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SPECIES BOUNDARIES AMONG THE *TELMATOBIUS* (ANURA: LEPTODACTYLIDAE) OF THE LAKE TITICACA BASIN: ALLOZYME AND MORPHOLOGICAL EVIDENCE

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ABSTRACT: The frog genus *Telmatobius* represents the radiation of 49 recognized species confined to high elevation aquatic habitats in the Andes Mountains of South America. The genus has not been shown to be monophyletic, and issues of species boundaries and relationships are poorly resolved. In this study, we sampled the four recognized subspecies of *T. albiventris* from Lake Titicaca, and other species (*T. crawfordi, T. culeus,* and *T. marmoratus*) from within the lake and the greater Titicaca Basin. We used allozyme characters and multivariate assessments of morphometric variation to test species boundaries in this complex. Our results suggest that four evolutionary species can be recognized: *T. culeus* (which includes *T. albiventris* and *T. crawfordi*), *T. marmoratus, T. affinis halli,* and *T.* sp. nov. Unique combinations of allozyme and morphometric variables can distinguish these, but only the sample of *T. culeus* is large enough, relative to the proportion of fixed characters, to evaluate distinctness of diagnostic characters with a high degree of statistical confidence ($\alpha = 0.05$). Phylogenetic analysis of 10 polymorphic allozyme loci revealed weak support for monophyly of Lake Titicaca *Telmatobius.* All taxa from Lake Titicaca examined here (under the name *T. culeus*) are interpreted as a single evolutionary species, but differences in body sizes and microhabitats provide circumstantial evidence for adaptation along ecological resource gradients. The possibility of ecological speciation among different morphs within the lake is considered, along with evidence needed to test this hypothesis.

Key words: *Telmatobius*; Allozymes; Null alleles; Morphometrics; Species boundaries; Resource niche polymorphism; Lake Titicaca

THE frog genus *Telmatobius* Wiegmann, 1835 contains 49 currently recognized species typically found in the Andes Mountain Range at altitudes above 2500 m (Lynch, 1986). Geographically, the genus extends from northern Ecuador (02° 05′ S) to northern Argentina (29° 39′ S) and Chile (22° 51′ S) (Benavides, 1999; Lavilla, personal communication). There is no clear evidence for monophyly of the group, as diagnosed by hypothesized synapomorphies (de la Riva, 1994; Formas et al., 1999; Lynch, 1978; Trueb, 1979; the possible synapomorphies suggested by Wiens, 1993, were rejected by Formas et al., 1999), and most species have been assigned to the genus on a phenetic basis and following typological criteria, increasing confusion regarding the identification of species (e.g., Laurent, 1970; Vellard, 1951, 1953, 1960).

Historically, Lake Titicaca was a primary destination of early expeditions to the central region of the Andes (Agassiz and Garman, 1876; D'orbigny, 1835–1847; Gilson, 1939; Pentland, 1848). Specimens collected during those trips have led to the description of four species of *Telmatobius*

from the Titicaca Basin. Two of these are primarily lacustrine species: *T. albiventris* (Parker, 1940) and *T. culeus* (Garman, 1876), whereas *T. marmoratus* (Dumeril and Bibron, 1841) and *T. crawfordi* (Parker, 1940) are semi-aquatic species inhabiting streams and shallow lagoons of the greater Titicaca Basin.

Although Vellard's thorough revision of Andean *Telmatobius* predates the current phylogenetic paradigm, he recognized the four above-mentioned species as a single, albeit highly polymorphic, evolutionary lineage (Vellard, 1951, 1953, 1960, 1969). The current taxonomic arrangement is based on a combination of polymorphic morphological characters and more stable ecological features (Vellard, 1953, 1960). Species of *Telmatobius* from the Titicaca Basin have also been affiliated to a presumably undifferentiated lowland taxon (2500 m; approximately 250 km east of Lake Titicaca), *T. marmoratus hintoni* (Parker, 1940), through a number of subspecies that represent intermediate morphs between each species group, often in questionable allopatry. The outcome of this arrangement was the rather arbitrary species boundaries among all taxa described, a point that has been repeatedly emphasized; "les coupes spécifiques et sous-spécifiques sont très difficiles à établir et deporvues de toute valeur réelle" ("specific and subspecific boundaries are hard to establish, and they lack any real value"; Vellard, 1952). All of the species in the Titicaca Basin are thought to be part of an *albiventris-crawfordi-culeus-marmoratus* complex (Cei, 1986), but within this group there has been no rigorous evaluation of species boundaries.

Within Lake Titicaca, *T. albiventris* is geographically restricted to shallow water areas around the periphery of the Lago Grande (Fig. 1). Initially described as a subspecies of *T. culeus,* it was later assigned to specific rank based on ecological and morphological differences (Vellard, 1951). It is confined to shallow water habitats, has a less emarginated toe webbing, possesses a dorsal disc [the thickened dorsal structure described by Angel, 1926 (pp. 108–109) and Parker, 1940 (p. 210)], paler ventral coloration, and smaller adult size than *T. culeus* (Table 1). In addition to the nominate form, *T. albiventris albiventris* (Parker, 1940) from the shoreline of the Isla de Taquiri in Lago Pequeño, three additional subspecies are currently recognized: *T. a. globulosus* Vellard, 1960, is confined to Playa Copani, in the southern embayment of the lake; *T. a. punensis* Vellard, 1951, applies to a form inhabiting Bahía de Puno; and *T. a. parkeri* Vellard, 1951, is restricted to the shallow Laguna Arapa (Fig. 1). Intraspecific diagnosis of these subspecies is ambiguous and based mainly on locality, although size and selected body proportions are presumably different between some taxa (Vellard, 1960). Furthermore, the subspecies in Lago Pequeño are, in all probability, sympatric (*T. a. albiventris* and *T. a. globulosus*; Vellard, 1960), and Laguna Arapa and Titicaca Lake may be connected in exceptionally rainy years (Cei, 1986).

Telmatobius culeus is the most distinct species within Lake Titicaca; it was described on the basis of specimens captured in deep water far from the shoreline (Garman, 1876), and it is characterized by unusual morphological features presumably related to life in this habitat (Hutchinson, 1982; Hutchinson et al., 1976; Macedo, 1960). The nominate form is well characterized by distinct phenotypic features such as large body size, a large flattened head with a round snout, a thick dorsal disc, smooth skin with a richly vascularized surface, and highly developed lateral dermal folds (bagginess). This form is found along the eastern coast of Lake Titicaca, but it extends partially into Lago Pequeño through the Estrecho de Tiquina (Fig. 1). However, it is absent from shallower areas in the southern basin, and from the Laguna Arapa and the Bahía de Puno, in the northern basin. Other subspecies are present in rivers flowing into the lake (*T. culeus fluviatilis, T. culeus dispar*) and in some small lagoons elsewhere within the basin (*T. culeus escomeli, T. culeus lacustris*). More distant from Lake Titicaca, *T. culeus exsul* was described on the basis of one specimen, taken from isolated warm springs near the Rio Yura at 2500 m, about

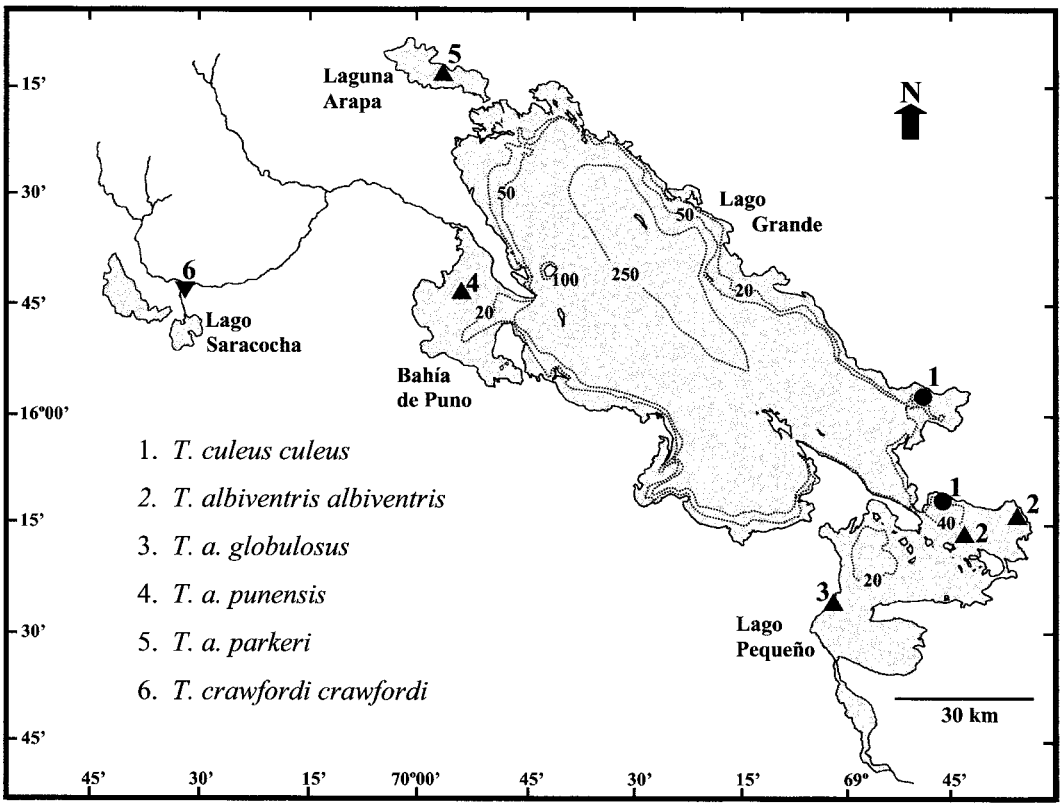

FIG. 1.—Map of Lake Titicaca, showing collection site for samples used in this study. Exact localities include: *T. culeus* (1; Achacachi Bay and Minor Lake), *Telmatobius albiventris albiventris* (2; Huatajata Bay and Taquiri Island), *T. a. globulosus* (3; Copani, Minor Lake); *T. a. punensis* (4; Puno Bay, Major Lake) and *T. a. parkeri* (5; Lake Arapa), and *T. crawfordi* (6; Saracocha Lake). Numbers correspond to those given in Table 1.

200 km east of Lake Titicaca. Sinsch et al. (1995) recently completed a multivariate morphometric assessment of these type specimens, and they placed all five subspecies, including *T. c. exsul*, as junior synonyms of *T. culeus*.

A third species, *T. crawfordi* Parker 1940, is found in small ponds near Lago Saracocha about 70 km northwest of Lake Titicaca, at 4200 m (Fig. 1). It is a dwarf form (Table 1), and diagnostic characters include a spotted ventral color pattern (Vellard, 1960, his plate IX), and the absence of dermal folds. Besides the nominate form, Vellard (1953) also described one additional subspecies: *T. crawfordi semipalmatus* from Laguna Chajchora, in the vicinity of the type locality (5 km east of Lago Saracocha), which is taxonomically inconsistent with the unequivocal sympatry of these two forms (Cei, 1986).

