Herpetologica, 45(1), 1989, 17–23

COLOR POLYMORPHISM IN NEOTROPICAL TREEFROGS: AN ALLOZYMIC INVESTIGATION OF THE TAXONOMIC STATUS OF *HYLA FAVOSA* COPE

TOM A. TITUS[1], DAVID M. HILLIS[2], AND WILLIAM E. DUELLMAN[1]

[1]*Museum of Natural History and Department of Systematics and Ecology, The University of Kansas, Lawrence, KS 66045, USA*
[2]*Department of Zoology, University of Texas, Austin, TX 78712, USA*

ABSTRACT: *Hyla favosa*, *Hyla leucophyllata*, and phenotypic intermediates between these species were discovered in microsympatry in southeastern Peru. The genetic composition of individuals of all three phenotypes was analyzed using starch-gel protein electrophoresis to test the validity of species level status for *H. favosa* and *H. leucophyllata*. Twenty-two presumptive gene loci were scored. No alleles diagnostic of the two pure phenotypes were found. Phenotypic intermediates were genetically indistinguishable from the two pure forms and could not be categorized as hybrids. Genetic distances among pairwise comparisons of phenotypes were low ($D^* = 0.031–0.081$). The three phenotypes lack diagnostic genetic differences, are morphologically similar, and seem to breed syntopically. The available data support the hypothesis that the three phenotypes belong to a single species.

Key words: *Hyla favosa*; *Hyla leucophyllata*; Color polymorphism; Protein electrophoresis

COLOR patterns are often used to distinguish among closely related species. As with other morphological attributes, the evolution of color patterns is often decoupled from the development of independent evolutionary lineages. In his discussion of variation in the evolution of morphological features, Mayr (1963) noted that morphological divergence may be slight or nonexistent, resulting in "sibling species," or show a confusing array of polymorphisms within a single species. Thus, strict reliance on morphological patterns of variation in taxonomic studies may result in over- or underestimation of the true number of species within a taxonomic group. Allozymic analyses often are useful in elucidating differences at the species level, particularly when the morphs in question are sympatric. The genetic discontinuity resulting from speciation generally leads to allozymic differentiation

FIG. 1.—Color morphs of *Hyla leucophyllata*; (top) *H. leucophyllata* pattern (KU 139227); (middle) intermediate pattern (KU 205614); (bottom) *H. favosa* pattern (KU 208997).

with or without the appearance of distinctive morphological attributes. These genetic markers provide an independent assessment of the number of reproductively isolated taxa and the extent of hybridization, if it occurs.

Although color patterns are used extensively to distinguish among species of most groups of anurans, it has been well established that color polymorphism characterizes many taxa. Intrapopulational pattern polymorphism has been documented in many species of *Eleutherodactylus* (Goin, 1954; Lynch, 1966; Savage and Emerson, 1970). African reed frogs of the genus *Hyperolius*, especially the *Hyperolius viridiflavus* "superspecies," are notorious for color polymorphism, which includes intrapopulational, geographic, sexual, and ontogenetic differences (Laurent, 1983; Schiøtz, 1971). Among neotropical hylids, sexual dimorphism in color pattern is characteristic of members of the *Hyla parviceps* group (Duellman and Crump, 1974), and ontogenetic color polymorphism is evident in several species of *Gastrotheca* (Duellman and Ruiz-C., 1986).

Many species of neotropical *Hyla* display a wide array of inter- and intraspecific variation in color pattern. In the absence of nonmorphological data, the potential for misclassification is high. This is especially evident in the *Hyla leucophyllata* group. For example, *Hyla triangulum* once was thought to be a complex of six species (Cochran and Goin, 1970). A detailed analysis of larval characteristics, vocalization, and color pattern revealed that all six "species" were actually color pattern morphs of a single species (Duellman, 1974).

