

## PHYLOGENY AND BIOGEOGRAPHY OF THE *RANA PIPIENS* COMPLEX: A BIOCHEMICAL EVALUATION

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**Abstract.**—The phylogeny of the *Rana pipiens* complex as supported by cladistic analysis of the enzymatic products of 50 gene loci is presented. Two broadly sympatric groups within the complex are identified and named the Alpha and Beta divisions. Within both of these divisions, a dichotomy exists between North American and Middle American species. The two groups in the Alpha division are recognized as the *R. montezumae* species group and the *R. areolata* species group; the two in the Beta division are referred to the *R. pipiens* species group and the *R. berlandieri* species group.

Most of the species within a species group are distributed parapatrically; some hybridization may take place within narrow zones of sympatry. Limited hybridization also occurs in the two narrow zones of sympatry that exist between members of different species groups in the same division. However, although sympatry between Alpha division species and Beta division species is extensive, interdivisional hybridization is relatively rare. [Biogeography; cladistics; electrophoresis; phylogeny; *Rana pipiens* complex.]

The systematics of the *Rana pipiens* complex (leopard frogs and related species) has been a modern biological enigma. Although a dozen species in this complex had been described before the start of the present century, this number was reduced to four through synonymy in association with development of the polytypic species concept during the 1940s and 1950s (Moore, 1944, 1946; Neill, 1957). However, extensive field work in the 1960s and 1970s led to the discovery of several zones of sympatry between different "morphotypes," in which little or no hybridization was evident (Post and Pettus, 1967; Mecham, 1968; Brown and Brown, 1972; Platz, 1972; Platz and Platz, 1973; Dunlap and Kruse, 1976; Frost and Bagnara, 1976, 1977a, b; Lynch, 1978; Hillis, 1981). The realization that these zones of sympatry exist, coupled with detailed morphological (Post and Pettus, 1966; Pace, 1974; Korky, 1978; Hillis, 1982), auditory (Littlejohn and Oldham, 1968; Frost and Bagnara, 1977a, b; Frost, 1982; Frost and Platz, 1983), biochemical (Salthe, 1969; Platz, 1972, 1976; Platz and Platz, 1973; Sage and Selander, 1979), and reproductive studies (Frost and Bagnara, 1977a; Hillis, 1981; Frost and Platz, 1983) has led to a return to the concept of numerous distinct

species of leopard frogs (Moore, 1975). Several of these species have recently been described (Mecham et al., 1973; Sanders, 1973; Frost and Bagnara, 1976; Platz and Mecham, 1979), and the descriptions of several others will be forthcoming (Frost and Hillis, unpubl. data).

Although each of the described species in the *R. pipiens* complex can be distinguished morphologically, the complex as a whole is morphologically conservative. The known morphological differences are too few to permit phylogenetic analysis based upon these traits. However, the complex is sufficiently diverse biochemically to lend this group to ready phylogenetic analysis through electrophoresis. Electrophoretic analyses of phylogenies are maximally informative when they are restricted to relatively closely related groups of organisms with known sister groups. Furthermore, because few synapomorphic electromorphs can be identified relative to the number that are autapomorphic, many loci must be examined in order to resolve fully a phylogeny if the group under consideration is highly speciose (Avice et al., 1980b).

The *R. pipiens* complex is an ideal group for electrophoretic analysis of phylogeny be-

cause: (1) Species in this group are relatively closely related. (2) Appropriate outgroups have been identified (Case, 1978). (3) Electrophoretic techniques for examining a large number of loci have been developed for this group and the genetic interpretations of the products of these loci have been confirmed through experimental breeding programs (Wright et al., 1980; Wright et al., 1983). (4) Heterozygosity levels are low within most populations of leopard frogs (R. D. Sage, pers. comm.), thus permitting the use of small sample sizes with little resultant loss in reliability or information (Nei and Roychoudhury, 1974; Sarich, 1977; Nei, 1978; Gorman and Renzi, 1979; Honeycutt et al., 1981).

We have assembled tissues from 20 species of the *R. pipiens* complex, as well as from 3 species of the *R. tarahumarae* group (a sister group of the *R. pipiens* complex according to Case, 1978). Of the 20 species in the former group, 12 have been formally described. Descriptions for the remaining eight species of leopard frogs, as well as for one of the species of the *R. tarahumarae* group, are either in press or in preparation. Most Mexican populations of leopard frogs considered in the present analysis represent those for which the results of earlier studies (laboratory crosses, morphological comparisons, and mating call analyses) by one of us (JSF) suggested probable distinctness. Throughout this paper we refer to the undescribed species by geographical or established common names; these names are used merely for convenient reference and do not reflect future scientific designations.

