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Nucleotide sequence data confirm diagnosis and local endemism of variable morphospecies of Andean astroblepid catfishes (Siluriformes: Astroblepidae)

SCOTT A. SCHAEFER[1]*, PROSANTA CHAKRABARTY[2], ANTHONY J. GENEVA[3,4] and MARK H. SABAJ PÉREZ[4]

[1]*American Museum of Natural History, Division of Vertebrate Zoology, Central Park West at 79th St., New York, NY 10024, USA*
[2]*Museum of Natural Science, Louisiana State University, 119 Foster Hall, Baton Rouge, LA 70803, USA*
[3]*Department of Biology, University of Rochester, Rochester, NY 14627, USA*
[4]*Academy of Natural Sciences, Department of Ichthyology, 1900 Ben Franklin Pkwy., Philadelphia, PA 19103, USA*

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Phylogenetic analysis based on nuclear and mitochondrial DNA sequences was used to test the validity of morphospecies of catfishes of the family Astroblepidae inhabiting the southern-most limit of their Andean distribution in the upper Ucayali and upper Madre de Dios river basins. Population samples of morphospecies designated a priori on the basis of morphological features were further diagnosed by the presence of unique and unreversed molecular synapomorphies, thereby confirming species validity for seven of nine cases. Although each are distinguished by unique combinations of morphological features, two morphospecies (designated F and H) cannot be diagnosed on the basis of apomorphic changes in molecular sequence that did not also occur in other astroblepid morphospecies or outgroup taxa. Further, one morphospecies (species G) was recovered as nested within the assemblage of populations sampled from morphospecies F, whose morphological diagnosis does not involve unique or apomorphic characters. In contrast, the absence of corroborating molecular apomorphies for species H, otherwise recognized by distinctive and uniquely derived morphological characters, suggests a history of rapid divergence and insufficient time for fixation of genetic differences. Species sharing syntopic distributions were not recovered as sister groups, and in some cases species distributed in adjacent river drainage basins were not more closely related to one another than to species distributed in more distant drainages. Three independent instances were observed of sister-group relationships involving species distributed in both the Apurimac and Urubamba rivers (Ucayali drainage). These observations combine to suggest that the current distribution of astroblepid species in the southern region may have arisen via a complex history involving both divergence between and dispersal amongst drainage basins that is probably repeated numerous times throughout the Andean distribution of the group.

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ADDITIONAL KEYWORDS: Andes – biogeography – evolution – ichthyology – South America – species concepts – taxonomy.

INTRODUCTION

Astroblepid catfishes represent a distinctive assemblage of species that live at moderate to high elevations in freshwaters of the tropical Andes. Their distribution extends from Panama to Bolivia and across nearly 28° of latitude. Within that range, astroblepids occur in all of the major river drainage systems of the Pacific, Caribbean, and Amazon-Orinoco basins. Most species are of moderate to small

*Corresponding author. E-mail: schaefer@amnh.org

size, typically less than 0.10 m in length, but occasionally reach about 0.30 m as adults. Astroblepids, commonly known as climbing catfishes, are easily recognized by their expanded fleshy oral disk and thickened, highly mobile pelvic fins, with which they adhere to the substratum and locomote in the high-gradient, rapidly flowing streams that characterize their montane habitats. In contrast to their sister group, the mega-diverse catfishes of the family Loricariidae (96 genera, 716 species; Ferraris, 2007), which are widespread in lowland rivers throughout the Neotropics, astroblepids are presently classified in a single genus (*Astroblepus*) and 54 species that are strictly Andean in distribution (Schaefer, 2003). There is no fossil record. With few exceptions, most species of *Astroblepus* have restricted geographical distributions, being limited to portions of single river drainage basins at elevations above 1000 m (Schaefer, 2003). In contrast, amongst the more species-rich genera of the Loricariidae having been the subject of recent taxonomic revisions involving comprehensive examination of material (e.g. *Panaque* – Schaefer & Stewart, 1993; *Otocinclus* – Schaefer, 1997; *Oxyropsis* – Aquino & Schaefer, 2002), a much larger proportion of the specific diversity is represented by species having broader geographical distributions (Ferraris, 2003; Fisch-Muller, 2003; Weber, 2003). The disparities in taxonomic diversity and distribution and the estimated age of divergence between astroblepids and their sister group (approx. 90 Mya; Sullivan, Lundberg & Hardman, 2006) relative to the much younger age (approx. 10 Myr) for higher elevations (above 2 km) in the Andes (Gregory-Wodzicki, 2000; Garzione *et al*., 2008) and rapid rates of recent species diversification observed for some plants at elevation (Hughes & Eastwood, 2006), pose several interesting questions regarding the timing of family-level divergence and rates of evolution within Neotropical catfishes. Furthermore, astroblepids themselves, as an important component of the poorly known and depauperate Andean fish fauna, are potentially important biotic indicators of the health of critically important source headwaters of the major rivers of the Neotropics.

Knowledge of the taxonomy, diversity, and ecology of astroblepid catfishes is rudimentary because there have been no synthetic revisionary studies of astroblepids since the monographic work of Regan (1904). Most of the species are known only from their original descriptions and all but four of the 54 nominal species were described before 1950. At present, it is difficult to distinguish species because most are defined only by single-character contrasts or by overlapping and non-unique combinations of external features that display high levels of inter- and intraspecific variation. During the course of a taxonomic revision of the family conducted by the first author, it became apparent that traits used in defining the morphological limits between astroblepid species, most notably, body shape, fin size and configuration, and pigmentation pattern, are confounded by variation on several levels. For example, observed patterns of morphological variation appear to be the result of complex contributions from multiple intrinsic and extrinsic sources, such as ontogeny, sexual dimorphism, and geographical variation. Pigmentation patterns on the head and trunk, in particular, are highly variable within and amongst species (Fig. 1) to an extent that application of independent sources of data are necessary for evaluating concepts of astroblepid morphospecies defined in part by coloration pattern.