The taxonomic status of two other taxa in the *Hyla leucophyllata* group, *H. leucophyllata* (Bereis) and *H. favosa* Cope, has remained problematic. These two species have been reported in microsympatry in the upper Amazon Basin in Ecuador, Peru, Bolivia, and western Brazil, but *H. leucophyllata* extends eastward to the mouth of the Rio Amazonas and to the Guianas (Duellman, 1974). *Hyla leucophyllata* displays a creamy white to pale yellow ground color with a large chocolate-brown hourglass-shaped middorsal mark and transverse bars on the limbs; *H. favosa* has a reticulate pattern of creamy-white lines on a chocolate-brown ground color (Fig. 1). Both species have orange flash colors on the flanks, hidden surfaces of the limbs, and webbing (Duellman, 1978:Pl. 2). Morphometrically, the two taxa are nearly identical. Duellman (1974: 17) stated: "In light of the pattern poly-

morphism displayed by *H. triangulum*, it is conceivable that *H. favosa* is a color morph of *H. leucophyllata*. However, in the absence of individuals with intermediate patterns and lack of data on tadpoles and mating calls of *H. favosa*, I prefer to recognize *H. favosa* as a distinct species."

While working in the rainforests of southeastern Peru in 1986, we discovered phenotypically "pure" individuals of *H. leucophyllata* and *H. favosa* in microsympatry, together with phenotypic intermediates between the two taxa. This provided a unique opportunity to examine the validity of the *H. leucophyllata-favosa* dichotomy using protein electrophoresis. Herein, we categorize genetically all three morphs to test the hypothesis that *H. leucophyllata* and *H. favosa* represent distinct species which hybridize occasionally to form intermediate phenotypes. Under this hypothesis, the predicted outcome is the presence of fixed allelic differences at one or more loci for phenotypically pure *H favosa* and *H. leucophyllata*, whereas the phenotypic intermediates should be heterozygous for the alternative alleles at these diagnostic loci. Although absolute genetic distance is not a reliable indicator of speciation, it provides a useful relative index of overall genetic similarity between populations. Therefore, if the intermediate phenotypes are hybrids, they should be genetically intermediate relative to the two pure phenotypes. Alternatively, *H. favosa*, *H. leucophyllata*, and the intermediates may be color morphs of a single species, or the three morphs may represent three species.

TABLE 1.—Allelic frequencies at 22 variable loci for three color morphs of *Hyla leucophyllata*. Acp-2, Ck, M-Aat-A, Ldh-B, M-Mdh-A, M-Me-A, and Pep-S were monomorphic.

Locus	*H. favosa* (*n* = 2)	Intermediate (*n* = 2)	*H. leucophyllata* (*n* = 22)
Acp-1	b (1.00)	b (1.00)	b (0.98)
	—	—	a (0.02)
Gpi-A	a (1.00)	a (0.75)	a (0.84)
	—	b (0.25)	b (0.14)
	—	—	c (0.02)
S-Aat-A	b (1.00)	b (0.75)	b (0.98)
	—	c (0.25)	a (0.02)
G3pdh-A	b (0.75)	b (1.00)	a (0.02)
	c (0.25)	—	b (0.98)
M-Icdh-A	—	—	a (0.02)
	b (1.00)	b (1.00)	b (0.98)
S-Icdh-A	—	—	a (0.02)
	b (1.00)	b (1.00)	b (0.96)
	—	—	c (0.02)
Ldh-A	—	a (1.00)	a (0.43)
	b (1.00)	—	b (0.57)
S-Mdh-A	—	—	a (0.07)
	b (1.00)	b (1.00)	b (0.93)
S-Me-A	—	a (0.25)	a (0.23)
	b (1.00)	b (0.50)	b (0.57)
	—	c (0.25)	c (0.20)
Pep-B	—	—	a (0.02)
	b (1.00)	b (0.50)	b (0.82)
	—	c (0.50)	c (0.16)
Pep-C	—	—	a (0.05)
	b (0.75)	b (1.00)	b (0.93)
	c (0.25)	—	c (0.02)
Pep-D	a (0.50)	a (0.25)	a (0.11)
	b (0.50)	b (0.75)	b (0.75)
	—	—	c (0.14)
Pgm-A	—	a (0.25)	a (0.07)
	b (1.00)	b (0.75)	b (0.82)
	—	—	c (0.11)
S-Sod-A	a (1.00)	a (1.00)	a (0.98)
	—	—	b (0.02)
Tpi-A	a (1.00)	a (1.00)	a (0.98)
	—	—	b (0.02)

MATERIALS AND METHODS

Frogs were collected in the Reserva Ecológica Cuzco Amazónico, approximately 15 km east of Puerto Maldonado on the northwest bank of the Río Madre de Dios, Departamento de Madre de Dios, Perú. Muscle and liver tissues were removed and frozen immediately in liquid nitrogen for transport to the United States, where they were stored at −90 C for up to 1 yr. Carcasses were fixed in 10% formalin and deposited in the herpetological collections of the Museum of Natural History, The University of Kansas (KU) and Museo de Historia Natural "Javier Prado," Lima, Perú. Sample sizes ranged from 22 (*H. leucophyllata*) to two (*H. favosa* and the intermediates).