#### MATERIALS AND METHODS

The collection localities of the 23 species that we studied are listed in Appendix I. For relatively well-known species, or in cases where species occur only in restricted areas, we obtained tissues from single specimens of the species. In three cases (*R. chiricahuensis*, *R. magnaocularis*, and the Chapala form), we obtained samples from the extremes of the species ranges in order to confirm the conspecificity of these populations. Samples of muscle and liver tissue were removed from freshly killed frogs in the field and were stored in liquid nitrogen until they were re-

turned to the laboratory, where they were maintained at  $-80^{\circ}\text{C}$  until use.

Liver and muscle tissue were separately minced on glass plates and then homogenized in ground-glass homogenizers. Muscle homogenates were diluted 1:1 (w:v) with 0.01 M tris-HCl, 0.001 M EDTA, and 0.001 M  $\beta$ -mercaptoethanol (pH 7.5); liver homogenates were diluted 1:3 with this solution. Homogenates were then refrozen at  $-80^{\circ}\text{C}$ , after which they were centrifuged for 20 min at 16,000 rpm.

We employed the procedures of vertical starch gel electrophoresis described by Siciliano and Shaw (1976). Either tris-citrate pH 7.0 (TC7) or tris-versene-borate pH 8.0 (TVB) buffer systems were used (Table 1). Gels were prepared from Connaught starch (90 g/600 ml buffer for TVB gels, 95 g/600 ml for TC7 gels); two drops of  $\beta$ -mercaptoethanol were added to the gel buffer mixture after boiling and degassing.

Gels were electrophoresed for 6 hr at 375 V (at about  $4^{\circ}\text{C}$ ). Each gel was sliced into eight 1-mm thick slabs for histochemical staining. The loci examined, as well as the buffer systems and tissues used, are listed in Table 1. The procedures for staining are described in Siciliano and Shaw (1976) or Harris and Hopkinson (1976).

Electromorphs were assigned letters according to their relative mobility, from cathode to anode. The enzyme nomenclature of Wright et al. (1980) was followed in naming multiple loci (numbered from anode to cathode). Only one locus was scored for aconitase and for phosphoglucomutase; these loci are ACO-2 and PGM-2 of Wright et al. (1980). The peptidase loci were designated according to their substrate specificity: LA for L-leucyl-L-alanine; LGG for L-leucylglycylglycine; and LP for L-leucyl-L-proline.

Electromorphs that were present in the outgroup were coded as ancestral (plesiomorphic) with respect to the *R. pipiens* complex, whereas those that were absent in the outgroup were coded as derived (apomorphic). The most parsimonious cladogram was constructed by standard Hennigian methods (Wiley, 1981) using procedures developed specifically for electrophoretic data (Avise et al., 1980a). Electromorphs were ordered into