Application of DNA-based approaches to taxonomic questions (Hebert *et al*., 2003) can be useful in these circumstances because the introduction of molecular criteria can supplement classic morphological and behavioural criteria in judging species boundaries and recognizing hitherto undiscovered diversity (DeSalle, Egan & Siddal, 2005). Population genetics approaches are often most appropriate in cases where putative species are highly polymorphic, suggesting that traits may have not become fixed and where gene flow via migration and hybridization operate to oppose segregation and differentiation. As these approaches can be demanding and time consuming, we are most interested in using simplified procedures for assessing species status that avoid making assumptions about divergence threshold (Hebert *et al*., 2003), divergence time (Pons *et al*., 2006), population size or number of generations required to achieve reciprocal monophyly (Hudson & Coyne, 2002), or other attributes of astroblepid populations that are unknown at present. Following DeSalle *et al*. (2005), we reject species delimitation on the basis of distance-based methods (e.g. based on amount or degree of divergence), as opposed to character-based approaches using DNA sequence data, because only the latter are compatible with current taxonomic principles and objective hypothesis tests of species diagnosis.

The goals of this study were to test a priori morphospecies designations of astroblepid catfishes using multigene nucleotide sequence data. We applied the phylogenetic species concept (Nixon & Wheeler, 1990) and used the criterion of autapomorphy (unique, unreversed derived change in molecular sequence; DeSalle *et al*., 2005) in testing the validity of putative species. A phylogenetic analysis of the molecular data set was used to infer the optimization of molecular characters on the tree, although, following DeSalle *et al*. (2005), we did not utilize the pattern of relationships amongst morphospecies in the test of species validity because species need not be

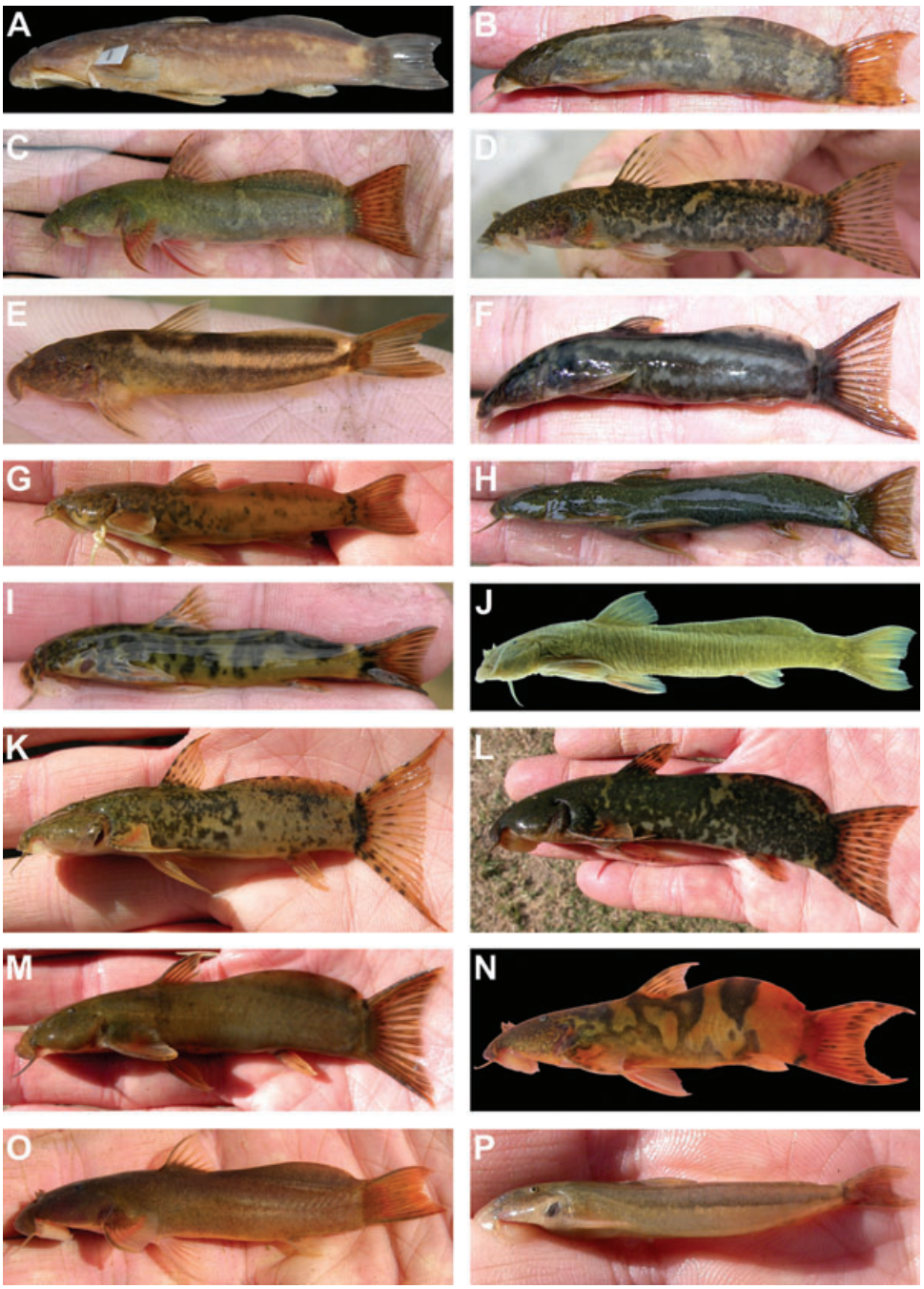

Figure 1. Variation in pigmentation in *Astroblepus* morphospecies A–I. A, morphospecies A, ANSP (Academy of Natural Sciences of Philadelphia) 180586 (4793), 51.6 mm standard length (SL), Araza River. B, morphospecies B, ANSP 180587 (4779), 75 mm SL, Araza River. C, morphospecies B, ANSP 180582 (4801), 80.4 mm SL, Araza drainage (Dr.) D, morphospecies B, ANSP 180582 (4800), 54.5 mm SL, Araza Dr. E, morphospecies C, ANSP 180581 (4805), 27.2 mm SL, Araza Dr. F, morphospecies C, ANSP 180586 (4794), 58 mm SL, Araza River. G, morphospecies D, ANSP 180599 (4822), 51.7 mm SL, Urubamba Dr. H, morphospecies D, ANSP 180602 (4499), 85 mm SL, Urubamba Dr. I, morphospecies H, ANSP 180618 (4423), 46.3 mm SL, Apurimac Dr. J, morphospecies H, ANSP 180616 (4436), 79.2 mm SL, Apurimac Dr. K, morphospecies E, ANSP 180595 (4785), 61.3 mm SL, Urubamba Dr. L, morphospecies E, ANSP 180605 (4490), 110.5 mm SL, Apurimac Dr. M, morphospecies F, ANSP 180606 (4487), 75.7 mm SL, Apurimac Dr. N, morphospecies F, ANSP 180601 (4759), 52.6 mm SL, Urubamba Dr. O, morphospecies G, ANSP 180588 (4787), 59.5 mm SL, Urubamba Dr. P, morphospecies I, ANSP 180607 (4477), 39.4 mm SL, Apurimac Dr. Photo in (A) by S. A. S.; photos in (B–P) by M. H. S. P.