Finally, the extremely polytypic *T. marmoratus* occupies a wide variety of habitats, and it can be found under stones in shallow pools, streams, and along rivers over a large elevational gradient on the high Andean Plateau, and including some lower-elevation montane environments (Cei, 1986; Vellard, 1969). The nominate form is clearly defined by the lack of a dorsal disc and dermal folds (no skin bagginess), the absence of completely webbing on the feet, the presence of a small, round metatarsal tubercle, a rugose dorsum, and a medium body size. Nine subspecies are currently recognized. *Telmatobius marmoratus marmoratus* is found in small ponds near Azangaro, about 75 km north

TABLE 1.—Localities, sample sizes (for allozymes and morphometric measurements), and selected ecological and morphological data for populations of *Telmatobius* sampled in this study. Values for snout–vent lengths (SVL) reported as mean ± SD and ranges (in parentheses). Locality numbers 1 to 6 correspond to those plotted in Fig. 1.

Locality (elevation in m)	Taxon	Allozyme/ total	Habitat	Water depth	SVL (mm)	Voucher numbers
(1) Bahia de Achacachi, Lago Menor (3800)	*T. culeus*	15/40	Lacustrine	>10 m	104.05 ± 16.20 (74.82–137.95)	BYU 46532–33, 46548–50; MZUC 15580, 15601–07, 15609–14, 25538, 25493, 25495–96, 25576–9, 25581–83, 25585-86, 25588–91, 25595–99,
(2) Isla Taquiri (3800)	*T. a. albiventris*	15/22	Lacustrine	<10 m	63.94 ± 9.06 (46.10–89.22)	BYU 46759–68; MZUC 25739–44, 25592–94, 25599, 25608, 25613
(3) Playa Copani (3800)	*T. a. globulosus*	24/42	Lacustrine	<2 m	58.61 ± 3.23 (47.27–66.66)	BYU 46534–47; MHNSM 7747; MZUC 25433–59
(4) Bahía de Puno (3800)	*T. a. punensis*	24/48	Lacustrine	<10 m	63.07 ± 5.79 (54.24–83.10)	BYU 46618–24, 46629–30, 46632; MHNSM 12421, 23; MZUC 25420–32, 25520–33, 25536–37, 25480, 25482, 25484–87, 25491
(5) Laguna Arapa (3800)	*T. a. parkeri*	23/28	Lacustrine	<10 m	65.23 ± 5.63 (56.43–76.81)	BYU 46609–17, 46633–35; MHNSM 7653–54; MZUC 25460–63, 25466, 68, 25472–75, 77, 79, 25481, 89
(6) Lago Saracocha (4500)	*T. crawfordi*	26/28	Pond, stream	<0.5 m	51.22 ± 2.59 (47.08–57.13)	BYU 46554–81
(7) Zongo (4200)	*T. marmoratus*	23/31	Stream	<0.5 m	53.52 ± 6.60 (36.95–74.61)	CBF 457–58, 466–70, 477–78, 486, 1009–1011, 3244–55; MZUC 25552–57
(8) Vilama (2500)	*T. affinis halli*	18*/20	Stream	<0.5 m	45.31 ± 4.57 (31.19–50.81)	CBF 3760–62; MZUC 25725–38, 25104–06
(9) Puquios (4150)	*Telmatobius* sp. nov.	19*/31	Thermal pond	<0.5 m	39.28 ± 2.56 (31.62–43.20)	CBF 03757–59; MZUC 25261–78, 25094–103

* These allozyme totals include tadpoles.

of Lake Titicaca. Three subspecies are thought to be present in different parts of Lake Titicaca: *T. m. angustipes, T. m. riparius,* and *T. m. rugosus,* all of which may be in contact with the widespread *T. m. marmoratus.* Three other forms are restricted to eastern slope drainage of the Andes (i.e., *T. m. microcephalus, T. m. pustulosus,* and *T. m. pseudojelski*), and another, *T. m. hintoni,* is confined to western drainages of the Andes. Finally, *T. m. gigas* Vellard, 1969, was described on the basis of a single female from Huallamarca, Oruro, Bolivia, about 175 km south of Lake Titicaca. This taxon shows all typical features of the deep-water morphs of *T. culeus* of Lake Titicaca, including large body size (SVL = 109 mm), flattened head with a rounded snout, well-developed dorsal disc, and prominent lateral skin folds. Recent exploration of the type locality has confirmed the continued existence of *T. m. gigas* (de La Riva, personal communication). Surprisingly, it is a stream-dwelling form found in ponds of 0.6–1.0 m deep, but its real identity cannot yet be ascertained.

In summary, neither Parker (1940), Cei (1986), nor Vellard (1953, 1960, 1991) reached firm conclusions regarding the species and subspecies status of the frogs of Lake Titicaca. All stressed the extensive morphological variation and the lack of distinct taxonomic boundaries among the widely intergrading populations of *Telmatobius* from the Titicaca Basin and surrounding region. In addition, the description of presumably "transitional" morphs such as *T. a. parkeri* (thought to represent an intermediate morph between *T. a. albiventris* and *T. culeus*; Vellard, 1953), and *T. m. angustipes* and *T. m. rugosus,* (considered intermediate between *T. culeus* and *T. marmoratus*; Vellard, 1953), renders even more difficult the interpretation of species boundaries among populations in Lake Titicaca.

Species of *Telmatobius* are generally conservative with respect to morphological characters, and adaptation to a fully aquatic life style has probably resulted in convergence in features such as coloration, general morphology, and behavior. This makes it difficult to find useful characters for sorting out species other than size, presumably body proportions, and to a certain extent, osteological characters. Trueb (1979) has shown that most of the morphological characters upon which species and subspecies diagnoses were made are sexually, seasonally, or ontogenetically variable, and as a rule, ambiguous descriptions, discordant patterns of intra- and interspecific variation, and inadequate diagnoses are typical for all but the most recently described taxa of *Telmatobius* (Salas and Sinsch, 1996; Trueb, 1979; Wiens, 1993).

In this context, the aim of this study is to test species boundaries in the completely lacustrine taxa of *T. albiventris* and selected other taxa of the *albiventris-crawfordi-culeus-marmoratus* "complex", by extensive populational sampling, and comparisons of multiple samples with specimens from type localities. We used allozyme characters and a multivariate assessment of morphometric variables, and we adopted the evolutionary species concept (Frost and Hillis, 1990; Frost and Kluge, 1994; Wiley, 1978) as the philosophical justification for species recognition.

Materials and Methods

Collection of Specimens

Samples (n = 290 frogs) from nine populations representing six putative species of *Telmatobius* were collected between August 1997 and February 1998. Populations from Lake Titicaca (14° 17′ S, 68° 71′ W) were collected from type localities for all recognized taxa except *T. marmoratus* (Table 1). One distant population of *T. m. marmoratus* was, however, collected from 75 km southeast of Lake Titicaca at 4200 m on the road to Valle de Zongo. Two other taxa were used as outgroups for the Lake Titicaca populations. *Telmatobius affinis halli,* previously confused with *T. halli* Noble, 1938 (Benavides, 1999), was collected from Arroyo Vilama (22° 51′ 432″ S to 68° 23′ 256″ W; 2600 m), near San Pedro de Atacama (Chile). A second population, *T.* sp. nov, was collected from a small, warm spring at Puquios (21° 00′ 42″

TABLE 2.—Enzymes, enzyme commission numbers (following recommendations of Murphy et al., 1996), enzyme abbreviations, number of putative loci, buffer systems (1. TBE-NAD, pH 9.1; 2. Tris-Citric, pH 7.0; 3. Lithium hydroxide, pH 8.0), and tissue sources (L = liver, M = skeletal muscle) used in this study.

Enzyme (E.C. number)	Abbreviations	No. of loci	Buffer	Tissue
Iditol dehydrogenase (1.1.1.14)	IDDH	1	2	M
Glucose-6-phosphate isomerase (5.3.1.9)	GPI	1	1	M, L
Phosphoglucomutase (5.4.2.2)	PGM	1	3	M, L
Glycerol-3-phosphate dehydrogenase (1.1.1.8)	G3PDH*	2	2	M
Malate dehydrogenase (1.1.1.37)	MDH	2	2	M, L
Superoxide dismutase (1.15.1.1)	SOD	2	3	M
Malate dehydrogenase ($NADP^+$) (1.1.1.40)	MDHP	2	2	M
Aconitate hydratase (4.2.1.3)	ACOH	2	2	M
Lactate dehydrogenase (1.1.1.27)	LDH	2	2	M, L
Esterase (3.1.1.—)	EST	2	2, 3	L
Leucine-aminopeptidase (3.4.11.1)	*LAP*	*2*	*3*	*M*
Aspartate aminotransferase (2.6.1.1)	*AAT*	*2*	*1, 2*	*L*
Alcohol dehydrogenase (1.1.1.1)	*ADH*	*1*	*1*	*M*
Phosphogluconate dehydrogenase (1.1.1.44)	*PGDH*	*1*	*2*	*M*
Isocitrate dehydrogenase (1.1.1.42)	*IDH*	*2*	*2, 3*	*M, L*

* This locus was fixed for a duplication in all samples, and displayed the expected three banded "heterozygote" isozyme pattern (see details in Sites and Murphy, 1991).

S to 68° 23′ 256″ W; 4150 m approximately) 15 km northwest of Ollague, on the Bolivian border; this taxon is currently being described by Benavides et al. (unpublished). The two last localities are part of the Provincia El Loa, II Region de Antofagasta, Chile. Most localities are shown in Fig. 1, and all are described in Table 1, along with some relevant ecological and morphological observations. Voucher specimens are cataloged in the Colección Boliviana de Fauna (CBF), Museo de Historia Natural Universidad Nacional Mayor de San Marcos (MHNSM), Museo de Zoología de la Universidad de Concepción (MZUC), and M. L. Bean Life Science Museum, Brigham Young University (BYU).

Protein Electrophoresis

Samples of liver and skeletal muscle were extracted from a subsample (n = 187) of freshly killed specimens and stored at −80 C. Small pieces of tissue were ground manually and diluted 1:1 (muscle) or 1:3 (liver) with 0.0099 M Tris-0.018 M EDTA, pH 6.8 plus 1% NADP (w/v). Homogenates were centrifuged at 15,000 rpm for 5 min, and supernatant was stored for later use. The tissue extracts were analyzed by standard techniques of horizontal starch gel electrophoresis (Murphy et al., 1996; Selander et al., 1971). Three buffer systems were used: (1) TBE-NAD 9.1 (electrode and gel): 175 mM Tris-17.5 mM boric acid, 2.75mM EDTA plus NAD 100 mg (gel), 60 mg (electrode); (2) TC-7, electrode: 0.135 M Tris, 0.043 M citric acid, gel: 1:9 electrode buffer; and (3) LiOH, electrode: 0.06 M lithium hydroxide, 0.3 M boric acid, pH. 8.1; gel: 0.03 M Tris-0.05 M citric acid, pH 8.5, 1:9 electrode; and procedures for staining followed Richardson et al. (1986), Morizot and Schmidt (1990), and Murphy et al. (1996). All enzymes, tissue sources, and buffer systems are listed in Table 2.