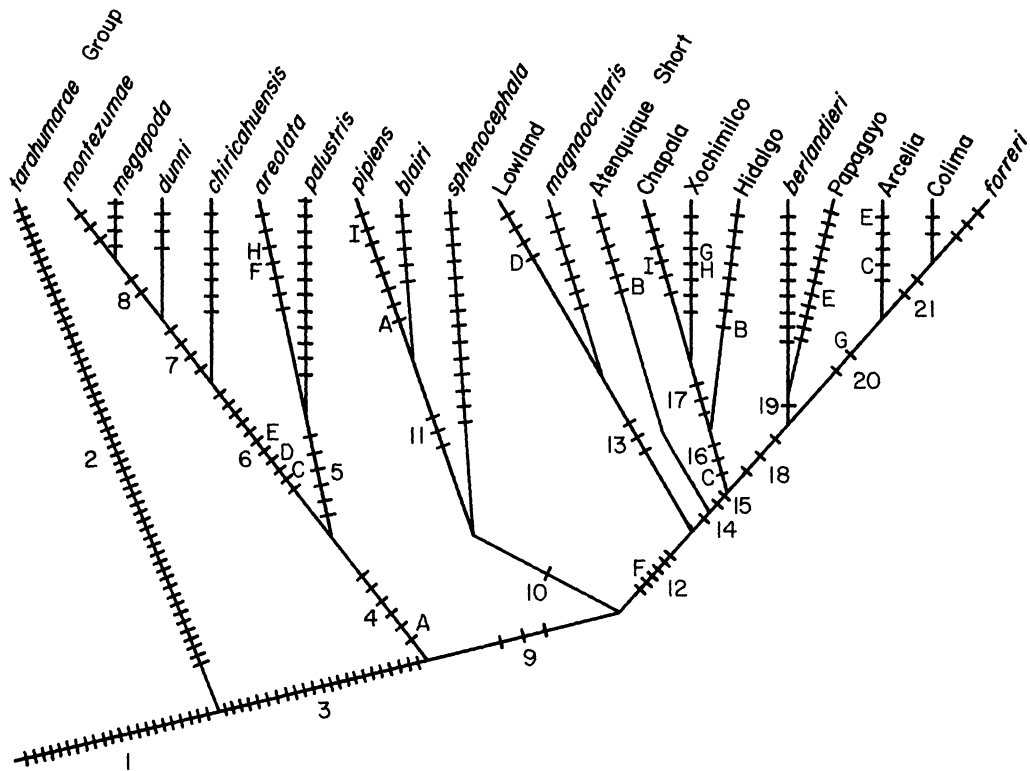


FIG. 1. Cladogram of 20 members of the *Rana pipiens* complex. Synapomorphies, symplesiomorphies, and autapomorphies for the numbered and labeled branch lengths are listed in Appendix II. Letters indicate convergence of electromorphs.

transformation series following the taxonomic outgroup and functional outgroup criteria of Watrous and Wheeler (1981), as expanded by Farris (1982). The assumptions and limitations of electrophoretic data with respect to systematics are discussed by Avise (1974).

#### RESULTS AND DISCUSSION

*Phylogeny.*—We identified 283 different electromorphs within the 50 gene loci examined among the 23 species (Table 2). The supported phylogeny of the *R. pipiens* complex based on this electrophoretic information is shown in Figure 1 (the synapomorphies are listed in Appendix II). A classification that identifies the major monophyletic groups is presented in Figure 2. This classification divides the *R. pipiens* complex into an Alpha division and a Beta division. A parallel dichotomy in each of these divi-

sions further divides the complex into two groups of North American species and two groups of Middle American species. These clades are identified with species-group names in Figure 2.

Because of the small sample sizes used in this study, some of the synapomorphies shown in Figure 1 may be misplaced. Examination of additional individuals and populations may show that some of the electromorphs should be placed lower in the cladogram. However, because of the large number of synapomorphies that support most of the clades that we recognize with group names in Figure 2, such corrections are unlikely to substantially change this classification.

The homoplasies indicated in Figure 1 represent fewer than 5% of the electromorphs. None of these homoplasies are congruently distributed, which provides

TABLE 1. Loci examined among 23 species of *Rana*. Abbreviations follow Wright et al. (1980) except as noted in the text. Enzymes Commission (E.C.) numbers follow the Commission on Biochemical Nomenclature (1979).