monophyletic (type-C monophyly of Rieppel, 2009). For reasons of efficacy and feasibility, we applied this test to the astroblepid species of southern Peru, the southern limit of the distribution of the family and a key region for understanding the historical and ecological factors that determine astroblepid distribution. The study region is physically and ecologically complex and includes a diversity of landforms and ecoregions, where biotic assemblages are greatly impacted by interactions amongst precipitation, temperature, and topography that vary greatly on regional scales (Killeen *et al*., 2007). These factors combine to define a transition zone in the pattern of distribution and endemism between the south-central and southern Andean biotas (Sarmiento, 1975; Kessler, 2002; López, 2003). Diversity and endemism of astroblepid species in this region is high, with eight nominal and 13 morphospecies distributed in the Madre de Dios, Beni, Ucayali, and Titicaca watersheds.

MATERIAL AND METHODS

Study region and specimens examined

The study region was defined as the freshwaters of the central portion of the Central Andes (Gregory-Wodzicki, 2000) of southern Peru and northern Bolivia between 10° and 18°S latitude (Fig. 2). The study region encompasses the major Andean headwater tributaries of the Amazon lowlands, including the inter-Andean upper Ucayali River and its southern tributaries (Apurimac and Urubamba), and the Madre de Dios and Beni/Madeira rivers of the Amazon fore slope to the south-east. Within the Ucayali drainage, the drainages of the Apurimac and Mantaro rivers on the west are separated from those of the Urubamba River on the east by the Cordillera Vilcabamba, whereas the combined Ucayali drainages are separated from the Amazon fore slope drainages by the Vilcanota, Carabaya, and Apolobamba ranges. Although astroblepids also occur in both the Pacific slope and isolated Titicaca drainages, there are extremely few verified locality records for astroblepid species in these portions of the study region and therefore these taxa were excluded.

Specimens examined were assembled from the major international ichthyological collections with holdings of Andean fishes (Appendix S1; codes for institutional repositories are as listed at http://www.asih.org/node/204). Veracity of locality data associated with the specimen records was checked against multiple gazetteers and literature sources. Locality records were geocoded and input to a geographical information system (ArcView, v. 9.3) and visualized on a three arc-sec digital elevation model

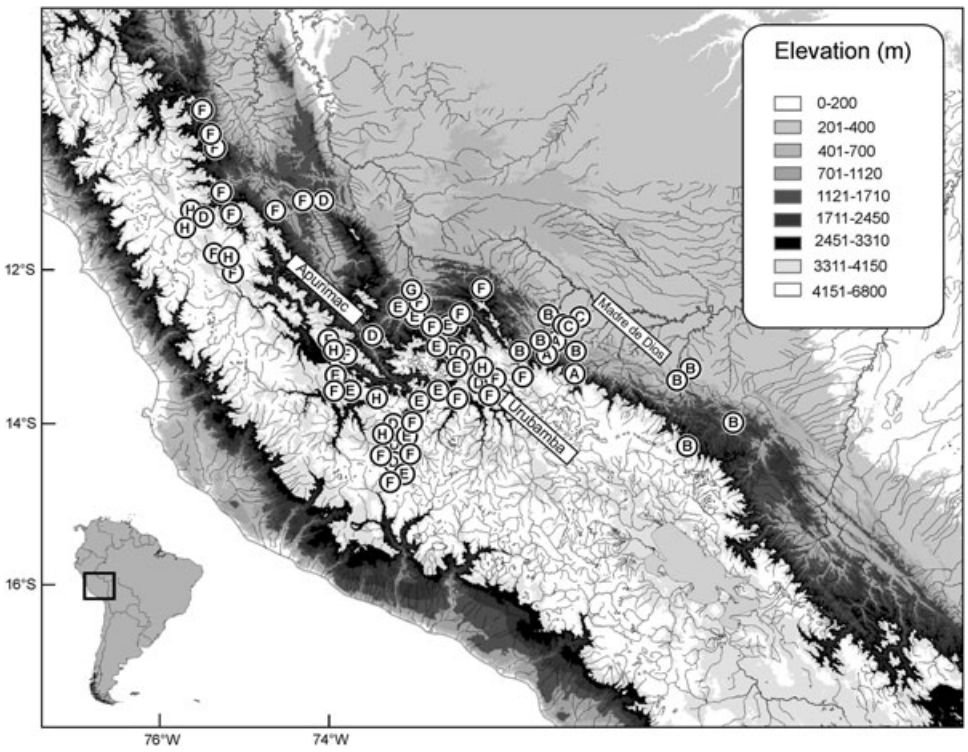

Figure 2. Distribution of astroblepid morphospecies and study region. Circled letters correspond with the morphospecies designations (Table 1) and may represent more than one lot or collection locality.

(DEM) obtained from the USGS/NASA Shuttle Radar Topography Mission (Jarvis *et al*., 2006). Additional specimens were obtained by fieldwork in 2004; these localities were coded in the field by a global positioning system.

Criteria for defining and testing morphospecies

Fixed and discrete states of homologous features were recorded from a variety of external morphological systems and used to assign astroblepid specimens to phenetic morphospecies. Specimens were treated as population samples and morphospecies were recognized by application of the diagnosability criterion (Nixon & Wheeler, 1990): those populations sharing the smallest mutually exclusive set of unique features and/or unique combinations of features. Geographical origin of specimens was ignored when assigning specimens to morphospecies. We used the phylogenetic species concept (Mayden, 1997; de Queiroz, 2007) in the test of morphospecies validity by application of the criterion of autapomorphy (Rosen, 1979; Wheeler & Platnick, 2000). Validity of morphospecies defined a priori on the basis of phenetic criteria was rejected when not further corroborated by the presence of unique and unreversed changes in the independent multigene molecular sequence data.

