in this clade). For the remainder of this contribution, we will refer to species traditionally placed in Amblyopinae (i.e., excluding Oxudercinae s.s.) by their common name of eel gobies.

4.3. Locomotory evolution

The evolution of amphibious and terrestrially capable fishes necessarily encompasses dealing with a wide range of anatomical and physiological demands, including body support, limb propulsion, respiration, water balance, and sensory acuity in addition to requisite favorable environments and available resources (Graham, 1997; Harris, 1960; You et al., 2014). Yet, extant fish lineages have evolved the capacity to foray onto land dozens of times. Ord and Cooke (2016) found that transitions to terrestriality were significantly more likely in lineages occupying the intertidal zone. However, these transitions were not more likely than expected among freshwater lineages, contra the prediction of Vermeij and Dudley (2000). This suggests a proclivity for taxa occupying marine littoral environments to be more likely to achieve terrestriality (Ord and Cooke, 2016), with mudskippers reinforcing this pattern,

The additional phylogenetic sampling provided here offers strong support for two convergent origins of fully-terrestrial locomotion from aquatic ancestors (Fig. 3). The *Periophthalmus*/*Periophthalmodon* clade is distantly related to *Boleophthalmus* and basal members of the *Boleophthalmus* group are aquatic eel gobies. Morphological evidence for convergence comes from several systems, including presence or absence of fusion in the pelvic fins that results in differing fin flexibilities (Wicaksono et al., 2016). Notably, the elongated eel gobies generally show anguilliform (sinuously undulating along the entire body length) rather than the carangiform (undulating only in the posterior body) locomotion typical of most gobies and are not strong open water swimmers. They build burrows subaquatically that they reside in when exposed at low tide (Graham, 1997; Murdy, 1989). Our ancestral reconstructions do not provide support for an ordered transitional model for the three aforementioned derived stages of locomotory specialization gleaned from previous morphological hypotheses. First, the *Periophthalmus* clade is unequivocally reconstructed as deriving directly from an aquatic ancestor (98% marginal likelihood). Second, within the phenotypically diverse sister-clade, ancestral reconstructions also indicate that the most likely states for these intermediate ancestors were aquatic with two independent transitions to mud-swimming (Fig. 3). The reconstruction however becomes equivocal with the evolution of the specialization of pectoral fins for either mud-swimming (state 2) or terrestrial crutching (state 3) in *Apocryptes*, *Scartelaos*, and *Boleophthalmus*. In order to further resolve the ancestral locomotory modes, additional sampling is required among the many species that presumably would fall in this region of transitions (*Taenioides* to *Scartelaos*), especially the eel gobies, some of which have one of the two intermediate locomotory modes. This region of the tree is the most under-sampled for both taxa and genes. In addition, there are several alternative explanations for these results even if the hypothesized transition sequence did occur. Key lineages retaining transitional states (e.g. mud-swimming [state 1] was hypothesized to precede pectoral mud-swimming [state 2]) may have gone extinct, most of the lineages that were at one time in an intermediate state may have subsequently evolved to a more derived condition, and there may have been more reversals among the eel gobies. Each of these alternatives would contribute to uncertain ancestral reconstructions.

Although the uncertain resolution of this complex region where the key transitions are hypothesized to have taken place precludes a decisive test of the transitional model proposed earlier, it appears that there were multiple transitions between locomotory modes, not just one. Such convergences provide more replications that actually increase our ability to understand the environmental processes and sequences of character shifts and needed to identify the morphological solutions to the same functional demands at each stage of the transition, not just the final stage. Although a direct reading of the ancestral reconstructions indicates only increasing terrestriality with no evidence of reversals, the model-fitting procedure among several combinations of character state ordering and differing forward and reverse rates fails to reject the simpler model of all rate being equal and provides weak evidence of

polarity, 17% of the AIC weights when summed across all three ordered models (Table 3, Appendix S2). These results reveal a complex radiation into multiple littoral niches, promising a richer field for ecomorphological and biomechanical exploration. The focus of future work should be as much on these transitional and alternative specializations as on the extreme terrestrialized forms.

5. Conclusions

The longstanding assumption based on morphology that mudskippers transitioned onto land in a single cladogenic event is rejected by our molecular analysis. Rather we find, as did all previous molecular studies with sufficient sampling, that terrestriality evolved on at least two occasions: once in the *Periophthalmus/Periophthalmodon* lineage and secondly in the *Boleophthalmus* lineage. We did not find support for the expected ecomorphological and locomotory transition from fully aquatic, to mudswimming, to pectoral-aided mudswimming to lobe-finned terrestrial locomotion implied by earlier morphological analyses. However, details of the transitions that did occur are ambiguous because of rapid cladogenesis and poor phylogenetic resolution in the critical region of the tree. Additional species from this clade of gobies, expanded sequence data, and improved ecomorphological observations and characterizations are needed to resolve the evolutionary transitions. Finally, Oxudercinae should be subsumed as a junior synonym under Amblyopinae.

CRedit authorship contribution statement

Scott J. Steppan: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Anna A. Meyer:** Investigation. **Lisa N. Barrow:** Investigation, Writing – review & editing. **Bader H. Alhajeri:** Resources, Writing – review & editing. **Amani S.Y. Al-Zaidan:** Resources, Writing – review & editing. **Paul M. Gignac:** Conceptualization, Writing – review & editing. **Gregory M. Erickson:** Conceptualization, Investigation, Funding acquisition, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2022.107416>.

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Glossary

Demersal: living close to the floor of the sea or a lake