	Locus and abbreviation	E.C. number	Tissue	Buffer system
1	Acid phosphatase-1 (AP-1)	3.1.3.2	Muscle	TC7
2	Acid phosphatase-2 (AP-2)	3.1.3.2	Muscle	TC7
3	Aconitase (ACO)	4.2.1.3	Muscle	TC7
4	Adenosine deaminase (ADA)	3.5.4.4	Muscle	TC7
5	Adenylate kinase (AK)	2.7.4.3	Muscle	TVB
6	Aldolase (ALD)	4.1.2.7	Muscle	TC7
7	Catalase (CAT)	1.11.1.6	Liver	TVB
8	Creatine kinase (CK)	2.7.3.2	Muscle	TVB
9	Enolase-1 (ENO-1)	4.2.1.11	Muscle	TVB
10	Enolase-2 (ENO-2)	4.2.1.11	Muscle	TVB
11	Fructose-1,6-diphosphatase (F-1,6-DP)	3.1.3.11	Liver	TVB
12	Fumarase (FUM)	4.2.1.2	Liver	TC7
13	Glucose-6-phosphate dehydrogenase (G-6-PD)	1.1.1.49	Muscle	TVB
14	Glucosephosphate isomerase (GPI)	5.3.1.9	Muscle	TVB
15	$\beta$ -Glucosidase ( $\beta$ -GSD)	3.2.1.21	Liver	TVB
16	$\beta$ -Glucuronidase ( $\beta$ -GUR)	3.2.1.31	Liver	TVB
17	Glutamate oxaloacetate transaminase-1 (GOT-1)	2.6.1.1	Liver	TVB
18	Glutamate oxaloacetate transaminase-2 (GOT-2)	2.6.1.1	Liver	TVB
19	Glyceraldehyde-3-phosphate dehydrogenase (G-3-PD)	1.2.1.12	Muscle	TC7
20	$\alpha$ -Glycerophosphate dehydrogenase ( $\alpha$ -GPD)	1.1.1.8	Liver	TC7
21	Glyoxalase I (GLY)	4.4.1.5	Liver	TVB
22	Glutamate pyruvate transaminase (GPT)	2.6.1.2	Muscle	TVB
23	Hexosaminidase-1 (HA-1)	3.2.1.30	Muscle	TVB
24	Hexosaminidase-2 (HA-2)	3.2.1.30	Muscle	TVB
25	Hexokinase (HK)	2.7.1.1	Liver	TVB
26	Isocitrate dehydrogenase-1 (IDH-1)	1.1.1.42	Muscle	TC7
27	Isocitrate dehydrogenase-2 (IDH-2)	1.1.1.42	Muscle	TC7
28	Lactate dehydrogenase-1 (LDH-1)	1.1.1.27	Liver	TC7
29	Lactate dehydrogenase-2 (LDH-2)	1.1.1.27	Liver	TC7
30	Malate dehydrogenase-1 (MDH-1)	1.1.1.37	Liver	TC7
31	Malate dehydrogenase-2 (MDH-2)	1.1.1.37	Liver	TC7
32	Malic enzyme-1 (ME-1)	1.1.1.40	Liver	TC7
33	Malic enzyme-2 (ME-2)	1.1.1.40	Liver	TC7
34	Mannosephosphate isomerase (MPI)	5.3.1.8	Muscle	TVB
35	$\alpha$ -Mannosidase ( $\alpha$ -MAN)	3.2.1.24	Liver	TVB
36	Peptidase(L-leucyl-L-alanine)-1 (PEP(LA)-1)	3.4.11 or 13	Liver	TVB
37	Peptidase(L-leucyl-L-alanine)-2 (PEP(LA)-2)	3.4.11 or 13	Liver	TVB
38	Peptidase(L-leucylglycylglycine) (PEP(LGG))	3.4.11 or 13	Liver	TVB
39	Peptidase(L-leucyl-L-proline) (PEP(LP))	3.4.13.9	Liver	TVB
40	6-Phosphogluconate dehydrogenase (6-PGD)	1.1.1.44	Muscle	TVB
41	Phosphoglucomutase (PGM)	2.7.5.1	Muscle	TC7
42	2,3-Phosphoglycerate mutase (PGAM)	5.4.2.1	Muscle	TVB
43	Pyruvate kinase-1 (PK-1)	2.7.1.40	Liver	TVB
44	Pyruvate kinase-2 (PK-2)	2.7.1.40	Muscle	TVB
45	6-Phosphofructokinase (PFK)	2.7.1.11	Liver	TVB
46	Phosphoglycerate kinase-1 (PGK-1)	2.7.2.3	Liver	TVB
47	Phosphoglycerate kinase-2 (PGK-2)	2.7.2.3	Muscle	TVB
48	Superoxide dismutase-1 (SOD-1)	1.15.1.1	Liver	TVB
49	Superoxide dismutase-2 (SOD-2)	1.15.1.1	Liver	TVB
50	Triosephosphate isomerase (TPI)	5.3.1.1	Liver	TC7

support for our contention that these electromorphs are not homologous but merely convergent in mobility. In the case of every homoplasy, treatment of the electromorph

as a synapomorphy would require the interpretation of many more congruent characters as homoplastic.

All but one of the species traditionally



TABLE 2. Continued.

Locus	Taxon																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
PEP(LA)-1	c,e	a	a	a	a	b	b	d	c	c	c	c	c	d	—	c	c	c	c	c	c
PEP(LA)-2	c,f	e	e	e	e	d	a,c	c	c	c	c	d	d	d	—	—	b	b,c	c	c,d	c
PEP(LGG)	a	d	d	c	d	d	e	d	d	d	d	d	d	d	d	d	c	d	d	d	d
PEP(LP)	b,g	f	k	k	i	i	i	i	a	e	j	j	j	d	j	j	h	d	h	h	h
6-PGD	f,h,i	c	c	c	c	c	c	a	a	b	e	b,e	b	d	d	d	d	d	d	d	g
PGM	a	b	b	b	a,b	b	b	a,b	a	a	a	a	b	b	b	b	a	b	b	b	b
PGAM	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
PK-1	a	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
PK-2	a	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
PFK	c,g	b	b	b	b	b	b	b	b	b	b	b,e	d	b	b	b	b	f	b	b	a
PGK-1	e	e	e	e	—	e?	e	c	e	a	—	e	e	d	—	f	a	e,g	e	e	e
PGK-2	b,f,i	h	h	h	h	h	h	a	a	b	g	g	j	e	c	g	d	g	g	g	g
SOD-1	h,i	b	b	b	b	g	g	b	a	c	b	b	b	b	d	f	b	e	b	b	b
SOD-2	a	c	c	c	a,c	b	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
TPI	a	a	a	a	a	b	c	a	a	a	a	a	a	a	a	a	a	a	a	a	a