Population Genetic Analysis

Electromorphs of any given locus were considered homologous if they had the same mobility. Loci were numbered from anode to cathode and electromorphs were labeled a, b, c, etc., in order of increasing anodal mobility. Individual genotypes were used to calculate electromorph frequencies, proportion of polymorphic loci, average heterozygosities, mean number of electromorphs per polymorphic locus, and genetic identity and distance coefficients (Nei, 1978). We then tested goodness of fit to Hardy-Weinberg proportions by means of a *U* exact test with heterozygote deficiency as the alternative hypothesis (Rousset and Raymond, 1995); the significance of this test was evaluated by a Mar-

kov chain method (Guo and Thompson, 1992). We estimated linkage disequilibrium using Fisher's exact test (Garnier-Gere and Dillman, 1992), and for those populations interpreted as possibly conspecific (see criteria below), we tested genetic differentiation by using an exact probability chi-square test (Raymond and Rousset, 1995*a*). We also calculated the *F* statistic ($\theta - P$) of Weir and Cockerman (1984), and from this we estimated the number of migrants per generation using the private allele method of Barton and Slatkin (1986). We implemented all calculations with the GENEPOP-3.1b (Raymond and Rousset, 1995*b*) and GDA-1.0 (Lewis and Zaykin, 1997; see http://lewis.eeb.uconn.edu/lewishome/gda.html) computer programs.

Phylogenetic Analysis

We performed a phylogenetic analysis of all samples, based on the allozyme matrix in which four loci with "fixed" alternative states (Mdh-2, Est-2, Acoh-2, and G3pdh–1 in Table 3) were entered into a PAUP matrix as characters, and with alternative electromorphs coded as unordered states. The remaining six polymorphic loci were coded by transforming the electromorph frequencies of each locus into Manhattan distances, which served as weighted values in a step matrix for each of these loci (da Silva and Sites, 2001; Wiens, 1995). This approach incorporates frequency information and permits the use of multistate characters, and frequency-based methods (distance, likelihood, or parsimony) appear to provide the most accurate estimates of phylogeny (Wiens, 2000; Wiens and Servedio, 1997, 1998).

Morphometric Data Analysis

We used 17 morphometric variables to quantify morphological differentiation among and within Lake Titicaca Basin samples. The two distinct southern taxa, *T. affinis halli* and *T.* sp. nov., were used as comparative groups in building principal components and discriminant functions. We assessed sexual maturity on the basis of the development of nuptial excrescences in males. Measurements of external features were taken with a digital Mitutoyo caliper to the nearest 0.05 mm, and abbreviations used throughout are: SVL (snout–vent length); TIBL (tibia length); FOOT (foot length); HLEN (head length); HWID (head width); LJW (lower jaw width); SED (snout–eye distance, anterior edge of eye to tip of snout); IOD (interorbital distance); ENOS (eye–nostril distance, anterior corner of eye to posterior edge of naris); IND (internarial distance); EYE (eye diameter); HNDL (hand length); RDL (radioulnar length, flexed elbow to proximal edge of outer palmar tubercle); FEML (femur length, from vent to flexed knee); HUML (humerus length, from the body central axis to flexed elbow); NMD (naris–mouth distance), and WEBL (web length, proximal edge of the inner metatarsal tubercle to distal edge of the III–IV toe web). We measured all bilateral characters on the right side. All measurements are illustrated in Fig. 2.

Because selection presumably acts upon whole phenotypes and not on single characters, we chose a multivariate approach to assess size and shape variation separately. Three procedures, Multivariate Analysis of Variance (MANOVA), Principal Component Analysis (PCA), and Discriminant Function Analysis (DFA) were used to answer three questions regarding morphometric variation among nine samples of *Telmatobius*. First, are samples a priori assigned to named taxa morphometrically distinct? Second, are taxa recognized by allozymic criteria also morphometrically differentiated? Finally, upon which characters is this differentiation based? To answer these questions, we assessed patterns of morphometric variation in a hierarchical design that was suggested by the existing taxonomy and results of the allozyme analyses. First, variation within and among subspecies of *T. albiventris* was summarized by MANOVA and PCA of non-transformed variables. Second, variation within and among samples of *T. albiventris, T. crawfordi,* and *T. culeus* was assessed by PCA of transformed variables. Third, variation within and among Lake Titicaca species (i.e., *T. albiventris, T. crawfordi,* and *T. culeus*), versus Lake Titicaca Basin spe-

TABLE 3.—Electromorph frequency for 10 polymorphic loci across nine samples of *Telmatobius* used in this study. The numbers correspond to those plotted in Figs. 1 and 2, and refer to the following populations: 1 = *T. culeus*, 2 = *T. a. albiventris*, 3 = *T. a. punensis*, 4 = *T. a. globulosus*, 5 = *T. a. parkeri*, 6 = *T. crawfordi*, 7 = *T. marmoratus*, 8 = *T. affinis halli*, 9 = *Telmatobius* sp. nov. The parameters summarized at the bottom of the table are *n* = mean sample size per locus; Ap = mean number of electromorphs per polymorphic locus; %P = percentage of polymorphic loci; H_e and H_0 expected and observed heterozygosity, respectively.

	Population								
Locus	1	2	3	4	5	6	7	8	9
MDH-2									
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
b								1.000	1.000
IDH-2									
a			0.022	0.021					
b	1.000	1.000	0.978	0.979	1.000	1.000	1.000	1.000	1.000
EST-1									
a			0.043			0.083			
b	1.000	1.000	0.957	1.000	1.000	0.917	0.975	1.000	1.000
c							0.025		
EST-2									
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000
n*								1.000	
MDHP-2									
a								0.115	
b								0.615	
c	0.125			0.175	0.091	0.176		0.077	1.000
d	0.458	0.556	0.731	0.475	0.409	0.412			
e	0.125	0.167	0.154	0.150	0.227	0.088	1.000		
n*	0.29	0.278	0.115	0.200	0.273	0.324		0.192	
ACOH-1									
a			0.021				0.033		
b	1.000	1.000	0.979	1.000	1.000	1.000	0.967	1.000	1.000
ACOH-2									
a	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000
b								1.000	
AAT-1									
a							0.136		
b	0.900	0.900	0.913	0.979	0.891	0.893	0.864		
c									1.000
d	0.100	0.100	0.087	0.021	0.109	0.107		1.000	
AAT-2								1.000	
a									
b	0.967	1.000	1.000	0.979	1.000	1.000			
c							0.023		
d							0.977		
e									1.000
f	0.033			0.021					
G3PDH-1									
a	1.000	1.000	1.000	1.000	1.000	1.000			
b							1.000	1.000	1.000
n	14.8	14.9	21.2	20.8	22.2	23.1	20.1	17.5	17.3
Ap	2.6	3.0	2.2	2.2	3.0	2.6	2.0	4.0	0.00
%P**	6.0	9.3	4.0	5.0	2.0	3.0	4.0	1.0	0.00
Ho	0.025	0.024	0.085	0.045	0.067	0.102	0.043	0.038	0.00
He	0.030	0.027	0.091	0.063	0.091	0.105	0.040	0.058	0.00

* n Hypothesized null electromorphs (see text).
** P A locus is considered polymorphic if the frequency of the most common electromorph does not exceed 0.99.

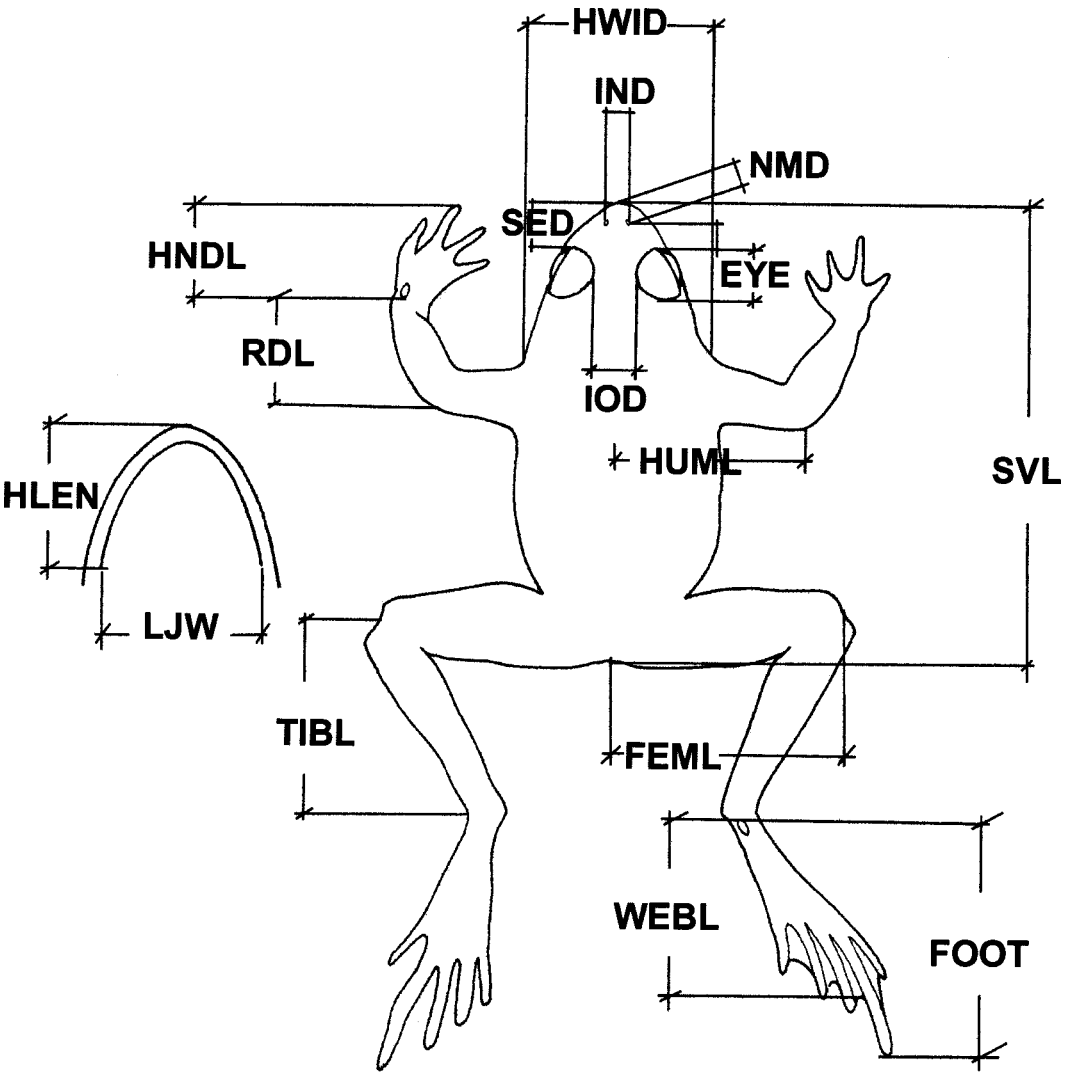

FIG. 2.—Morphometric variables measured from all specimens of *Telmatobius* used in this study (Table 1); see text for abbreviations.

cies (i.e., *T. marmoratus*), versus those outside of the Titicaca Basin (i.e., *T. affinis halli,* and *T.* sp. nov.) was examined by PCA, and DFA was applied to ascertain which combination of morphometric variables best defined the groups.

Ordination techniques are greatly influenced by body size differences (Humphries et al., 1981), and several correction procedures intended to partition size variability into within and between group components have been devised (Burnaby, 1966; Humphries et al., 1981; Somers, 1986). However, none of these is consistently used and there is no consensus on which is the best method (Klingenberg and Froese, 1991). Although there is no automatic partitioning of size and shape onto different axes (Reist, 1985), we chose to partially remove the effect of individual size by log-transforming variables, and then using residuals of the regression of each character on SVL (Arntzen and Garcia-Paris, 1995; Tarkhnishvili et al., 1999). Success in removing size was evaluated by a single linear regression between the first

principal component, PC I (the dependent variable), and SVL (the independent variable). Fit of the data to critical assumptions was assessed by two methods. Data distribution was checked by univariate normal distribution tests, which can be taken as partial proof of normal multivariate distribution. Violations of homoscedasticity were checked with Bartlett's (univariate) and Box *M* (multivariate) tests. All statistical procedures were performed using STATISTICA 5.1 (StatSoft, 1995).