Molecular data and phylogenetic analyses

A total of 37 samples representing nine astroblepid morphospecies collected from 24 field sites was used in this study (Table 1). Tissues (fin clips, liver, or muscle) were sampled and preserved in 95% ethanol prior to specimen fixation in 10% formalin, or subsequently transferred to 95% ethanol (for long-term storage at –80 °C) from specimens field-preserved in 70% ethanol. Additional voucher specimens were fixed in formalin and transferred to 70% ethanol. Additionally, six samples of astroblepid species collected from localities external to the study area were included, along with four species of Loricariidae as outgroups. Tissue, GenBank, and voucher specimen numbers for all taxa examined are listed in Table 1.

We obtained a total of 3217 base pairs (bp) of DNA sequence from the following genes: recombination activating gene 1 (*Rag-1*; 1355 bp), cytochrome *c* oxidase subunit I (*COI*; 658 bp), cytochrome *b* (*cytb*; 629 bp), and 16S rRNA (*16S*; 575 bp). Total DNA was extracted using a Qiagen DNEasy tissue extraction kit following the manufacturer's protocol. The *Rag-1* fragment was amplified and sequenced using the primers F74, R1333, F354, and R798 as specified in Sullivan *et al*. (2006: Table 1). The *COI* fragment was amplified and sequenced using the primers LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 5′-TAAACTTCAGGGTGACCAAAAA ATCA-3′ (Folmer *et al*., 1994) or Pros1Fwd 5′-TTC TCGACTAATCACAAAGACATYGG-3′ and Pros2Rev 5′-TCAAARAAGGTTGTGTTAGGTTYC-3′ ('COIfor' and 'COIrev' from Chakrabarty, 2006). The *cytb* fragment was amplified and sequenced using the primers ICyt*b*-F1 5′-TTCCTTYCACCCCTATTTCT-3′ and ICyt*b*-R1 5′-CTGGGGTGAAGTTTTCTGGG-3′ (Hardman & Page, 2003). The *16S* fragment was amplified and sequenced using the primers 16S ar-L 5′-CGCCTGTTTATCAAAAACAT-3′and 16S br-H 5′-CCGGTCTGAACTCAGATCACGT- 3′ (Kocher *et al*., 1989; Palumbi, 1996). Double-stranded amplification products were desalted and concentrated using AMPure (Agencourt Biosciences Corp.) or ExoSAO-IT (USB Corp.). Both strands of the purified PCR fragments were used as templates and directly cycle sequenced using the original amplification primers and an ABI Prism Big Dye Terminator Reaction Kit (versions 1.1, 3.1). The sequencing reactions were cleaned and desalted using cleanSEQ (Agencourt Biosciences Corp.) or BigDye X-Terminator (Applied Biosystems Corp.). The sequencing reactions were electrophoresed on an ABI 3730xl automated DNA sequencer. Contigs were built in SEQUENCHER version 4.8 (Gene Codes, Ann Arbor, MI, USA) using DNA sequences from the complementary heavy and light strands. Sequences were edited in SEQUENCHER and BIOEDIT (Hall, 1999), aligned using ClustalX (Larkin *et al*., 2007), and modified by eye. All novel sequences have been deposited in GenBank under accession numbers HM048988-49165 (Table 1).

A total of 3217 aligned bp from the four gene fragments was analysed. Our multigene data set represents an approximate 50 : 50 assemblage of bp drawn from mitochondrial and nuclear markers. Although data derived from mitochondrial genes can be readily obtained and have proven to be effective in diverse studies of fishes (Farias *et al*., 1999; Miya *et al*., 2003), these data are less reliable than nuclear gene markers under situations involving rapid divergence and incomplete lineage sorting of mtDNA haplotypes over relatively short branches, and horizontal transfer of genes across populations (Hudson & Coyne, 2002). Given the absence of pre-existing information on the performance of genomic markers for astroblepid catfishes and lack of insight on their population biology, we therefore adopted a conservative approach and compared the phylogenetic signals provided by the nuclear and mitochondrial data sets both separately and combined under a total-evidence approach (Eernisse & Kluge, 1993; Nixon & Carpenter, 1996; Frost *et al*., 2001) using both maximum likelihood (ML) and parsimony (MP) optimality criteria. ML analyses and bootstrap calculations were

Table 1. Letter designation, relevant specimen and sequence identifiers, and collection localities for the astroblepid morphospecies and outgroups used in the phylogenetic analyses. Tissue number refers to individual specimen tag, voucher number references the ANSP catalogue number unless specified otherwise.