identified as a "leopard frog" belong to the Beta division of the *R. pipiens* complex. The single exception is *R. chiricahuensis*, a Sierra Madrean relative of three large species of the Mexican Plateau—*R. dunni*, *R. megapoda*, and *R. montezumae*. These four species make up the *R. montezumae* species group of the Alpha division. In addition to sharing 11 electrophoretic synapomorphies (Fig. 1), this species group also is characterized by at least one morphological synapomorphy: the presence of small yellow spots on the posterior surfaces of the thighs. The northern *R. areolata* species group of the Alpha division contains *R. palustris* and *R. areolata*, and (although none was examined electrophoretically in our study) undoubtedly includes *R. capito* as well. These three species also share at least one morphological synapomorphy, namely the presence of thick, glandular dorsolateral folds. *Rana palustris* is the only member of the Alpha division that does not have a short, stocky body and a broad head. Probably because of its relatively attenuated body, *R. palustris* has been more commonly placed with frogs of the Beta division than has either *R. areolata* or *R. capito*.

On the basis of ventral pigmentation, Sanders and Smith (1971) divided leopard frogs (essentially the species of the Beta division) into two groups—"those of the United States . . . as *Rana pipiens* with its subspecies and those of Mexico as *R. berlandieri* with its subspecies." Subsequent field work has shown that many of the "subspecies" of each of the groups occur sympatrically with little or no hybridization and, therefore, are now considered to be distinct species. However, the basic division of leopard frogs made by Sanders and Smith (1971) is supported by the electrophoretic data; these two groups are identified as the *R. pipiens* species group and the *R. berlandieri* species group in Figure 2. In addition to the relative absence of ventral melanism in the *R. pipiens* species group, all of these species also have a distinct white supralabial stripe. Sanders and Smith (1971) considered the presence of ventral melanism to be primitive (based on outgroup comparisons), so there is no known morphological synapomorphy that defines the *R. berlandieri* species group. Instead, the support for its

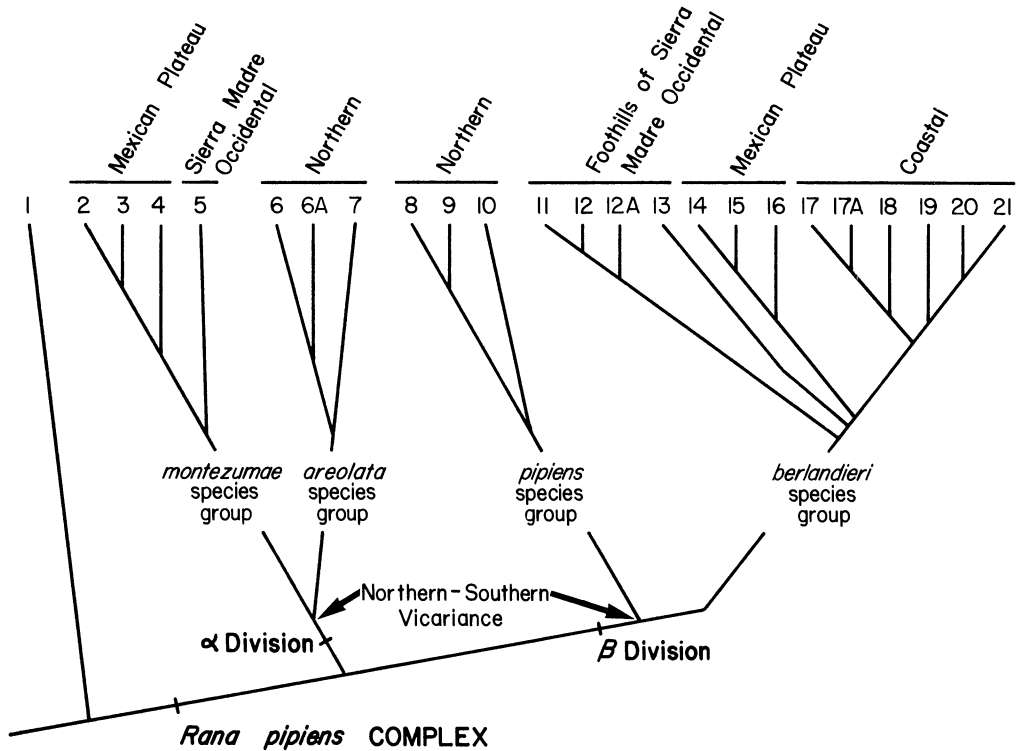


FIG. 2. Classification and general distribution of clades of the *Rana pipiens* complex. Numbers refer to the following taxa: (1) *R. tarahumarae* group; (2) *R. montezumae*; (3) *R. megapoda*; (4) *R. dunni*; (5) *R. chiricahuensis*; (6) *R. areolata*; (6A) *R. capito*; (7) *R. palustris*; (8) *R. pipiens*; (9) *R. blairi*; (10) *R. sphenoccephala*; (11) lowland form; (12) *R. magnaocularis*; (12A) Atenquique long form (relationship based on morphology); (13) Atenquique short form; (14) Chapala form; (15) Xochimilco form; (16) Hidalgo form; (17) *R. berlandieri*; (17A) *R. brownorum* (relationship based on morphology); (18) Papagayo form; (19) Arcelia form; (20) Colima form; (21) *R. forreri*.

monophyly rests with the six electrophoretic synapomorphies shown in Figure 1.