Species Diagnosis

Methodological aspects of testing species boundaries have received little attention, in spite of considerable focus on the species–concept problem (Frost and Hillis, 1990; Frost and Kluge, 1994; Wiens, 1999). Although differences among species concepts are often rooted in different ontological views, de Queiroz (1998) suggested that all species concepts agree fundamentally on what species are (distinct evolutionary entities), but differ in criteria for their recognition. Sites and Crandall (1997) suggested greater clarity would be achieved if investigators would provide a priori operational criteria by which species boundaries may be tested as a hypothesis, and empirically accepted or rejected.

From an operational perspective, several authors have emphasized that the issue of deciding which characters are polymorphic and which are "fixed" is certainly the most relevant, because some authors consider only fixed characters to be properly diagnostic of species boundaries (Davis and Nixon, 1992). Recently, Wiens and Servedio (2000) demonstrated that documenting true fixation of a character requires sampling every individual in a species, while arguing probabilistically that a character is present only at a low frequency only requires sampling many individuals. In order to overcome this limitation, Wiens and Servedio (2000) described a more realistic procedure, which allows characters to be diagnostic for species if they can be shown to be below a certain frequency cut-off with a high degree of statistical confidence. In this approach, the H_0 states that low frequency character states are present at a frequency greater than p (the pre-determined cut-off) even if they are undetected. Rejection of the H_o suggests that one or more characters are below the frequency cut-off, which is taken as evidence of reduced or absent gene flow. We employ this approach here, using a character state frequency cut-off of 10%, with a 5% confidence interval ($\alpha = 0.05$), for a statistically supported delimitation of species boundaries. In the discussion, we return to this issue with respect to caveats and potential limitations of the method.

In the absence of a statistical test, we think diagnosis of independent evolutionary trajectories is strongest if it is based on a concordant pattern of differentiation in unlinked markers (Avise and Ball, 1990; Avise and Wollenberg, 1997), and this criterion is applied here. Therefore, we explicitly diagnose taxa as species by the presence of "fixed" electromorphic differences based on the Wiens-Servedio test, and also the presence of unique electromorphic combinations (non-fixed private alleles) if they are concordant with other diagnostic markers, and/or morphometric differences (see Gergus, 1998; Mink and Sites, 1996, for non-statistical applications of a concordance approach).

Results

Allozymes: Patterns of Variation

We examined 15 enzymes encoded by 25 presumptive loci (Table 2), and 10 loci were variable within or between populations (Mdh-2, Idh-2, Est-1, Est-2, Mdhp-2, Acoh-1, Acoh-2, Aat-1, Aat-2, G3pdh–1). The electromorph frequencies for the variable loci, percentage of polymorphic loci, observed and expected heterozygosity, and mean number of electromorphs per polymorphic locus are shown in Table 3. Of the 10 variable loci, four (Mdh-2, Est-2, Acoh-2, and G3pdh–1) revealed among-sample variation in the form of seemingly fixed, alternative electromorphs. One of these, Est-2, is hypothesized to be fixed for a null electromorph (Katoh and Foltz, 1989) in *T. affinis halli* (sample 8), because no product was ever resolved for any of the 18 individuals included in this sample

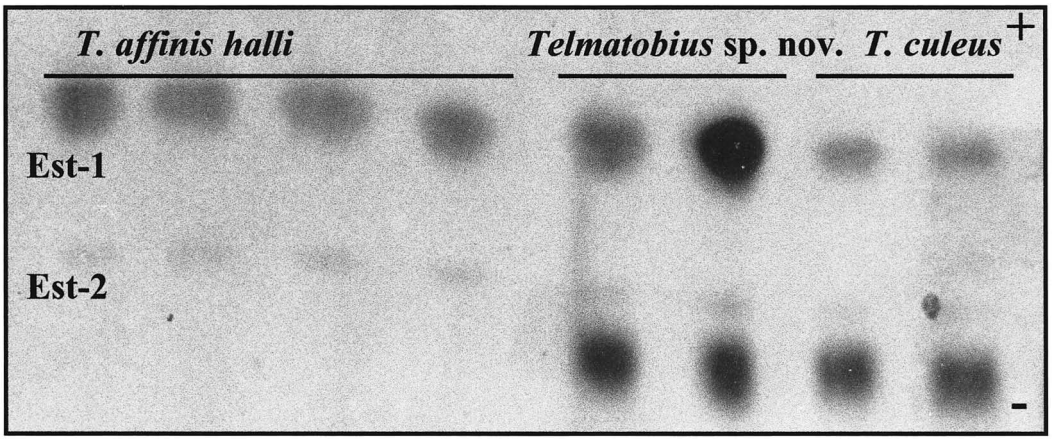

FIG. 3.—Isozyme patterns showing homozygotes for a presumed null electromorph at the Est-2 locus in *T. affins halli*, and normal enzyme phenotypes in *Telmatobius* sp. nov. and *T. culeus*; the "normal" pattern seen in these two species was resolved in all other samples.

(Fig. 3). We observed consistent expression of the Est-1 locus in this sample, and an intensely stained product for the Est-2 locus in all samples from the eight other localities. The fact that all tissue samples were treated the same way, and that the samples of *T. affinis halli* showed clear expression of all other enzyme products, strongly suggests that the Est-2 null pattern in *T. affinis halli* is not a manifestation of enzyme degradation.

The remaining six loci were variable at the level of within-population sample polymorphism, and some populations are characterized by unique electromorphs. When these six loci were tested for conformance to Hardy-Weinberg expectations, all were found to have genotypic ratios consistent with Hardy-Weinberg equilibrium except the Mdhp-2 locus. Examination of the isozyme patterns at this locus revealed many population samples with a high proportion of alternative homozygotes, and no heterozygotes. If a null electromorph is postulated again, as for Est-2 (see also Foltz, 1986; Gaffney et al., 1990; Gardner, 1992), and the genotypes scored so that the light bands are heterozygous for the null (Fig. 4), then all the population samples but one conform to Hardy–Weinberg expectations (the exception being *T. a. globulosus, P* = 0.036, in the direction of heterozygote de-

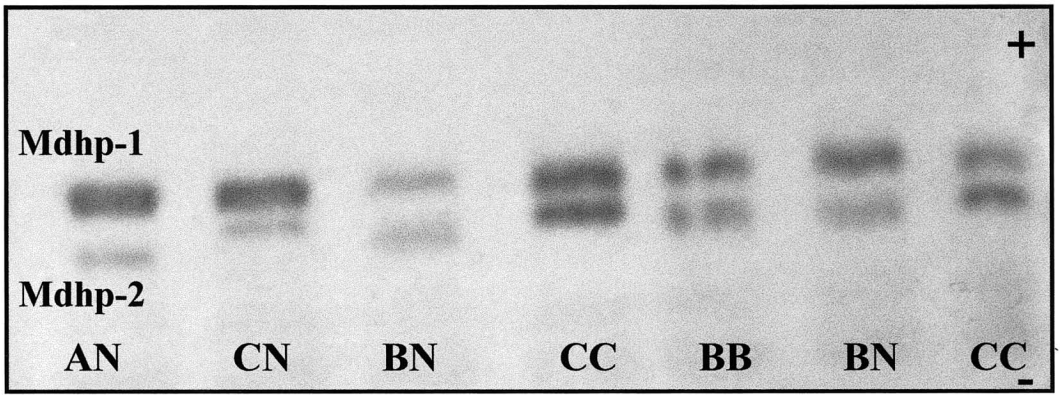

FIG. 4.—Isozyme patterns showing monomorphic Mdhp-1 locus, and hypothesized null electromorphs (N) and genotypes scored at polymorphic Mdhp-2 locus. All individuals represent samples of the *culleus* group.

ficiency). Because the alternative interpretation would require postulation of extreme negative heterosis (selection against the heterozygote) at this locus only, while all other loci conform to random mating expectations, we retain the Mdhp-2 locus in our analysis under the assumption that a null electromorph is segregating in most populations (Table 3).

Observed values of heterozygosity ranged from 0.058 for pooled populations of the *T. culeus* group (*T. albiventris, T. culeus,* and *T. crawfordi,* nos. 1–6 in Table 3; $n = 129$), to 0.043 in *T. m. marmoratus* ($n = 22$), 0.038 in *T. affinis halli* ($n = 18$), and 0.00 in *T.* sp. nov. ($n = 19$). The percentage of polymorphic loci was 4.8 for the *T. culeus* group, 4.0 for *T. m. marmoratus,* 1.0 for *T. affinis halli,* and 0.00 in *T.* sp. nov. (Table 3). A test of association between genotypes across all populations of the *culeus* "group" revealed three cases of significant linkage disequilibrium (Acoh-1 × Idh-2, Est-1 × Aat-1, and Aat-1 × Mdhp-2; $P < 0.05$); no significant association between genotypes was found for any combination of polymorphic loci in *T. marmoratus.*

Populations of the *T. culeus* group share at least one electromorph for each of the 10 variable loci; this group shares electromorphs at eight variable loci with *T. marmoratus,* five with *T. affinis halli,* and five with *T.* sp. nov. (Table 3). The three latter species share one seemingly "fixed" difference (G3pdh–1^{bb}) distinguishing them from the *culeus* group. Finally, *T. affinis halli* and *T.* sp. nov. are fixed for a second unique electromorph (Mdh-2^{bb}). At a finer level of resolution, *T. m. marmoratus* is diagnosed by non-fixed but exclusive (= private) electromorphs in three loci (Aat-1^{a}, Aat-2^{cd}, and Est-1^{c}). Alternatively, three fixed electromorphs (Est-2^{nn}, Acoh-2^{bb}, Aat-2^{aa}) and polymorphism for two other private electromorphs at one locus (Mdhp-$2^{a,b}$) diagnose *T. affinis halli.* Lastly, fixation for unique electromorphs at two loci (Aat-1^{cc} and Aat-2^{ee}) diagnoses *T.* sp. nov.

Indices of Nei's pairwise distance (D) and identity (I) values reflect very low levels of divergence between all combinations of the samples from within Lake Titicaca (Benavides, 1999; data not shown). For example, values of D range from 0.0–0.02 within the lake, while including the three taxa taken from outside of the lake increases the range of values from 0.31 (*T. m. marmoratus–T. a. albiventris*) to 0.67 (*T. m. marmoratus–T.* sp. nov.). Given the low levels of divergence among the recognized taxa in Lake Titicaca, it is not surprising that the six "*culeus* group" populations also showed no significant allelic differentiation ($\chi^2_{12} = 12.38$, $P > 0.41$). Consequently, the value of $\theta - P$ (F_{st}) was quite low ($\theta - P = 0.0017$) and the number of migrants, after correction for sample size, was estimated as $Nm = 12.29$ per generation.