Tissue #	Taxon	Morpho-species	Voucher catalog #	GenBank accession #				Locality
				NCBI Rag1	NCBI CytB	NCBI CO1	NCBI 16s	
4793	*Astroblepus*	A	180586	HM049147	HM049103	HM049061	HM049015	Madre de Dios, R. Araza NE of Marcapata on road to Quince Mil
4799	*Astroblepus*	B	180582	HM049150	HM049106	HM049064	HM049018	Madre de Dios, Q. Miraflores NE of Marcapata
4800	*Astroblepus*	B	180582	HM049151	HM049107	HM049065	HM049019	Madre de Dios, Q. Miraflores, NE of Marcapata
4801	*Astroblepus*	B	180582	HM049152	HM049108	HM049066	HM049020	Madre de Dios, Q. Miraflores, NE of Marcapata
4779	*Astroblepus*	B	180587	HM049140	HM049097	HM049054	HM049008	Madre de Dios, R. Araza, NE of Marcapata
4780	*Astroblepus*	B	180587	HM049141	HM049098	HM049055	HM049009	Madre de Dios, R. Araza, NE of Marcapata
4791	*Astroblepus*	B	180587	HM049145	HM049101	HM049059	HM049013	Madre de Dios, R. Araza, NE of Marcapata
4806	*Astroblepus*	B	180578	HM049154	HM049110	HM049068	HM049022	Madre de Dios, trib R. Araza, vicinity of Quince Mil
4805	*Astroblepus*	C	180581	HM049153	HM049109	HM049067	HM049021	Madre de Dios, Q. Cadena, SW of Quince Mil
4795	*Astroblepus*	C	180583	HM049149	HM049105	HM049063	HM049017	Madre de Dios, Q. Miraflores, NE of Marcapata
4816	*Astroblepus*	C	180569	HM049157	HM049113	HM049071	HM049025	Madre de Dios, Q. Huadjiumbie, vicinity of Quince Mil
4792	*Astroblepus*	C	180586	HM049146	HM049102	HM049060	HM049014	Madre de Dios, R. Araza, NE of Marcapata
4794	*Astroblepus*	C	180586	HM049148	HM049104	HM049062	HM049016	Madre de Dios, R. Araza, NE of Marcapata
4808	*Astroblepus*	C	180579	HM049155	HM049111	HM049069	HM049023	Madre de Dios, trib R. Araza, vicinity of Quince Mil
4809	*Astroblepus*	C	180579	HM049156	HM049112	HM049070	HM049024	Madre de Dios, trib R. Araza, vicinity of Quince Mil
4822	*Astroblepus*	D	180599	HM049158	HM049114	HM049072	HM049026	Urubamba, small creek SE of Quillabamba
4496	*Astroblepus*	D	180602	HM049134	HM049092	HM049048	HM049002	Urubamba, small creek SE of Quillabamba
4499	*Astroblepus*	D	180602	HM049135	HM049093	HM049049	HM049003	Urubamba, small creek SE of Quillabamba
4731	*Astroblepus*	D	180602	HM049136	HM049094	HM049050	HM049004	Urubamba, small creek SE of Quillabamba
4427	*Astroblepus*	E	180428	HM049125	–	HM049039	HM048993	Apurimac, R. Antabamba above confluence with R. Chalhuanca
4490	*Astroblepus*	E	180605	HM049133	–	HM049047	HM049001	Apurimac, R. Apurimac, Cconoc, WSW of Limatambo
4785	*Astroblepus*	E	180595	HM049143	–	HM049057	HM049011	Urubamba, Q. Rosariomayo, W of Quelluono
4736	*Astroblepus*	E	180600	HM049137	–	HM049051	HM049005	Urubamba, R. Amaybamba SE of Quillabamba on road to Ollantaytambo
4436	*Astroblepus*	F	180616	HM049126	HM049085	HM049040	HM048994	Apurimac, Q. Muyu-Muyu 20 km ENE of Chalhuanca
4453	*Astroblepus*	F	180613	HM049127	HM049086	HM049041	HM048995	Apurimac, Q. Pichirhua ca. 30 km ENE Colcabamba (km 417)
4460	*Astroblepus*	F	180611	HM049128	HM049087	HM049042	HM048996	Apurimac, R. Pachachaca S of Abancay
4487	*Astroblepus*	F	180606	HM049132	HM049091	HM049046	HM049000′	Apurimac, R. Sotccomayo/Pincus 25km E of Andahuaylas
4483	*Astroblepus*	F	180608	HM049131	HM049090	HM049045	HM048999	Apurimac, R. Pampas W of Chincheros
4759	*Astroblepus*	F	180601	HM049139	HM049096	HM049053	HM049007	Urubamba, R. Coribeni vicinity Kiteni
4782	*Astroblepus*	F	180594	HM049142	HM049099	HM049056	HM049010	Urubamba, R. Yanatili near confluence with R. Urubamba
4839	*Astroblepus*	F	180594	HM049159	HM049115	HM049073	HM049027	Urubamba, R. Yanatili near confluence with R. Urubamba
4787	*Astroblepus*	G	180588	HM049144	HM049100	HM049058	HM049012	Urubamba, R. Mapitunari N of Kiteni
4750	*Astroblepus*	G	180588	HM049138	HM049095	HM049052	HM049006	Urubamba, R. Mapitunari N of Kiteni
4470	*Astroblepus*	H	180609	HM049129	HM049088	HM049043	HM048997	Apurimac, R. Chimbao upstream of Andahuaylas
4416	*Astroblepus*	H	180618	HM049123	HM049083	HM049037	HM048991	Apurimac, R. Lucre near town of Lucre, NE of Colcabamba
4423	*Astroblepus*	H	180618	HM049124	HM049084	HM049038	HM048992	Apurimac, R. Lucre near town of Lucre, NE of Colcabamba
4477	*Astroblepus*	I	180607	HM049130	HM049089	HM049044	HM048998	Apurimac, R. Pampas,W of Chincheros
6641	*Astroblepus* sp		188865	HM049160	HM049117	HM049074	HM049029	Magdalena, R. San Francisco, Cundinamarca, Colombia
P6058	*Astroblepus* sp		AUM46559	HM049164	HM049121	HM049078	HM049033	Maranon, Q. Siasme, Condorcanqui, Amazonas, Peru
P6059	*Astroblepus* sp		AUM46559	HM049165	HM049122	HM049079	HM049034	Maranon, Q. Siasme, Condorcanqui, Amazonas, Peru
P6041	*Astroblepus* sp		AUM46536	HM049162	HM049119	HM049076	HM049031	Maranon, R. Almendro, Chiriaco, Amazonas, Peru
P6042	*Astroblepus* sp		AUM46536	HM049163	HM049120	HM049077	HM049032	Maranon, R. Almendro, Chiriaco, Amazonas, Peru
P6020	*Astroblepus* sp		AUM46522	HM049161	HM049118	HM049075	HM049030	Maranon, R. Huancabamba, Piura-Cajamarca, Peru
–	*Liposarcus multiradiatus*		INHS54585	DQ492605	HM049116	–	HM049028	Orinoco, C. Maraca, Portuguesa, Venezuela
–	*Farlowella nattereri*		182779	DQ492578	HM049082	HM049036	HM048990	Amazon, R. Solimões, Amazonas, Brazil
4181	*Loricaria simillima*		180498	DQ492607	HM049080	–	HM048988	Madre de Dios, R. Inambari, Cuzco, Peru
4182	*Lamontichthys stibaros*		180635	DQ492602	HM049081	HM049035	HM048989	Madre de Dios, R. Inambari, Cuzco, Peru

ANSP, Academy of Natural Sciences of Philadelphia; AUM, Auburn University Museum; C, Caño; E, east; INHS, Illinois Natural History Survey; N, north; NCBI, U.S. National Center for Biotechnology Information; Q, Quebrada; R, Río; S, south; trib., tributary; W, west.

conducted on individual gene partitions as well as on the concatenated data set in RaxML 7.0.4 using the Cipres Portal v. 1.15 implementing a general time reversible (GTR) + gamma model as recommended (Stamatakis, Hoover & Rougemont, 2008). Partitions were based on gene fragments and codon position, when applicable. The number of bootstrap replicates (250 *Rag-1*, *cytb*; 200 *COI*, 400 *16S*; 150 concatenated data set) was automatically determined during the runs as adequate and rigorous by RaxML for each data set. MP analyses were conducted on the concatenated data set using TNT v. 1.1 (Goloboff, Farris & Nixon, 2008) using traditional heuristic searches, ten random taxon addition sequences, tree bisection reconnection (TBR) with 30 replicates and ten trees per replicate. Indels and substitutions were weighted equally.