**Biogeography.**—The members of the Alpha division of the *R. pipiens* complex (distributions in Fig. 3) are, in many cases, broadly sympatric with the members of the Beta division (distributions shown in Fig. 4). However, the members within each of the two major divisions are distributed essentially parapatrically (Figs. 3, 4), although most of these species occur sympatrically with another species in their division in at least a small zone of overlap. No natural hybrids have been reported within the intradivisional overlap zones of the Alpha division. However, limited hybridization has been reported in five of the six zones of sympatry of Beta division species that have been studied (Platz, 1972; Pace, 1974; Axtell, 1976; Kruse

and Dunlap, 1976; Sage and Selander, 1979; the one exception was reported by Frost and Bagnara, 1977a). Isolated events of hybridization have been reported between *R. palustris* of the Alpha division and both *R. sphenoccephala* and *R. pipiens* of the Beta division (Hardy and Gillespie, 1976; Salthe, 1969). In addition, *R. chiricahuensis* of the Alpha division hybridizes to a limited extent with two of three species of the Beta division with which it is partially sympatric in southern Arizona (*R. pipiens* and the lowland form; Platz and Platz, 1973; Frost and Bagnara, 1977b).

Both the Alpha and Beta divisions contain a North American clade (the *R. areolata* group and the *R. pipiens* group, respectively) and an essentially Middle American clade (the *R. montezumae* group and the *R. berlandieri*

group, respectively). The eastern North America-central Mexico vicariant pattern is a common one, exhibited by such diverse groups as certain plants, fishes, salamanders, snakes, birds, and mammals (see review in Rosen, 1978). However, two species of the *R. berlandieri* group (*R. berlandieri* and the lowland form) depart somewhat from the usual distribution of the Mexican vicariants. These two species range into the deserts (Chihuahuan and Sonoran) that separate the mesic areas of North America from those of Mexico. *Rana berlandieri* also occurs in mesic areas to the south in Mexico, as does the sister species of the lowland form, *R. magnaocularis* (Fig. 4). Therefore, the distributions of *R. berlandieri* and the lowland form in the United States may represent relatively recent range expansions. Because widespread sympatry does not exist between species within a division, the northward expansion of these two species may be limited, in part, by the distributions of members of the *R. pipiens* species group. This interpretation seems especially probable for *R. berlandieri*, which has come into contact and established narrow hybrid zones with both *R. blairi* and *R. sphenoccephala* (Hillis, 1981).

The *R. areolata* species group consists of three largely parapatric species that occur in eastern North America. *Rana palustris* and *R. areolata* have curious distributions. The former is found in the northeastern United States and southeastern Canada and the Ozark Plateau south to the Gulf of Mexico, whereas the latter occurs in an arc that encircles the eastern, northern, and western boundaries of the Ozark Plateau. The areas of sympatry between *R. palustris* and *R. capito* are even more limited; these two species occur together only on the coastal plain of North and South Carolina. The interactions of these species in the zones of sympatry have not been studied.

The *R. montezumae* species group consists of four described species that occupy the Mexican Plateau and the Sierra Madre Occidental from southern Arizona to Jalisco. An additional undescribed species in this group occurs in east-central Mexico and appears to be most closely related to *R. chiricahuensis* (based on morphology). The only two species

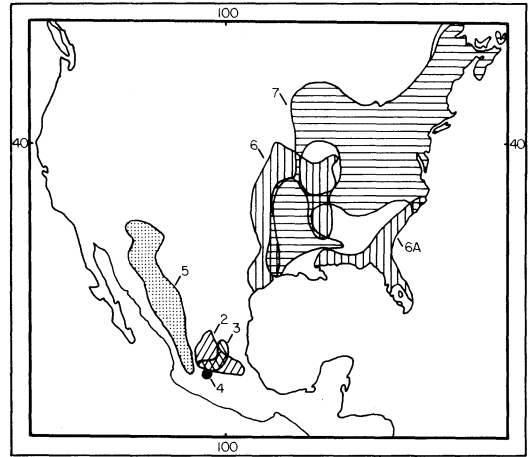


FIG. 3. Distribution of members of the Alpha division of the *Rana pipiens* complex. Numbers refer to the same taxa as in Figure 2.

in this group that are known to occur in sympatry are the sister taxa *R. montezumae* and *R. megapoda*; the range of the latter species is virtually contained within the range of the former (Fig. 3).