Allozymes: Statistical Tests of "Fixation"

The application of the Wiens–Servedio test revealed two things, on the basis of the total number of characters resolved in this study (25 loci). First, the number of seemingly "fixed" characters and the mean sample size per locus screened (row N in Table 3), suggests that the *T. culeus* group (samples 1–6 in Table 3) can be inferred to have at least one character state with a frequency <10% at a confidence level of $\alpha = 0.05$ ($P = 0.00063$; see Appendix A of Wiens and Servedio, 2000). However, this test does not identify which character this might be, thus either electromorph Aat-2^{b} or G3pdh–1^{a} could be diagnostic. Second, application of the same test to the three taxa outside of the lake (*T. m. marmoratus, T. affinis halli,* and *T.* sp. nov) revealed that sample sizes are not large enough to rule out the possibility of alternative alleles present at a frequency of 10% or more, in those loci that appear diagnostic (i.e., "fixed" in Table 3) for each species.

Phylogenetic Analysis of Allozyme Characters

Figure 5 shows the strict consensus of step-matrix maximum parsimony searches based on the 10 isozyme loci, and rooted alternatively with *T. affinis halli* or *T.* sp. nov. Both topologies recovered a monophyletic Lake Titicaca clade, albeit with weak bootstrap support, and with *T. marmoratus* as the first outgroup. If this topology is retained with stronger support,

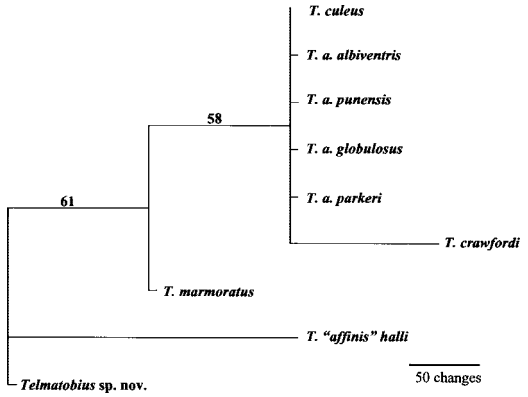

FIG. 5.—Strict consensus of 945 equally parsimonious trees from step-matrix weighted analysis of allozyme data (TL = 548, CI = 0.81, RI = 0.75); numbers on internal nodes are bootstrap values based on 1000 pseudoreplicates.

as future studies add more characters and sample other species of *Telmatobius* on the high Andean plateau, then the possibility of nonmonophyly of the Lake Titicaca forms can be rejected with greater confidence.

Hierarchical Analysis of Morphometric Variation

The summary statistics for all morphometric variables taken from the nine samples of *Telmatobius* are shown in Table 4. The first hierarchical grouping assessed variation among the subspecies of *T. albiventris* (*T. a. albiventris, T. a. globulosus, T. a. punensis,* and *T. a. parkeri*) by MANOVA, and revealed significant differences with non-transformed variables (Wilks' Lambda = 0.199, $F_{51,355.087} = 5.00$, $P < 0.001$). This result is supported by a Kolmogorov-Smirnov test for each character, which showed that only the variable ENOS differed significantly from normality ($P < 0.01$). However, homogeneity of variance-covariance matrices is not supported by the Box *M* test ($M = 969.3820$, $\chi^2_{459} = 737.2872$, $P = 0.0001$), and a Bartlett's test showed homogeneity only in the EYE variable ($\chi^2_3 = 4.084$, $P = 0.252$). Therefore the results of the Principal Component Analysis should be preferred. The first three components explained 78.35% of the variation of the original variables (Table 5), and a scree test of the PCs indicated that only the two first components contain nontrivial information. The first principal component is largely a size axis, with most variables showing high positive loadings, while PC II is a shape axis, with positive loadings (albeit low in many cases) for IOD, IND, SED, NMD, and EYE, and negative loadings for FEML, TIBL, FOOT, ENOS, and WEBL (Table 5). All four subspecies of *T. albiventris* showed extensive overlap when scores were plotted in a bivariate space along PC I and PC II (Fig. 6), and were therefore grouped together in the next round of analysis.

A second hierarchical grouping included the four subspecies of *T. albiventris* as a single sample, *T. crawfordi,* and *T. culeus.* The three samples are consistently different in SVL (Kruskall-Wallis ANOVA, $H = 135.912$, df = 2; $P < 0.0001$; Table 1, Fig. 7). However, a preliminary bivariate approach on the regression of each log-transformed variable against log-SVL showed heterogeneity of slopes in only four variables (HUML, IOD, SED, and WEBL; $P < 0.05$; data not shown, see Benavides, 1999), suggesting a common growth pattern for the remaining variables. On the other hand, variation of non-transformed variables assessed by PCA was largely explained by variation in overall body size (Fig. 8; $R^2 = 89.1\%$), which distinguished the three taxa. The use of residuals as variables eliminates the influence of SVL, while preserving the assumption of normality in all but four (HLEN, FEML, HUML, and ENOS) adjusted characters. High positive loadings of FEML, TIBL, and FOOT, indicated the strong influence of limb proportions on PC I. Positive loadings of HLEN, LJW, and HWID, coupled with negative loadings of FOOT and WEBL on PC II, revealed shape differences (Table 6). Nevertheless, when the first three PCs were used to summarize morphometric variation, all three taxa collapsed into a single group in multivariate space (Fig. 8). This pattern suggests an absence of major differences in proportion or shape among these taxa.

In a third hierarchical design, we treat-

TABLE 4.—Morphometrical characteristics of all nine populations of *Telmatobius* used in this study. Measurements are in mm (illustrated in Fig. 3), numbers for each variable indicate mean ± standard deviation, and range in parentheses.

Variable	*T. a. albiventris* $n = 22$	*T. a. globulosus* $n = 42$	*T. a. parkeri* $n = 28$	*T. a. punensis* $n = 48$	*T. crawfordi* $n = 28$	*T. culeus* $n = 40$	*T. affinis halli* $n = 20$	*T. marmoratus* $n = 31$	*T.* sp. nov. $n = 31$
SVL	63.94 ± 9.06 (46.10–89.22)	58.61 ± 3.23 (47.27–66.60)	65.23 ± 5.63 (56.43–76.81)	63.07 ± 5.79 (54.24–83.10)	51.22 ± 2.59 (47.08–57.13)	104.05 ± 16.20 (74.84–137.95)	45.31 ± 4.57 (38.36–50.81)	53.52 ± 6.60 (36.95–74.61)	39.28 ± 2.56 (31.62–43.20)
HLEN	19.11 ± 3.12 (13.50–28.29)	17.09 ± 1.20 (14.38–19.38)	17.85 ± 1.36 (14.83–20.75)	18.10 ± 2.03 (15.12–25.37)	14.43 ± 0.97 (12.61–17.15)	35.66 ± 6.52 (23.49–54.37)	12.46 ± 0.93 (10.50–13.89)	15.69 ± 2.23 (10.80–25.10)	12.95 ± 1.38 (10.09–15.22)
FEML	26.23 ± 4.48 (16.53–37.22)	23.92 ± 1.97 (16.99–28.38)	27.38 ± 2.66 (23.38–32.04)	27.04 ± 2.70 (20.78–34.19)	21.66 ± 1.52 (18.29–25.16)	45.31 ± 7.97 (29.47–58.11)	20.40 ± 2.27 (14.20–23.28)	22.40 ± 2.54 (15.09–29.98)	17.48 ± 1.08 (14.77–19.67)
TIBL	26.46 ± 4.37 (16.78–37.37)	23.86 ± 1.69 (19.44–26.73)	27.71 ± 2.55 (22.28–32.40)	26.62 ± 2.70 (22.47–34.12)	20.75 ± 1.38 (18.11–24.07)	45.12 ± 7.65 (28.76–57.27)	20.49 ± 2.16 (14.11–23.54)	21.97 ± 2.48 (14.91–29.95)	16.66 ± 1.16 (13.59–19.20)
FOOT	43.68 ± 7.66 (26.86–62.40)	39.02 ± 3.57 (23.61–44.70)	45.10 ± 3.95 (37.77–52.43)	43.54 ± 4.73 (34.16–58.11)	33.05 ± 2.17 (29.64–38.43)	73.28 ± 11.59 (47.58–93.24)	32.71 ± 3.50 (22.02–36.83)	35.22 ± 3.80 (24.66–46.03)	25.37 ± 1.59 (21.81–28.74)
HUML	25.04 ± 4.07 (18.25–36.32)	23.27 ± 1.82 (18.97–27.95)	25.50 ± 2.68 (22.04–32.27)	24.59 ± 2.29 (20.59–31.70)	18.95 ± 1.27 (16.29–22.34)	43.51 ± 7.92 (28.32–56.79)	17.07 ± 1.35 (13.82–18.56)	19.94 ± 2.60 (13.47–2.47)	15.79 ± 1.23 (13.27–10.08)
RDL	13.50 ± 3.57 (1.38–20.57)	12.61 ± 1.00 (9.92–14.70)	14.01 ± 1.32 (11.61–16.76)	13.45 ± 1.63 (10.59–18.76)	10.70 ± 0.86 (8.84–12.63)	23.34 ± 5.14 (8.90–32.18)	9.55 ± 1.04 (7.19–11.20)	11.63 ± 1.39 (7.42–15.56)	9.54 ± 0.65 (7.77–10.56)
HNDL	14.78 ± 2.20 (10.03–20.28)	13.86 ± 1.00 (10.67–16.17)	15.20 ± 1.21 (12.55–18.06)	14.48 ± 1.70 (11.70–19.87)	11.41 ± 0.96 (8.68–13.15)	23.74 ± 3.48 (16.63–30.94)	10.15 ± 0.92 (7.69–11.41)	12.42 ± 1.38 (9.88–17.43)	9.07 ± 0.54 (7.77–10.12)
LJW	21.27 ± 4.25 (13.13–33.23)	19.98 ± 1.50 (17.39–23.89)	21.60 ± 2.32 (18.42–27.47)	21.45 ±; 2.57 (16.86–30.83)	16.84 ± 1.18 (15.04–20.56)	39.59 ± 6.97 (26.45–54.37)	14.15 ± 1.19 (10.89 –15.96)	18.68 ± 2.88 (12.28–30.97)	13.98 ± 0.94 (11.13–15.71)
HWID	21.51 ± 4.00 (13.30–32.98)	20.23 ± 1.36 (17.64–24.41)	22.54 ± 2.02 (18.22–26.80)	22.08 ± 2.75 (16.83–32.12)	17.97 ± 1.36 (15.57–22.80)	41.08 ± 7.23 (27.82–59.94)	15.42 ± 1.43 (11.31–17.54)	20.07 ± 2.85 (12.28–31.74)	14.94 ± 0.88 (11.92–16.15)
IOD	7.64 ± 1.27 (5.13–11.32)	7.30 ± 0.70 (4.74–8.19)	7.57 ± 0.66 (6.43–9.00)	7.49 ± 0.76 (5.40–10.01)	6.19 ± 0.45 (5.41–7.46)	13.05 ± 2.60 (8.77–18.48)	4.72 ± 0.39 (3.72–5.32)	6.65 ± 0.79 (6.16–9.77)	4.98 ± 0.44 (4.16–6.34)
IND	3.05 ± 0.41 (2.12–4.17)	3.47 ± 0.28 (2.70–4.12)	3.24 ± 0.45 (2.44–4.00)	3.19 ± 0.44 (2.16–4.53)	2.95 ± 0.25 (2.51–3.66)	5.29 ± 1.08 (3.24–7.61)	3.25 ± 0.35 (2.32–3.81)	3.20 ± 0.46 (2.11–4.85)	2.45 ± 0.28 (1.98–3.25)
ENOS	4.20 ± 0.84 (2.48–6.55)	3.84 ± 0.30 (3.19–4.52)	4.00 ± 0.41 (3.17–4.80)	4.02 ± 0.47 (3.09–5.17)	3.26 ± 0.27 (2.62–3.76)	7.15 ± 1.20 (4.39–9.89)	3.27 ± 0.25 (2.55–3.73)	3.55 ± 0.46 (2.84–4.93)	2.67 ± 0.24 (2.34–3.29)
SED	10.52 ± 1.60 (7.14–14.81)	10.13 ± 0.70 (8.03–11.64)	10.09 ± 0.98 (8.01–12.06)	10.07 ± 1.31 (8.04–14.51)	8.91 ± 0.70 (7.78–10.78)	16.90 ± 2.62 (12.17–23.03)	8.58 ± 1.04 (5.32–10.00)	10.39 ± 1.08 (7.54–14.67)	7.74 ± 0.59 (6.24–8.91)
NMD	4.17 ± 0.86 (1.99–5.7)	4.09 ± 0.40 (3.26–5.06)	4.30 ± 0.44 (3.44–5.17)	4.12 ± 0.54 (3.09–6.14)	3.78 ± 0.33 (3.06–4.52)	7.07 ± 1.05 (4.52–9.20)	3.19 ± 0.41 (2.01–3.80)	4.01 ± 0.53 (2.59–6.05)	3.15 ± 0.40 (2.11–3.85)
EYE	5.04 ± 0.60 (3.63–6.23)	5.10 ± 0.43 (4.12–5.99)	5.04 ± 0.54 (3.96–6.24)	4.89 ± 0.57 (4.06–6.62)	4.59 ± 0.29 (3.71–5.12)	6.81 ± 0.88 (4.51–8.45)	4.00 ± 0.26 (3.40–4.43)	4.56 ± 0.56 (3.24–5.82)	4.21 ± 0.32 (3.61–4.86)
WEBL	17.47 ± 3.70 (8.90–27.11)	15.86 ± 1.54 (10.63–18.70)	19.26 ± 1.80 (16.78–24.87)	18.21 ± 2.43 (12.56–23.87)	13.11 ± 1.30 (10.96–15.46)	31.48 ± 5.59 (18.48–42.36)	13.25 ± 1.62 (8.16–15.49)	12.99 ± 1.48 (10.07–16.05)	9.36 ± 0.75 (7.89–10.65)