RESULTS

Our survey of astroblepid external morphology resulted in the recognition of nine morphospecies (Fig. 1; species designated A–I, material examined listed in Appendix S1). A tenth morphospecies, corresponding to the nominal *Astroblepus longiceps*, was recognized as the sole representative of the genus in Bolivia, but was excluded from the test of morphospecies status because of a lack of tissue samples. Four of the nine morphospecies (A, B, C, G) are restricted in distribution to a single drainage basin, with three species (A, B, C; Fig. 1) occurring sympatrically at multiple localities within the Madre de Dios river system. The remaining five morphospecies (D, E, F, H, I) each have a wider geographical distribution and occur in more than one drainage basin within the study region (Fig. 2).

For the combined data set of 3217 nucleotides, 1007 sites were variable and 766 of these were parsimony informative. ML analysis of the concatenated sequence data run with joint branch length optimization yielded the highest likelihood score of ln –13710.612764 (Fig. 3). For the partitioned data sets, amongst individual trees (not shown), the best scores were ln –2065.012655 (*16S*), ln –3010.527911 (*COI*), ln –3635.814303 (*cytb*), ln –4652.939822 (*Rag-1*). MP analyses on the concatenated data set yielded 28 equally most-parsimonious trees of length = 2186, consistency index = 0.64, retention index = 0.83. The strict consensus amongst these trees yielded a topology identical to that obtained from the ML analysis in terms of recovered species assemblages and relationships amongst the morphospecies. Monophyly of Astroblepidae was strongly supported in all analyses, but the morphospecies of the study region were not recovered as monophyletic because sample 6020 *Astroblepus* sp. (Marañon River) nested within the ingroup at an identical position amongst the ML and MP trees.

Six of nine astroblepid morphospecies designated a priori on the basis of morphological characteristics were recovered as monophyletic in all analyses (Fig. 3). Two of nine morphospecies (A, I) were both represented in the phylogenetic analyses by a single specimen, and therefore monophyly of these species cannot be falsified. Morphospecies G was recovered as nested within a monophyletic assemblage that also included individuals of morphospecies F (Fig. 3). Within the ingroup, most nodes, including those indicative of morphospecies monophyly, were well supported in the bootstrap analyses (bootstrap proportions > 80%). The combined species F+G clade was recovered as the sister group to a well-supported species E. Species B and C were each recovered as monophyletic and placed in a well-supported clade including species E and F+G; that clade sister to one composed of species A, I and sample 6020 from the Marañon. Sister species D and H were recovered as the sister group to the clade inclusive of all other morphospecies and sample 6020.

Seven of the nine morphospecies were each associated with one or more unique and unreversed bp changes amongst the molecular sequences examined. These uniquely derived molecular characters, combined with the unique morphological features or unique combinations of characters, serve to diagnose these seven morphospecies (Table 2). Two of the nine morphospecies (F, H) are not diagnosed by any autapomorphic molecular characters, and therefore fail our test of species status.

DISCUSSION

Our analysis recovered a monophyletic *Astroblepus*, but the nine morphospecies of the study region do not represent a monophyletic assemblage, exclusive of species from other geographical regions. Despite the occurrence of unique combinations of morphological features useful for the identification of all nine morphospecies, our analysis of combined mitochondrial and nuclear gene sequence data sets failed to identify unique molecular characters for two of the nine morphospecies (F and H). Applying the criterion of apomorphy under the phylogenetic species concept (Wheeler & Platnick, 2000), and in the absence of corroboration provided by the molecular data, we would reject species status for these two morphospecies. This outcome is both surprising and illuminating with respect to the utility of the morphological features hypothesized at the outset to define these particular morphospecies.