The *R. pipiens* species group contains the most widely distributed members of the complex. *Rana pipiens* is the northern and montane species, *R. blairi* the plains species, and *R. sphenoccephala* (= *R. utricularia* of Pace, 1974) the southeastern representative. The status of peninsular Florida populations (which differ in the males retaining vestigial oviducts; Moore, 1944) and eastern seaboard populations (which differ in LDH expression [Salthe, 1969] and karyotype [Corcoran and Travis, 1980]) of *R. sphenoccephala* is unresolved.

The *R. berlandieri* species group is the most diverse clade of leopard frogs. Members of this group extend from the southern United States south to central Panama. Among the species that we examined, we identified several additional subgroupings. One clade consists of six species (17-21 on Fig. 4) that are distributed along the Atlantic and Pacific coasts of Mexico. Additional members of this clade occur along the coasts of Central America southeastward to Costa Rica. A second clade consists of montane forms that occur in relatively high areas of the Mexican Pla-



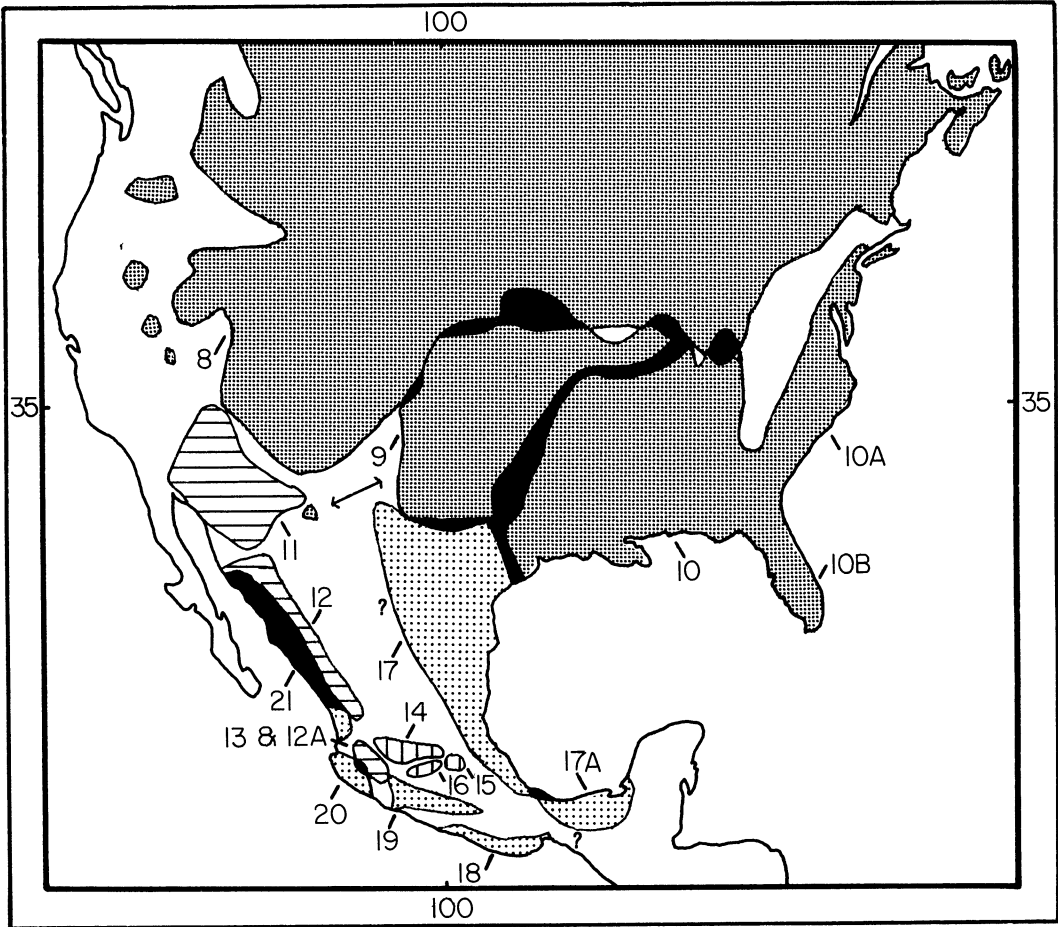


FIG. 4. Distribution of members of the Beta division of the *Rana pipiens* complex. Numbers refer to the same taxa as in Figure 2; 10A and 10B refer to portions of the range of *R. spheniocephala* where populations of questionable taxonomic status are located (see text). Zones of sympatry are indicated in black; clades within the Beta division are indicated by the same type of shading.

teau (14–16 on Fig. 4). The remaining two clades occur in the foothills of Sierra Madre Occidental. The Mexican Plateau clade and the coastal clade are more closely related to each other than either is to the two Sierra Madrean clades (Figs. 1, 2).