TABLE 5.—Eigenvectors, eigenvalues, and percent variance explained for the first three axes of principal component analysis of non-transformed data of four populations of *T. albiventris* (*T. a. albiventris, T. a. globulosus, T. a. punensis,* and *T. a. parkeri*).

Variable	PC I	PC II	PC III
SVL	0.921	−0.076	0.053
HLEN	0.861	0.094	0.060
FEML	0.798	−0.349	−0.108
TIBL	0.917	−0.266	−0.070
FOOT	0.909	−0.253	−0.049
HUML	0.899	−0.013	−0.061
RDL	0.869	0.008	−0.038
HNDL	0.877	−0.005	−0.134
LJW	0.937	−0.033	−0.026
HWID	0.928	−0.053	0.043
IOD	0.835	0.124	−0.020
IND	0.564	0.516	0.013
ENOS	0.331	−0.113	0.917
SED	0.721	0.321	0.211
NMD	0.733	0.162	−0.056
EYE	0.490	0.640	−0.076
WEBL	0.898	−0.210	−0.091
Eigenvalues	11.20	1.15	0.95
Percent of variance	65.925	6.801	5.626

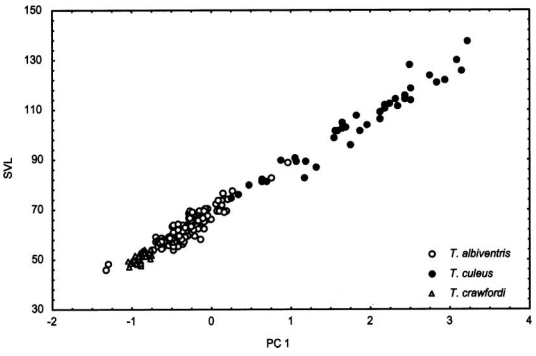

FIG. 7.—Regression of principal component one (PC I) on snout–vent length (SVL) for three recognized species in Lake Titicaca; *T. albiventris* (circles), *T. culeus* (solid circles), and *T. crawfordi* (triangles).

ed *T. albiventris, T. crawfordi,* and *T. culeus* as a single group (*T. culeus* sensu lato) as indicated both by the present morphometric analysis and by a shared pattern of electromorphic variants. The PCA of the non-transformed variables on the four effective "species" (*T. culeus* sensu lato, *T.*

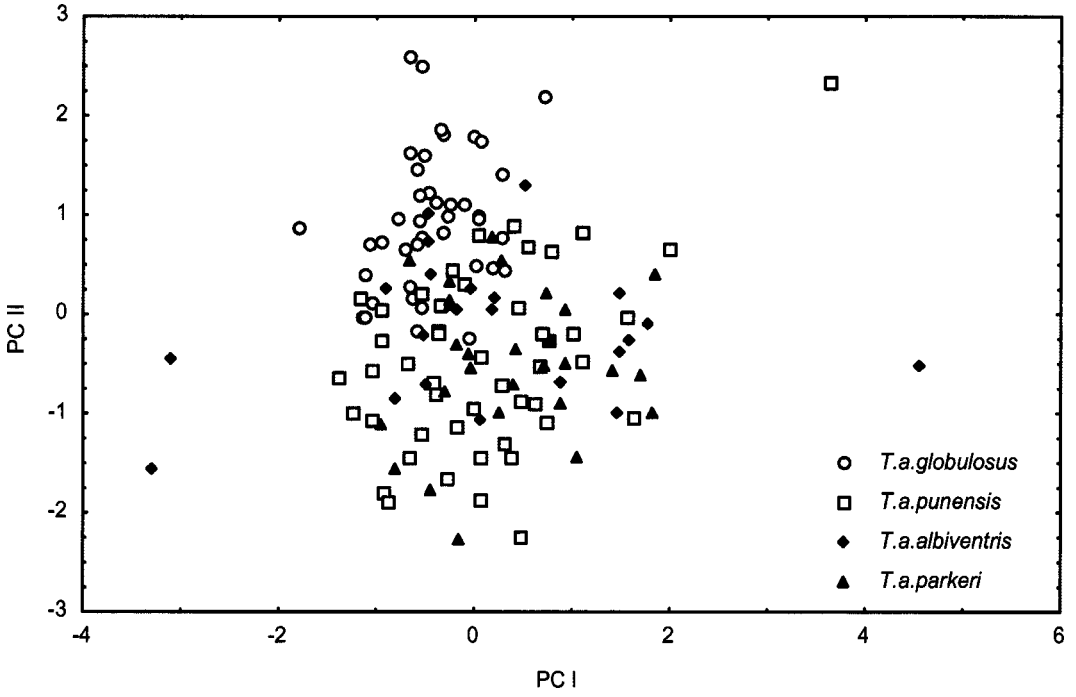

FIG. 6.—Bivariate plot of morphometric variation within and among four subspecies of *T. albiventris*; as summarized in the PCA (diamonds = *T. a. albiventris*; triangles = *T. a. globulosus*; squares = *T. a. parkeri*, and circles = *T. a. punensis*); eigenvectors, eigenvalues, and percent variance explained for the first three principal components are summarized in Table 5.

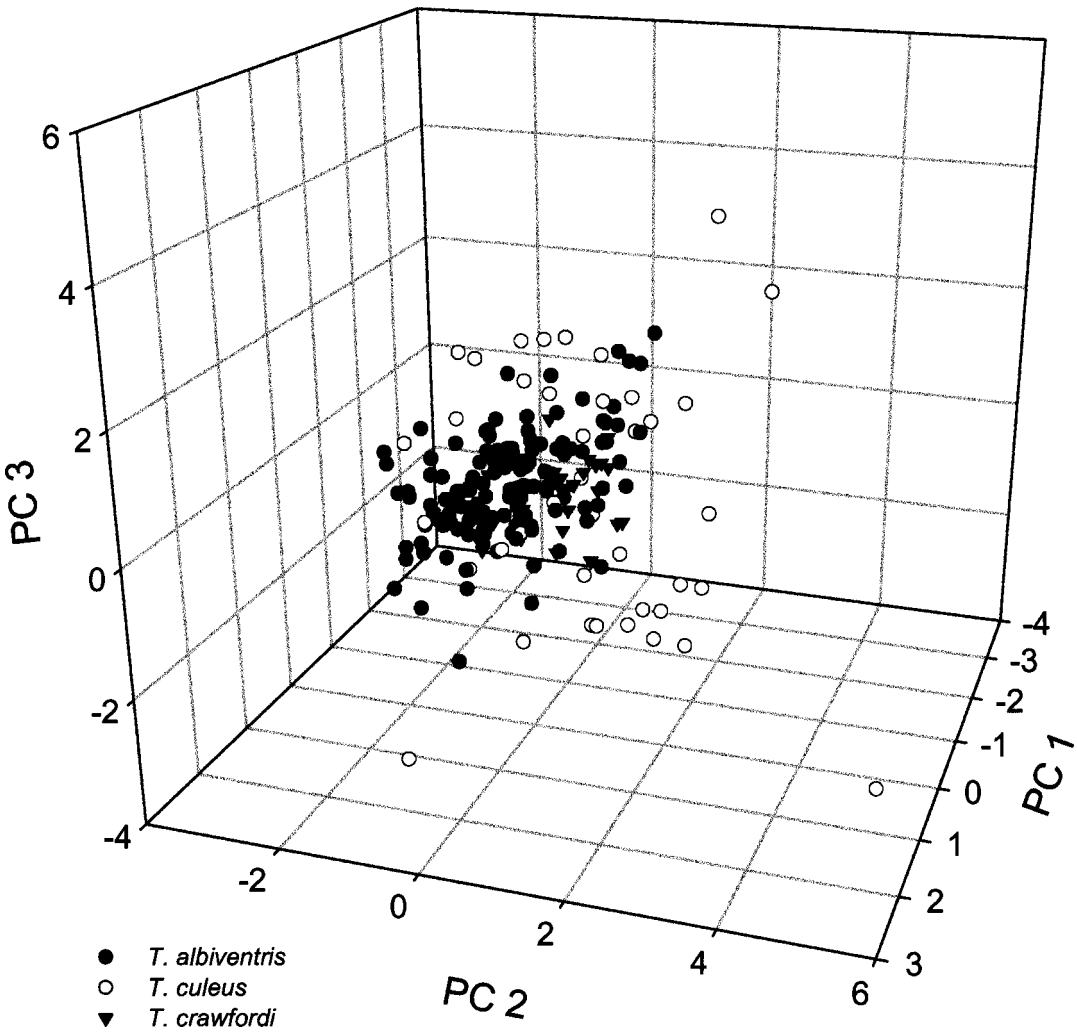

FIG. 8.—Plot of the first (x), second (y), and third (z axis) principal components of three recognized species in Lake Titicaca; *T. albiventris* (solid circles), *T. culeus* (circles), and *T. crawfordi* (inverted triangles); all variables are residuals of the regression of each variable on the snout–vent length (SVL).