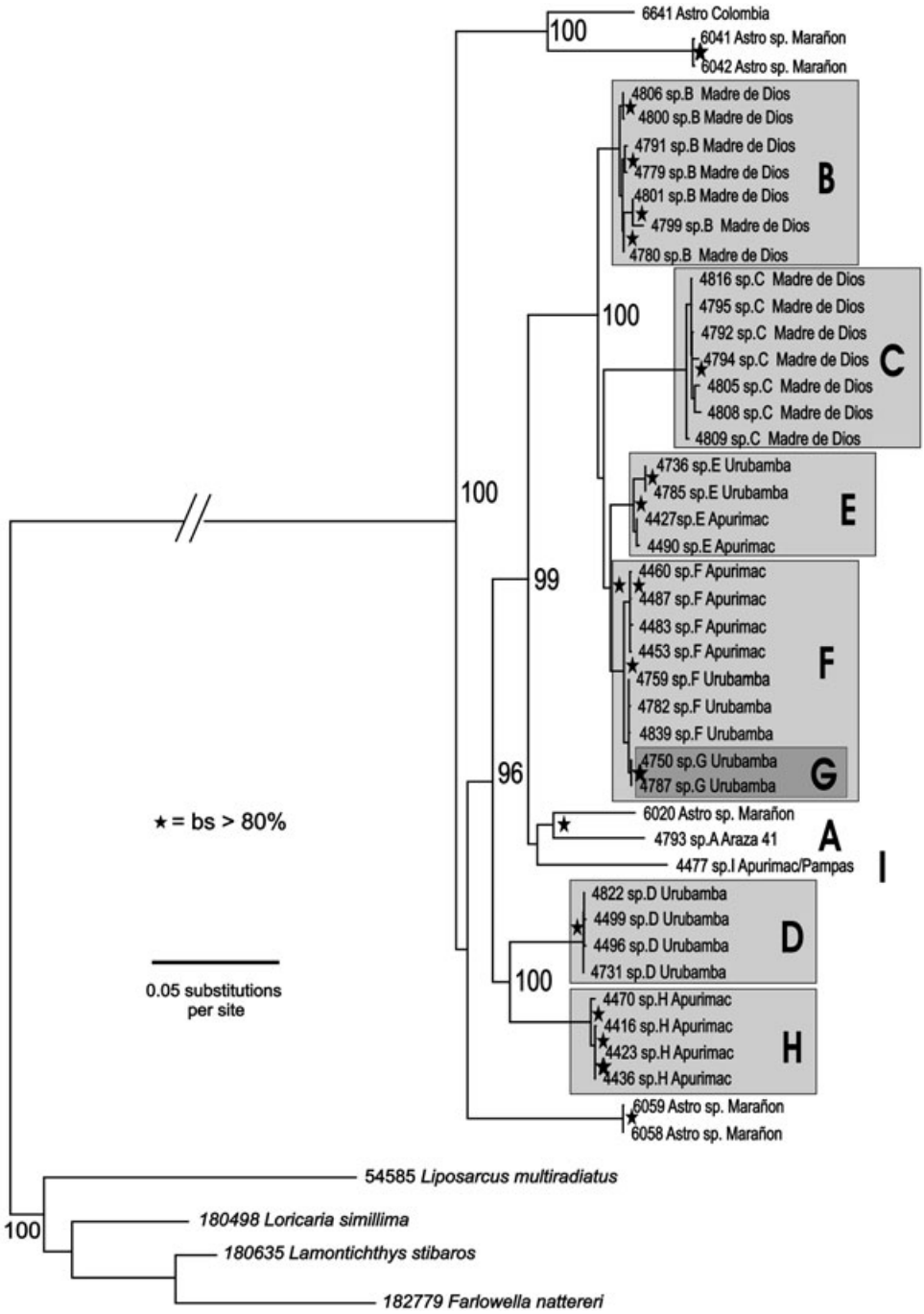

Figure 3. Results of the phylogenetic analysis of astroblepid morphospecies obtained from maximum likelihood analysis of the combined DNA sequence data set. Numerals at nodes represent bootstrap proportions (values less than 50% not shown); stars represent nodes supported by bootstrap values of 80% or greater. Sample numbers correspond with materials listed in Table 1. Letters designate morphospecies; shaded boxes denote monophyletic assemblages of population samples.

The phylogenetic analyses uniformly recovered a nonmonophyletic species F, because of the fact that individuals assigned a priori to species G were recovered as nested within the assemblage of population samples for species F. Although the finding of nonmonophyly for species F does not factor into our test of morphospecies status, because species need not be type-C monophyletic (Rieppel, 2009), the absence of

Table 2. Morphological and molecular diagnosis of *Astroblepus* morphospecies

Taxon	Morphology	*COI* (1–658)	*Cytb* (659–1287)	*16S* (1288–1862)	*Rag1* (1863–3217)
A	Broad symmetrically bifid premaxilla teeth, adipose spine absent, maxillary barbel not to posterior lip margin	(94:T), (277:T), (401:T), (508:G), (571:C), (589:T)	(744:T), (812:A), (815:T), (839:A), (932:G), (1083:G), (1151:T), (1169:C), (1169:G)	(1556:T), (1574:T) (1617:T), (1695:C)	(2010:T), (2180:C), (2274:A), (2416:A), (3133:T)
B	Premaxillary teeth unicuspid, adipose-fin membrane tall, adipose spine absent, maxillary barbel extended beyond posterior lip margin, trunk mottled	(536:T)	(966:T), (1046:G)	–	(2345:G), (2605:A)
C	Maxillary barbel not to posterior lip margin, pectoral-fin rays 11–12	(70:G), (193:T), (532:T), (542:T)	(785:T), (797:T), (824:T), (999:G), (1000:C), (1284:C)	(1400:A), (1705:C)	(2162:A), (2595:A), (2605:G)
D	Maxillary barbel extended beyond posterior lip margin, pore soc3 separated from soc2 by distance less than posterior naris diameter	(40:C), (430:G), (544:T), (586:G)	(763:T), (773:T), (824:G), (833:G), (890:C), (1058:G), (1133:T), (1138:G)	(1715:T)	(2474:T), (2583:A), (2646:G), (2822:T), (2996:A), (3028:T), (3097:C), (3120:C)
E	Dentary covered ventrally by extension of lower lip	(299:A), (359:C)	–	–	(2332:A), (3111:T)
F	Adipose membrane not truncated, continued onto caudal peduncle; adipose spine present; pmx teeth eight to ten; maxillary barbel long, reaching beyond posterior margin of lower lip; dentary not covered by extension of lower lip	–	–	–	–
G	Premaxillary and dentary teeth asymmetrically bifid; adipose spine absent	–	–	–	(2173:G)
H	Mandibular ramus narrow; posterior lip lamina wide, extremely deep	–	–	–	–
I	Adipose membrane absent, adipose spine separate, elevated, maxillary barbel short, not to lip margin, bicoloured pigmentation	(334:A), (550:T),	(671:G), (672:T), (1169:G)	(1530:G), (1561:A), (1574:A), (1637:A), (1704:A)	(1991:I), (2113:C), (2467:T), (2472:C), (2473:A), (2495:A), (2557:A) (2663:A), (2739:A), (2892:A), (3111:T), (3116:A)

Letter designations for morphospecies follow Table 1. Diagnostic features represent unique combinations of morphological characters and unique nucleotide base-pair changes (i.e. unreversed autapomorphies) occurring in the diagnosed taxon and in no other astroblepid or outgroup taxon examined in this study. Sequence position : state specifying molecular characters given in parentheses.

16S, 16S rRNA; A, adenine; C, cytosine; *COI*, cytochrome *c* oxidase subunit I; *Cytb*, cytochrome *b*; G, guanine; pmx, premaxillary; *Rag-1*, recombination activating gene 1; soc, supraoccipital; T, thymine.

molecular synapomorphies for species F is consistent with the finding of paraphyly. Species G is a distinctive, but rare (undescribed) species known only from two proximate collection sites separated by 2 km distance in tributaries of the Río Consebidayoc of the upper Urubamba River drainage. It is diagnosed amongst morphospecies by the presence of distinctive asymmetrically bifid teeth and absence of an adipose spine. These features are absent in representatives of species F, which in turn is distinguished by a combination of morphological characters (Table 2), none of which alone represent apomorphies or features unique to morphospecies F. Our a priori hypothesis of species F distinction is not corroborated by the presence of autapomorphic molecular characters. Although our samples of species F and G do not represent strictly sympatric populations, the two species nevertheless co-occur in a relatively short (21.4 km) section of the same upper Urubamba tributary and therefore sympatry of these two species is likely (as occurs for multiple astroblepid species elsewhere in their distribution range) and could be tested upon additional fieldwork.