Tissues were prepared for allozyme electrophoresis following standard methods (Hillis, 1985) and histochemically stained for 22 presumptive gene loci following the methods of Harris and Hopkinson (1976), Selander et al. (1971), and Siciliano and Shaw (1976). Loci, IUBNC

TABLE 2.—Genotypic frequencies at 22 variable loci for three color morphs of *Hyla leucophyllata*. Acp-2, Ck, M-Aat-A, Ldh-B, M-Mdh-A, M-Me-A, and Pep-S were monomorphic. Sample sizes in parentheses.

Locus	Genotype	*H. favosa*	Intermediate	*H. leucophyllata*
Acp-1	ab	—	—	0.05 (1)
	bb	1.0 (2)	1.0 (2)	0.95 (21)
Gpi-A	aa	1.0 (2)	0.50 (1)	0.73 (16)
	ab	—	0.50 (1)	0.18 (4)
	ac	—	—	0.05 (1)
	bb	—	—	0.05 (1)
S-Aat-A	ab	—	—	0.05 (1)
	bb	1.0 (2)	0.50 (1)	0.95 (21)
	bc	—	0.50 (1)	—
G3pdh-A	ab	—	—	0.05 (1)
	bb	0.50 (1)	1.0 (2)	0.95 (21)
	bc	0.50 (1)	—	—
M-Icdh-A	ab	—	—	0.05 (1)
	bb	1.0 (2)	1.0 (2)	0.95 (21)
S-Icdh-A	ab	—	—	0.05 (1)
	bb	1.0 (2)	1.0 (2)	0.90 (20)
	bc	—	—	0.05 (1)
Ldh-A	aa	—	1.0 (2)	0.18 (4)
	ab	—	—	0.50 (11)
	bb	1.0 (2)	—	0.32 (7)
S-Mdh-A	ab	—	—	0.14 (3)
	bb	1.0 (2)	1.0 (2)	0.86 (19)
S-Me-A	aa	—	—	0.09 (2)
	ab	—	—	0.23 (5)
	ac	—	0.50 (1)	0.05 (1)
	bb	1.0 (2)	0.50 (1)	0.36 (8)
	bc	—	—	0.18 (4)
	cc	—	—	0.09 (2)
Pep-B	ab	—	—	0.05 (1)
	bb	1.0 (2)	—	0.68 (15)
	bc	—	1.0 (2)	0.23 (5)
	cc	—	—	0.05 (1)
Pep-C	ab	—	—	0.09 (2)
	bb	0.50 (1)	1.0 (2)	0.86 (19)
	bc	0.50 (1)	—	0.05 (1)
Pep-D	ab	1.0 (2)	0.50 (1)	0.23 (5)
	bb	—	0.50 (1)	0.55 (12)
	bc	—	—	0.18 (4)
	cc	—	—	0.05 (1)
Pgm-A	ab	—	0.50 (1)	0.14 (3)
	bb	1.0 (2)	0.50 (1)	0.64 (14)
	bc	—	—	0.23 (5)
S-Sod-A	aa	1.0 (2)	1.0 (2)	0.95 (21)
	ab	—	—	0.05 (1)
Tpi-A	aa	1.0 (2)	1.0 (2)	0.95 (21)
	ab	—	—	0.05 (1)

numbers, tissue sources, and running conditions are as follows:

Tris-citrate 6.7, muscle (Acp-1, 3.1.3.2; Acp-2, 3.1.3.2; Ck, 2.7.3.2; M-Icdh-A, 1.1.1.42; S-Icdh-A, 1.1.1.42; M-Me-A, 1.1.1.40; S-Me-A, 1.1.1.40; Tpi-A, 5.3.1.1); Tris-citrate 6.7, liver (G3pdh-A, 1.1.1.8; Ldh-A, 1.1.1.27; Ldh-B, 1.1.1.27; M-Mdh-A, 1.1.1.37; S-Mdh-A, 1.1.1.37; Pgm-A, 5.4.2.2; S-Sod-A, 1.15.1.1); Poulik, muscle (Gpi-A, 5.3.1.9; M-Aat-A, 2.6.1.1; S-Aat-A, 2.6.1.1); and Poulik, liver (Pep-B, 3.4.13.11; Pep-C, 3.4.13.11; Pep-D, 3.4.13.9; Pep-S, 3.4.13.11).

Locus nomenclature, IUBNC numbers, and abbreviations follow the recommendations of the International Union of Biochemistry Nomenclature Committee (1984). Multiple loci were numbered from cathode to anode. Peptidase loci were given letter designations following Harris and Hopkinson (1976) corresponding to their substrate specificities. Electromorphs were designated by letters with "a" corresponding to the most cathodal electromorph. Electromorphs are labelled relative to this study only and are not meant to correspond to those of any previous studies.

Allelic frequencies, heterozygosity, and genetic distances were calculated for each phenotype using LYNS (L. Loveless, unpublished). Genetic distances were calculated for three possible pairwise comparisons of phenotypes following Nei (1972) as modified by Hillis (1984).

RESULTS

Forty-six electromorphs were identified among 22 presumptive gene loci. Allelic frequencies are presented in Table 1 and genotypic frequencies are presented in Table 2. Seven loci were monomorphic in all phenotypes (Acp-2, Ck, M-Aat-A, Ldh-B, M-Mdh-A, M-Me-A, and Pep-S). No alleles were diagnostic of the *H. favosa* or *H. leucophyllata* phenotypes. Average number of alleles per locus ranged from 1.16 in *H. favosa* to 1.23 in the intermediates. Mean heterozygosity was similar for all phenotypes, ranging from 0.091 (1 SD = 0.04) in *H. favosa* to 0.159 (1 SD = 0.06) for the intermediates.

At all loci, the common allele was the same in all three phenotypes. Both individuals of the intermediate phenotype were heterozygotes for the "ab" genotype at Pep-B. Although the "b" allele is found only in the homozygous condition in *H. favosa*, the "a" allele is rare in *H. leucophyllata*, indicating that heterozygosity in

the intermediate phenotype is not a result of hybridization.

Genetic distances between each pair of phenotypes do not display a pattern consistent with the prediction that the intermediates are hybrids. The largest distance (0.081) occurs between *H. favosa* and the intermediate phenotype, whereas the distances between *H. favosa* and *H. leucophyllata* and between *H. leucophyllata* and the intermediates (0.031 and 0.033, respectively) are nearly equal. Thus, the intermediate phenotype is not genetically intermediate between the pure forms. In addition, all genetic distances are exceedingly low.

Discussion

We found no evidence of genetic divergence between *H. leucophyllata* and *H. favosa* or of hybridization between the two taxa. No diagnostic alleles for the two "pure" phenotypes are present at the 22 loci examined. As a result, no loci were found representing the expected heterozygous condition if the intermediate were the product of hybridization. The large number of alleles shared among the three phenotypes resulted in low genetic distances. The smallest distance is between *H. favosa* and *H. leucophyllata* (indicating little genetic divergence between the two "species"), whereas the largest distance is between *H. favosa* and the intermediate phenotype. Therefore, even if the genetic distances are considered significantly different from zero, the pattern of genetic distances also is inconsistent with the hybridization hypothesis. Thus, the genetic data support the alternative hypothesis, that *H. favosa*, *H. leucophyllata*, and the intermediate phenotype are color pattern morphs of a single species.

Recently (1987), other investigators discovered *H. leucophyllata* and *H. favosa* in the same breeding chorus 32 km south of Altimira on the Río Xingú, Pará, Brazil (J. P. Caldwell, personal communication). This observation corroborates our data from Cuzco Amazónico that breeding activity is not separated spatially or temporally in these morphs. Furthermore, among 18 specimens of *H. leucophyllata* from 3–5 km east of Belém, Estado Pará, Brazil, one (KU 128429) has the fragmented dorsal pattern like the intermediate phenotypes from Cuzco Amazónico, but no individuals having the *H. favosa* pattern are known from Belém.