m. marmoratus, *T. affinis halli*, and *T.* sp. nov.) revealed poor discrimination (Fig. 9), because PC I (which explains 93.15% of the total variation) is, again, primarily a size axis and most of these taxa overlap in body size (Table 7). Treating residuals as variables in the manner described above revealed discrete groupings, and in this case the first three components of size-adjusted variables explain 70.30% of the original variability (Benavides, 1999; data not shown). However, when samples are plotted in a bivariate space defined by the size-adjusted PC I and PC II, overlap is still extensive (Benavides, 1999; data not shown). Here, lack of group differentiation and failure of the PCA to reduce variable dimensionality is not correlated with the effectiveness of orthogonal components, but with the strong reduction of correlation coefficients produced by this data-set transformation. There are $p\,(p - 1)/2 = 120$ distinct pairwise correlations among $p = 16$ variables in the correlation matrix (Benavides, 1999; data not shown), and only eight (6.6%) of these correlations are

TABLE 6.—Eigenvectors, eigenvalues, and percentage of variance explained for the first three principal components from transformed variables in three putative species: *T. albiventris, T. culeus,* and *T. crawfordi.*

Variable	PC I	PC II	PC III
HLEN	0.000	0.725	−0.142
FEML	0.330	0.000	0.869
TIBL	0.343	0.000	0.145
FOOT	0.722	−0.229	−0.396
HUML	0.201	0.000	0.000
RDL	0.186	0.000	0.000
HNDL	0.134	0.000	−0.106
LJW	0.203	0.380	−0.124
HWID	0.174	0.443	0.000
IOD	0.000	0.103	0.000
IND	0.000	0.000	0.000
ENOS	0.000	0.000	0.000
SED	0.000	0.135	0.000
NMD	0.000	0.000	0.000
EYE	0.000	0.000	0.000
WEBL	0.281	−0.183	0.000
Eigenvalues	4.29	2.61	1.94
Percent of variance	44.678	16.508	9.127

TABLE 7.—Eigenvalues, variable loadings for principal components I to III, and percent of variance explained for non-transformed data from *T. culeus* sensu lato, *T. marmoratus, T. affinis halli,* and *Telmatobius* sp. nov.

Variable	PC I	PC II	PC III
SVL	0.990	−0.020	−0.050
HLEN	0.967	0.036	−0.012
FEML	0.978	−0.062	−0.068
TIBL	0.987	−0.072	−0.074
FOOT	0.982	0.092	−0.100
HUML	0.987	0.019	−0.047
RDL	0.973	0.018	0.042
HNDL	0.981	−0.020	−0.092
LJW	0.991	0.008	−0.014
HWID	0.988	0.009	0.012
IOD	0.979	0.009	0.017
IND	0.912	0.037	0.314
ENOS	0.899	−0.219	0.187
SED	0.964	0.061	0.113
NMD	0.949	0.100	0.074
EYE	0.892	0.385	−0.054
WEBL	0.972	−0.097	−0.125
Eigenvalues	15.837	0.242	0.207
Percent of variance	93.159	1.423	1.220

over 0.50. This indicated that the correlation structure among the 17 original variables was strongly dependent on size. Hence, if the size effect is removed, the remaining variables show an almost complete independence among themselves. In this context, the multivariate approach by PCA offers little resolution. The clustering pattern observed in a PCA of non-transformed variables also suggests that within-group variability largely exceeds between-group variability (Fig. 9).

To further clarify this point, we carried out a Discriminant Function Analysis (DFA), in which group membership was determined a priori (*T. culeus* complex, *T. m. marmoratus, T. affinis halli,* and *T.* sp. nov.), and group discrimination is based on the maximization of group differences. Unlike PCA of either adjusted or non-transformed data, DFA showed group separation (Fig. 10) and was highly significant (Wilks' Lambda = 0.2400, $F_{48,803}$ =

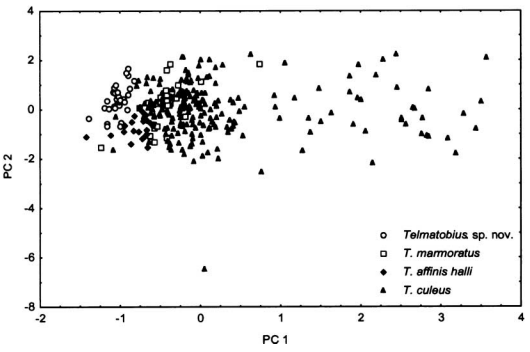

FIG. 9.—Plot of principal component scores for *T. culeus* sensu lato, *T. marmoratus, T. affinis halli,* and *Telmatobius* sp. nov.; eigenvectors, eigenvalues, and percent variance explained for the first three principal components are summarized in Table 7.

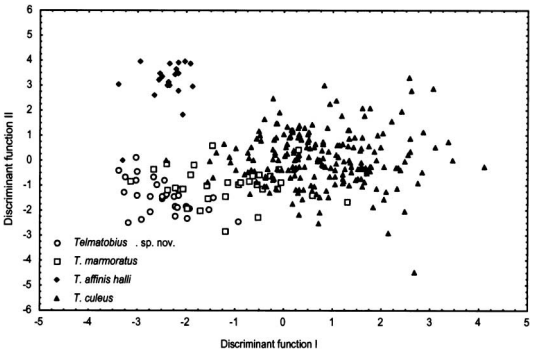

FIG. 10.—Plot of the first and second discriminant functions from a DFA of four effective species of *Telmatobius*; coefficients of multiple regressions between 17 morphometric variables and the first three discriminant functions are summarized in Table 9.

TABLE 8.—Discriminating analysis classification matrix for non-transformed data of four effective species of *Telmatobius*.

Actual species	Number of cases	% classified correctly	Predicted classification			
			1	2	3	4
1. *T.* sp. nov.	31	100.0000	31	0	0	0
2. *T. m. marmoratus*	31	74.1936	1	23	0	7
3. *T. affinis halli*	20	95.0000	1	0	19	0
4. *T. culeus*	208	97.5962	1	3	1	203

10.30992, $P < 0.0001$). Discrimination functions were most successful in correctly assigning individuals to their groups based on non-transformed data. Despite large within-group variability, misclassification of *T. culeus* sensu lato occurs in less than 3.0% of the cases, whereas *T. m. marmoratus* was more often wrongly classified either as *T. culeus* (22.5%) or *T.* sp. nov. (3.22%). Erroneous classification was nonexistent in *T.* sp. nov. and low in *T. affinis halli* (5.0%; Table 8). Although DFA is an a posteriori classification procedure, it nevertheless provides an indication of the extent of variable influence on group discrimination. Table 9 summarizes regression coefficients of each variable on the first three discriminant functions, suggesting that SVL, HWID, and IOD are the most effective variables in discriminating groups along DF I, and RDL, HWID, IOD, and WEBL along DF II. It is noteworthy that although DF I is strongly influenced by body size differences among taxa (regression coefficient = 3.647), loadings from DFA calculated for DF I and DF II had positive and negative values, indicating that both axes summarize shape rather than size discrimination alone (Table 9). Most importantly, our results show that even though overlap is considerable among some groups, each species occupies a fairly well defined area in the discriminant space of 17 measured variables (Fig. 10).

TABLE 9.—Coefficients from multiple regressions between each of the first three discriminant functions (DF) and 17 morphometric variables from four effective "species" of *Telmatobius*. Loadings for characters with the highest discriminatory power for each DF are typed in bold face.

Variable	DF I	DF II	DF III
SVL	**3.64758**	0.31493	**−2.75519**
HLEN	−1.18222	0.92335	**2.35155**
FEML	0.05262	−0.21115	0.20048
TIBL	−1.20847	1.48304	0.68699
FOOT	0.79378	1.45396	−1.05496
HUML	−0.37061	−0.81803	**2.47079**
RDL	−1.07674	**−1.63040**	0.40403
HNDL	1.33202	−0.97730	−1.31428
LJW	−0.17001	−0.55040	−0.22812
HWID	**−1.78716**	−1.78262	−1.24335
IOD	**1.79383**	**−2.05293**	0.11156
IND	−0.71357	1.89704	−0.37643
ENOS	−0.07465	0.23865	0.28968
SED	−1.14034	0.00576	−2.04936
NMD	0.18722	−0.37437	0.05740
EYE	0.39217	−0.02934	0.68652
WEBL	−0.08126	**2.31233**	1.87503

DISCUSSION

Species Diagnosis and Statistical Tests

The main objective of this study was to test taxonomic boundaries of the recognized subspecies of *T. albiventris,* and other species within and outside of the Lake Titicaca Basin. The absence of either isozyme or morphometric characters for discriminating among the named subspecies of *T. albiventris* was anticipated, but the lack of discrimination between this and the other two species from Lake Titicaca was unexpected. This is because differences in adult body sizes would appear to provide a mechanism for reproductive isolation, at least between the large *T. culeus* (average SVL = 104.5 mm), and the smaller *T. crawfordi* (average SVL = 51.22 mm) and *T. albiventris* (average SVL = 62.71 mm). However, as long as allopatry and vertical isolation along depth gradients cannot be safely argued, and estimates of population structure reveal high gene flow (populations could be interconnected by gene flow around the lake's perimeter, and along river courses), we conclude that the three former species conform to a single evolutionary lineage in Lake Titicaca. This con-

clusion is conditional given several caveats (see next section), but further study designed to disentangle historical from ongoing demographic components of population structure will ultimately be needed to clarify this point.

At the most inclusive hierarchical level, both allozyme and morphometric data provide corroborating evidence for diagnostic differences among at least four species: the *T. culeus* group, *T. m. marmoratus, T. affinis halli,* and *Telmatobius* sp. nov. However, Wiens (1999) has revisited the issue of species delimitation and character polymorphism, and he pointed out that unless "fixed" characters are surveyed from the entire population, they cannot be considered as truly invariant characters. This is one of the requirements of the PAA aggregation method (Davis and Nixon, 1992), and as shown by Wiens and Servedio (2000), determining absolute fixation of a character with a conventional level of statistical confidence ($\alpha = 0.05$) is almost unattainable. Our data suggest that using a frequency cutoff of 10%, while maintaining the $\alpha = 0.05$ degree of statistical confidence, will not be feasible in empirical studies of populations characterized by low levels of character divergence. In this case, only a single locus of 25 screened was "fixed" between the Lake Titicaca samples and *T. marmoratus* (Table 3), and our sample sizes of both characters and specimens (Table 1) are probably within the range of those used in most conventional studies of geographic variation, metapopulation structure, and species boundaries. However, all but the largest sample (the combined *T. culeus* group; $n = 127$) are too small to achieve conventional statistical power, primarily because the Wiens-Servedio test is sensitive to both sample sizes and the number of fixed character differences (sample sizes of 25 individuals are adequate if populations are distinguished by at least seven fixed differences; Wiens and Servedio, 2000). We stress that this limitation is not restricted to the Wiens-Servedio test, because recently derived species will be the hardest to diagnose by any criterion (Avise, 2000).