The case involving morphospecies H is even more surprising, given the nature of its definition on the basis of distinctive and unique morphological features (i.e. narrow mandibular ramus and wide, deep posterior lip; Table 2) and characteristic distribution in high-elevation streams. Although monophyly of the population samples of species H was well supported in both ML and MP analyses of the sequence data, we found no apomorphic molecular characters with which to diagnose this species. As suggested by the relatively long branch length associated with the species H assemblage, this implies the presence of numerous homoplastic (non-unique, reversed) changes in the molecular sequences in the lineage leading to the node inclusive of all species H samples (Fig. 3). Both species D and species H are occupants of extreme headwater, high elevation habitats. Species H is known to occur at elevations from 2530 to 3900 m within the Apurimac drainage, whereas species D has a much broader distribution range, known from 1500 to 4200 m elevation and occurring in both the Apurimac and Urubamba drainages. The apparent allopatry of these sister species between the Apurimac and Urubamba drainages, combined with the presence of unique morphological characters in both species, suggests that the absence of corroborating molecular apomorphies in species H may be the result of rapid divergence from a common ancestor shared with species D and insufficient time for fixation of genetic differences between incipient species. Alternatively, this finding may represent little more than our failure to capture the genomic divergence between species in the particular gene fragments targeted by our analyses. These hypotheses, as well as the proposition of separate status for species H, must be subjected to further analysis using additional sources of data.

Although our phylogenetic analysis was restricted to a small portion of the species diversity of the group (nine of approximately 70 species), a number of interesting phylogeographical patterns were discovered. First, those species sharing sympatric distributions within a particular drainage system were not always recovered as sister taxa. Species A, B, and C co-occur in multiple locations within the Madre de Dios river system and all three were collected at a single site in one particular tributary, the Araza River. In the phylogenetic analyses, all three species were each recovered as more closely related to species assemblages with representatives inhabiting river systems external to the Madre de Dios (i.e. the Marañon and Apurimac/Urubamba, respectively) than to other Madre de Dios species. Second, we recovered three independent instances of sister-group relationship involving species distributed in both the Apurimac and Urubamba rivers (species D+H, F, E). We discuss each of these patterns in turn.

In the ML analysis (Fig. 3), species A was recovered as sister to a representative of a species from the Marañon River, collected from a locality well outside the study region and separated by some considerable geographical distance to the north-west. That species pair is most closely related to species I, although recovered without strong support. This result suggests broader clade membership of at least a portion of the southern astroblepid fauna. In the MP analysis, the inter-relationships amongst these three species was not resolved. Both species A and I were each represented in our phylogenetic analysis by a single sample. Species A is known from four localities and a total of 30 preserved specimens, whereas species I is known from four localities and a total of four specimens. Although we would obviously prefer to judge species validity on the basis of more complete sampling of these morphospecies, we note nevertheless a relatively large number of unique and unreversed molecular sequence changes as additional support for the recognition of these two species (Table 2). Species A differs from all congeners in the study region in the presence of highly distinctive chisel-shaped symmetrically bifid jaw teeth, whereas species I differs from congeners in the presence of a highly distinctive adipose fin, spine configuration, and bicoloured pigmentation (Fig. 1P; dusky above lateral line, pale below). Samples of both species are associated with relatively long branch lengths in the ML tree (Fig. 3).

Species C (Madre de Dios) was recovered (although with low support) as the sister group to a well-supported clade comprised of species E+F+G

(Apurimac+Urubamba rivers), with species B (Madre de Dios) recovered as the sister group to that assemblage. Species D (Urubamba) and H (Apurimac) were recovered as sister species and that clade was strongly supported as the sister group to all other ingroup species. Separate reciprocal geographical clades (Apurimac, Urubamba) were recovered for the population samples of species E and F+G, although without strong support in all analyses. Species E inhabits low to middle elevations, occurring from 689 m in the Urubamba drainage to 2297 m in the Apurimac. Urubamba representatives of species E inhabited small streams, whereas Apurimac representatives were found along the margins of larger rivers. Species F also occurred largely below 2300 m to as low as 560 m in the Urubamba, although a few records in the Apurimac exceeded 2500 m elevation (e.g. as high as 2643 m in Sotccomayo/Pincus River). Intraspecific coloration pattern in species F varied widely in the Urubamba, from uniform grey or brown (Fig. 1M) to boldly mottled or marbled with reddish-orange undertones (Fig. 1N). High levels of variation are perhaps most exemplified by the presence of the full range of coloration patterns exhibited by specimens collected together at a single location (e.g. ANSP 180594, 180601; images showing additional examples of intraspecific variation in coloration are archived at http://silurus.acnatsci.org/ACSI/field/Peru2004/fish/Astroblepidae/index_22-36.html).

Our results provide independent character evidence that support the hypothesis of morphospecies in seven of nine cases represented in the study area. These results, evaluated within the context of the distribution of the species, further indicate that astroblepid species are typically restricted in geographical distribution and endemic to single or adjacent river systems of the Andes Mountains. As also observed for the astroblepid fauna of the northern and central portions of the Andean Cordilleras (e.g. *Astroblepus orientalis*, *Astroblepus phelpsi, Astroblepus frenatus*; Schaefer, 2003), species distributions generally do not cross the major headwater divides amongst drainage basins (e.g. those separating the Ucayali and Madre de Dios watersheds), many of which involve elevations above the altitudinal limits of the Andean fish fauna. Likewise, astroblepid species are limited at the opposite, lower extreme of their altitudinal range by ecological conditions and physiological limits to life in warm water (Schaefer, 2011). Of the six species endemic to the Ucayali watershed, only three species (D, E, and F) have relatively broader distributions that include both the Apurimac and Urubamba drainages within the more inclusive Ucayali system. Constrained distributions at both extremes of the elevation range combine to limit astroblepid species to drainage islands within the Andean cordilleras, thereby promoting isolation and divergence on relatively small spatial scales. The temporal scales of astroblepid divergence and speciation have yet to be directly examined in detail.

These observations combine to suggest that the current distribution of astroblepid species in the southern region may have arisen via a complex history involving both divergence between and dispersal among drainage basins that is probably repeated numerous times throughout the Andean distribution of the group. Upon inclusion in future analyses of additional representatives of species from other geographical regions, we would expect to recover additional clades and expanded sets of relationships amongst groups of species beyond those recovered in this limited analysis. The sorting of population samples by drainage within morphospecies (E, F) indicates that these particular species should be re-evaluated for the presence of undetected morphological differences that are potentially congruent with the observed geographical pattern of divergence within species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Material examined.

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