Limited morphological data suggest no differences between *H. favosa* and *H. leucophyllata*. Duellman (1974) presented data based on five standard measurements demonstrating a high degree of morphometric similarity between these taxa. However, the small samples of *H. favosa* and intermediates currently available from a single locality preclude the use of multivariate analyses of morphometric data. Future analyses based on large sample sizes and more measurements would be useful in clarifying further the relationships among these three phenotypes.

The data from protein electrophoresis should be interpreted with caution because of the small sample sizes obtained for *H. favosa* and the intermediates. Species-level divergence may have occurred at loci not associated with the protein-encoding loci utilized in this study. Also, these species may be characterized by frequency differences at protein-encoding loci that are not fixed. Under these circumstances, the small samples used in this study may not provide the statistical power necessary for discerning patterns of hybridization. Further electrophoretic work should be undertaken utilizing larger sample sizes. Unfortunately, efforts to increase the number of specimens examined will certainly be hampered by the extremely low frequency at which *H. favosa* and intermediates occur. Of 37 individuals collected in approximately 15 wk of field work at Cuzco Amazónico, 29 were *H. leucophyllata*, six were *H. favosa*, and two were intermediates.

Although alternative hypotheses certainly exist, the most conservative interpretation of all available data is that *H. leucophyllata*, *H. favosa*, and the intermediate phenotype are color pattern morphs of a single species. The three morphs are genetically indistinguishable at the 22 loci examined so far. All three occur in microsympatry and breed syn-

chronously. The only character supporting the recognition of *H. favosa* as a distinct species from *H. leucophyllata* is color pattern, and the presence of color pattern intermediates renders this character ambiguous. Therefore, until data are gathered supporting the distinct nature of *H. favosa*, we recommend that *Hyla favosa* Cope, 1886, be considered a junior synonym of *Hyla leucophyllata* (Bereis, 1783).

The color pattern polymorphism observed in *H. leucophyllata* is not unique among anurans. Zimmerman and Zimmerman (1987) reported greater color pattern polymorphism within and among six generations of captive-bred *Hyperolius marmoratus* than we observed in *H. leucophyllata*. Furthermore, striking differences in dorsal color pattern also occur in the hylid genus *Gastrotheca*. Two color morphs occur in *Gastrotheca aureomaculata*—black with yellow flecks and plain green; no immunological genetic distance was found between the two morphs (Duellman, 1983). Individuals of *Gastrotheca helenae* may be brownish-black with yellow flecks or tan with dark brown longitudinal markings; a female with the former pattern gave birth to young all having the latter pattern (Duellman and Ruíz-C., 1986). The genetic systems controlling coloration may be simple; Resnick and Jameson (1963) found coloration differences in *Hyla regilla* to be the result of allelic variants at only two loci. These few examples suggest strongly that in the absence of congruent morphological, developmental, or behavioral differences the use of color pattern alone to distinguish sympatric species can lead to erroneous conclusions. In these cases, the "specific" status of the color polymorphisms may be determined by allozyme electrophoresis.

Resumen

Hyla leucophyllata, *H. favosa*, y un fenotipo intermedio occurren en microsimpatría en el sureste de Perú. Productos enzimáticos correspondientes a 22 loci fueron examinados electroforéticamente. No se encontraron alelos diagnósticos para cada especie y los fenotipos intermedios son genéticamente de los indistinguibles fenotipos puros. La ausencia de diferencias genicas dentro de los fenotipos intermedios sugiere que *Hyla favosa* Cope, 1886 es un sinónimo de *Hyla leucophyllata* (Bereis, 1783).

Acknowledgments.—Field studies at the Reserva Ecológica Cuzco Amazónico were made possible by the generosity of the owners, José E. Koechlin von Stein and Jorge E. Seoane M. of Lima, Perú, logistics provided by the staff of the reserve, and financial support from the National Geographic Society (grants 3196-85 and 3405-86 to W. E. Duellman). Laboratory work was supported by NSF grants BSR 8657640 and BSR 8614622 (to D. M. Hillis). We are indebted to our field companions—Patricia A. Burrowes, Alan Channing, Rafael de Sá, Victor Morales, John E. Simmons, and Linda Trueb—for their efforts in obtaining specimens. Permits were issued by Ing. Marco Romero Pastor, Blga. Rosario Acero, and Blga. Mariza Falero Sánchez of the Dirección General Forestal y de Fauna, Ministerio de Agricultura, Lima, Perú.