Beyond the sampling limitation, *T. marmoratus* can be unambiguously separated from all samples of *culeus* in a nonstatistical sense by a single diagnostic locus (Aat-2^{cd}), as well as two private, low frequency electromorphs at two other loci (Est-1^{c} and Aat-1^{a}). Differentiation is even stronger for *T. affinis halli* and *T.* sp. nov. by the criteria of character diagnosability and allopatry. *Telmatobius* sp. nov. is diagnosed by unique "fixed" electromorphs at Aat-1^{cc} and Aat-2^{dd}, while *T. affinis halli* is "fixed" for unique electromorphs at Est-2^{nn}, Acoh-2^{bb}, Aat-2^{aa}, and segregates for the unique electromorphs Mdhp-2^{ab}. *Telmatobius marmoratus, T. affinis halli,* and *T.* sp. nov. can therefore be unambiguously diagnosed on the basis of unique concordant allozyme character states. Thus, concordance among presumably independent markers still can be taken as strong evidence of long-term isolation (i.e., sufficient time for these lineages to accumulate novel electromorphs; Avise, 2000). We recognize that in terms of statistical power, we cannot make a strong case that these electromorphs are strictly unique to the species that they appear to diagnose (i.e., they might be present at low frequencies in other species), but the evidence provided by allozymes is also supported by the assessment of morphometric variability. The DFA identified the same four groups of *Telmatobius* as those identified by diagnostic electromorphs (Fig. 10), and in most studies based on modest sample sizes, evidence from concordant patterns of variation across different data sets will continue to provide a valid assessment of species boundaries.

Telmatobius *in Lake Titicaca: Niche Polymorphism and Ecological Speciation*

Although we interpret three named forms within Lake Titicaca Basin as conspecific, the differentiation in body size morphs along a depth gradient (Table 1) appears to be similar to that for several freshwater fish species flocks, in which ecological and trophic specializations correlate strongly with phenotypic divergence, but not genetic structure (Lu and Bernatchez, 1999; Nagel and Schluter, 1998; Rundle et al., 2000). Many of these

examples in fishes provide evidence for ecologically driven adaptive divergence, in which phenotypic divergence among populations within a lake is promoted by relaxed resource competition and use of alternative niches (Lu and Bernatchez, 1999; Schluter, 1998). If reproductive isolation follows as a consequence of the same forces causing phenotypic and ecological divergence, ecological speciation may result in the absence of allopatry (Schluter 1996*a,b*).

In other words, resource-based divergent natural selection may play a major role in the evolution of reproductive isolation over very short geographic distances (Schluter, 1998), and recent empirical evidence has also suggested that this kind of divergence, or life history shift, may occur over extremely short time frames (Bridle and Jiggins, 2000; Orr and Smith, 1998; Reznick et al., 1990). Moreover, several key predictions dealing with the model of sympatric speciation have been successfully tested. First, phenotypic differences may accumulate between populations in different but adjoining habitats despite continued gene flow (Blondel et al., 1999; Lu and Bernatchez, 1999; Schneider et al., 1999; Smith et al., 1997). Second, distinct ecotypes do have differential fitness and hybrids often show reduced fitness and viability (Nagel and Schluter, 1995; Schluter, 1993). Finally, assortative mating may lead to reinforcement mechanisms among ecologically differentiated populations in the face of gene flow (Nagel and Schluter, 1995; Saetre et al., 1997).

The pattern of widely shared electromorph frequencies among *Telmatobius* "ecotypes" in Lake Titicaca Basin, which we take here as evidence of interconnectedness by gene flow, is also consistent with the hypothesis of a recent origin and ongoing ecological speciation among these populations. Speciation in *Telmatobius* has long been regarded as a rapid adaptive process, facilitated by colonization of new and unoccupied niches in the recently uplifted Andes (Cei, 1986; Lynch, 1986). However, the mechanisms underlying the processes of speciation by *Telmatobius* are not well understood, and current Andean amphibian diversity cannot be predicted from a purely geographic perspective (Lynch, 1986). However, two lines of evidence seem to support, the possibility of differential niche use (Skúlason and Smith, 1995; Smith and Skúlason, 1996) by adult anurans, and ongoing processes of ecological speciation.

First, the Titicaca Basin is geologically relatively young, on the order of about 3.0×10^6 yr old (Dejoux, 1994; Lavenu, 1981), and Lake Titicaca has had a very recent history of dramatic oscillations in water and salinity-levels (approximately the last 20,000 yr; Mourguiart et al., 1995*a,b*; Wirrmann et al., 1991). Such changes have promoted several widespread extinction and recolonization events, as shown by detailed palynological evidence (Dejoux, 1994; Wirrmann et al., 1991) and by the actual composition of the aquatic macroflora in Lake Titicaca when compared to the rest of the basin (Ybert, 1991). Massive cyclic turnovers in the lake macrophyte community were likely to have heavily impacted the animal communities, and may have contributed to repeated extinction and recolonization events of unoccupied niches; the most recent such turnover may have occurred as recently as 3000 yr ago (Ybert, 1991). In parallel to *Telmatobius,* the native fish genus *Orestias* Valenciennes, 1839 (Cyprinodontiformes: Cyprinodontidae) displays the very same pattern of extensive phenotypic plasticity (Villwock, 1986) and differential habitat use (littoral, pelagic, and benthic; Lauzanne, 1991). Furthermore, the taxonomy of the species in Lake Titicaca is controversial, and the lake is unexpectedly rich in terms of number of fish species (Lauzanne, 1982, 1991, recognized 24 species while Parenti, 1984, recognized 28).

Second, regardless of the absolute age of the *T. culeus* group, we think that the conditions described above for the "resource-based divergence" version of ecological speciation (Schluter 1996*a,b*) may be met by the ecotypes of *Telmatobius* in Lake Titicaca. The large frogs are confined to deep water (they are collected by fishermen with nets), while the early-maturing, small-bodied morphs are restricted to

shallow embayments (Table 1). This is correlational evidence only, and given that some amphibians are characterized by disassortative mating, and virtually all have indeterminate growth (Duellman and Trueb, 1986; Castellano and Giacoma, 1998), such a pattern could be explained by two non-exclusive alternatives. If our interpretation of conspecificity of the Lake Titicaca populations is correct, then *T. culeus* is characterized by extreme phenotypic plasticity and niche polymorphism (Skúlason and Smith, 1995; Smith and Skúlason, 1996), with low heritability for body size. This hypothesis can be tested by common garden experiments designed to estimate heritabilities of different body sizes, and studies of ecological performance of the morphotypes and their intermediates, in different microhabitats. One circumstantial line of evidence, suggesting high heritability of body size, is the recent rediscovery of a large-bodied *T. marmoratus gigas* (holotype SVL = 109.0 mm) in very shallow streams 150 km south of Lake Titicaca (de la Riva, personal communication). This taxon is a probable relict from an ancient lake, and its restriction to shallow streams suggests that gigantism is not readily lost despite a shift to dramatically different ecological conditions.

Another possibility is that the different size morphs of *T. culeus* represent independent colonization events into Lake Titicaca by previously differentiated lineages, in which case the present niche differences are unrelated to in situ adaptive differentiation among conspecific populations along resource gradients. This interpretation, however, would require sufficient post-colonization hybridization and introgression to homogenize electromorph frequencies across all ecotypes. This hypothesis could be falsified by a phylogenetic study demonstrating unambiguous monophyly of the Lake Titicaca populations and extreme recency of the clade's origin within the lake (i.e., corroboration of the pattern shown in Fig. 5). Populations currently inhabiting the lake could yet represent a monophyletic assemblage if all previously differentiated forms themselves formed a clade prior to colonization of Lake Titicaca, but in this case we would expect some evidence for older allopatric divergence in taxa outside of the lake.

At a more general level, the study of speciation processes and patterns ultimately centers on disentangling evidence based on correlation from that based on causation. Under the assumptions of habitat variability, open niches, relaxation of interspecific competition, and strong selection (Smith and Skúlason, 1996), equivalent evolutionary outcomes, or parallel speciation, has been cited as the strongest correlational argument of speciation by adaptation or habitat shift (Losos et al., 1998; Rundle et al., 2000; Schluter, 1996*a,b*). In this context, studies by Sinsch and Juraske (1995) and Sinsch et al. (1995) are relevant to this work. These investigators taxonomically reassessed the two recognized species of the genus *Batrachophrynus* (*B. brachydactylus* and *B. macrostomus*) and showed that significant differences in habitat between the small stream-dwelling *B. brachydactylus* (SVL = 56.9; $n = 53$) and the larger lake-dwelling *B. macrostomus* (SVL = 131.5; $n = 13$) are positively correlated with morphometric differentiation (Sinsch et al., 1995). However, an allozyme survey revealed the lowest pairwise genetic distance ever recorded for two recognized species of amphibians [Nei (1978) $D = 0.031$; Sinsch and Juraske, 1995]. Furthermore, both species of *Batrachophrynus* can occur in sympatry and apparently do not interbreed (Sinsch and Juraske, 1995). Whether this example of microgeographic phenotypic differentiation is adaptive remains to be demonstrated (but see Blondel et al., 1999; Lambrechts et al., 1997; Schilthuizen, 2000), but it does suggest a common pattern of recent morphological differentiation in high Andean populations of telmatobiine frogs (for another possible example, see Laurent, 1973, 1977, and Cei, 1986).

In summary, although the paradigm of Pleistocene changes and speciation has been taken as the most general model of speciation in many groups, many lines of evidence are suggesting that the origin of living species may either pre-date the Pleistocene (Klicka and Zink, 1997) or be

due to recent divergence along ecological gradients (Schilthuizen, 2000; Schneider et al., 1999; Smith et al., 1997). We suggest that a relatively continuous history of pre- and post-Pleistocene speciation for *Telmatobius,* based both on ecological opportunity and vicariant events, may better explain the radiation of the genus in the high Andes.

Resumen

Las diversidad específica del género *Telmatobius* es el resultado de una radiacion adaptativa que actualmente alcanza a 49 especies, confinadas en ambientes acuáticos de altura en la Cordillera de los Andes. El género carece de una hipótesis filogenética que sustente su monofilia y en general, las relaciones y límites interespecíficos han sido escasamente abordados. En este trabajo, colectamos las cuatro subespecies reconocidas de *T. albiventris* del Lago Titicaca, más ejemplares asignados a *T. culeus* (Lago Titicaca), *T. crawfordi* y *T. marmoratus* de la cuenca circundante. Estas poblaciones han sido comparadas mediante caracteres aloenzimaticos y a través de un estudio multivariado de variables morfométricas para determinar su estatus específico. La delimitación propuesta está basada en la utilización de un concepto evolutivo de especie y en el uso de un criterio de concordancia entre caracteres independientes como método explícito de reconocimiento de especies evolutivas. Nuestros resultados fundamentan el reconocimiento de cuatro especies evolutivas; *T. culeus* (que incluye a *T. albiventris* y *T. crawfordi*), *T. marmoratus, T. affinis halli* y *Telmatobius* sp. nov. Los cuatro taxa anteriores pueden ser discriminados por combinaciones únicas de caracteres morfométricos y aloenzimáticos. Sin embargo, sólo *T. culeus* cuenta con un tamaño muestral suficiente en relación al número de caracteres fijos como para evaluar con un grado de confianza significativo ($\alpha = 0.05$) los caracteres diagnóstico propuestos. Los tres taxa considerados como parte de una sola especie evolutiva muestran diferencias significativas en la talla corporal y microhabitat que pueden ser interpretadas como evidencia circunstancial de adaptación a un gradiente ecológico continuo. Finalmente, se discute la posibilidad de un fenómeno de especiación ecologica y las predicciones que deben ser testeadas para comprobar tal hipótesis.

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