Literature Cited

Cochran, D. M., and C. J. Goin. 1970. Frogs of Colombia. Bull. U.S. Natl. Mus. 288:1–655.

Duellman, W. E. 1970. The hylid frogs of Middle America. Monogr. Mus. Nat. Hist. Univ. Kansas 1: 1–753.

———. 1974. A reassessment of the taxonomic status of some neotropical hylid frogs. Occas. Pap. Mus. Nat. Hist. Univ. Kansas 27:1–27.

———. 1978. The biology of an equatorial herpetofauna in Amazonian Ecuador. Misc. Publ. Mus. Nat. Hist. Univ. Kansas 65:1–352.

———. 1983. *Gastrotheca aureomaculata*: Systematic resolution of a case of intrapopulational polychromatism in a hylid frog. Herpetologica 34: 105–110.

Duellman, W. E., and M. L. Crump. 1974. Speciation in frogs of the *Hyla parviceps* group in the upper Amazon Basin. Occas. Pap. Mus. Nat. Hist. Univ. Kansas 23:1–40.

Duellman, W. E., and P. M. Ruíz-C. 1986. Ontogenetic polymorphism in marsupial frogs (Anura: Hylidae). Caldasia 15:617–627.

Goin, C. J. 1954. Remarks on evolution of color pattern in the *gossei* group of the frog genus *Eleutherodactylus*. Ann. Carnegie Mus. 33:185–195.

Harris, H., and D. H. Hopkinson. 1976. Handbook of Enzyme Electrophoresis in Human Genetics. North-Holland, Amsterdam.

Hillis, D. M. 1984. Misuse and modification of Nei's genetic distance. Syst. Zool. 33:238–240.

———. 1985. Evolutionary genetics of the Andean lizard genus *Pholidobolus* (Sauria: Gymnophthalmidae): Phylogeny, biogeography, and a comparison of tree construction techniques. Syst. Zool. 34: 109–126.

International Union of Biochemistry Nomenclature Committee. 1984. Enzyme Nomenclature, 1984. Academic Press, New York.

Laurent, R. F. 1983. La superespece *Hyperolius*

viridiflavus (Dúmeril et Bibron, 1841) (Anura Hyperoliidae) en Afrique centrale. Monit. Zool. Italiano, N.S., Supple. 1:1–93.

LYNCH, J. D. 1966. Multiple morphotypy and parallel polymorphism in some neotropical frogs. Syst. Zool. 15:18–23.

MAYR, E. 1963. Animal Species and Evolution. Harvard University Press, Cambridge.

NEI, M. 1972. Genetic distance between populations. Am. Nat. 106:283–292.

RESNICK, L. E., AND D. L. JAMESON. 1963. Color polymorphism in Pacific treefrogs. Science 142: 1081–1083.

SAVAGE, J. M., AND S. B. EMERSON. 1970. Central American frogs allied to *Eleutherodactylus bransfordii* (Cope): A problem of polymorphism. Copeia 1970:623–644.

SCHIØTZ, A. 1971. The superspecies *Hyperolius viridiflavus* (Anura). Vidensk. Medd. Dansk Naturhist. Foren. 134:21–76.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON, AND J. B. GENTRY. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). Stud. Genetics IV, Univ. Texas Publ. 7103:49–90.

SICILIANO, M. J., AND C. R. SHAW. 1976. Separation and localization of enzymes on gels. Pp. 184–209. *In* I. Smith (Ed.), Chromatographic and Electrophoretic Techniques, Vol. 2, 4th ed. Williams Heinemann Medical Books, London.

ZIMMERMAN, H., AND E. ZIMMERMAN. 1987. Mindestanforderungen für eine artgerechte Haltung einiger tropischer Anurenarten. Zeit. Kolner Zoo 30:61–71.

Accepted: 12 March 1988
Associate Editor: John Iverson