#### CONCLUDING REMARKS

The phylogeny presented here provides a valuable framework for interpreting many studies of the comparative biology of leopard frogs. Members of this group are common, one relatively easily obtained, and can be handled, raised, and bred in the laboratory. Species in the complex occur in virtually all

major biotic regions of North and Middle America, and in many cases individual species are limited to specific biotic regions. Many of the species can be hybridized artificially. In short, the group provides an ideal system for the study of many evolutionary questions. If these studies are based on a solid systematic framework, their relevance and importance to biology will be increased manifold.

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## APPENDIX I

### Collection Localities of Specimens Examined

**R. areolata**: 8 km N Galena, Cherokee County, Kansas, USA (94°38'W, 37°06'N). **R. berlandieri**: Dark Canyon, Eddy County, New Mexico, USA (104°15'W, 32°18'N). **R. blairi**: Lawrence, Douglas County, Kansas, USA (95°09'W, 38°54'N). **R. chiricahuensis**: Three Forks, Apache National Forest, Apache County, Arizona, USA (109°22'W, 33°50'N); Río Chico at Mex. Hwy. 40, Durango, Mexico (104°50'W, 23°57'N). **R. dunnii**: Lago Patzcuaro, near Tzintzuntzan, Michoacán, Mexico (101°38'W, 19°36'N). **R. forreri**: 38 km S Escuinapa, Sinaloa, Mexico (105°38'W, 22°36'N). **R. magnaocularis**: 10 km SW El Batel, Sinaloa, Mexico (105°54'W, 23°30'N); Arroyo Hondo, 15 km N Nuri, Sonora, Mexico (108°16'W, 28°10'N). **R. megapoda**: Rodeo, San Luis Potosí, Mexico (101°01'W, 22°15'N). **R. montezumae**: Xochimilco, Distrito Federal, Mexico (99°07'W, 22°15'N). **R. palustris**: Cave River, 5 km N Campbellsburg, Washington County, Indiana, USA (86°16'W, 38°39'N). **R. pipiens**: Near Alburg, Grande Isle County, Vermont, USA (73°19'W, 44°58'N). **R. pustulosa**: 10 km SW El Batel, Sinaloa, Mexico (105°54'W, 23°30'N). **R. sphenoccephala**: 5 km E Bastrop, Bastrop County, Texas, USA (97°15'W, 30°10'N). **R. tarahumarae**: 15 km E Yécora, Sonora, Mexico (108°44'W, 28°22'N). **Arcelia form**: Arcelia, Guerrero, Mexico (100°26'W, 18°14'N). **Atenquique short form**: Atenquique, Jalisco, Mexico (103°29'W, 19°32'N). **Chapala form**: 10 km NW Zamora, Michoacán, Mexico (102°20'W, 20°08'N); Zurumbueno, Michoacán, Mexico (100°04'W, 19°22'N). **Colima form**: 16 km SW Colima, Colima, Mexico (103°50'W, 19°03'N). **Hidalgo form**: 11 km E junction Mex. Hwy. 51 and 15, Michoacán, Mexico (100°50'W, 19°40'N). **Lowland form**: Redington Pass, Pima County, Arizona, USA (110°37'W, 32°19'N). **Papagayo form**: Río Papagayo at Mex. Hwy. 95, Guerrero, Mexico (99°38'W, 17°10'N). **Southern "pustulosa"**: Santa Fe, Morelos, Mexico (99°14'W, 18°40'N). **Xochimilco form**: Xochimilco, Distrito Federal, Mexico (99°07'W, 19°16'N).

## APPENDIX II

### Plesiomorphic, Synapomorphic, and Autapomorphic Alleles (See Fig. 1)

Alleles in parentheses indicate some subsequent evolutionary change at that locus. A "p" after an allele indicates its evolution in a polymorphic state, with some retention of the primitive allele.

1. Plesiomorphic alleles: (ADA<sup>a</sup>), (AK<sup>b</sup>), CK<sup>a</sup>, ENO-1<sup>a</sup>, ENO-2<sup>a</sup>, (F-1,6-DP<sup>b</sup>), (FUM<sup>a</sup>), (GOT-1<sup>a</sup>), GOT-2<sup>a</sup>, GLY<sup>a</sup>, HA-1<sup>a</sup>, HA-2<sup>a</sup>, (LH-1<sup>a</sup>), (LDH-2<sup>b</sup>), (MDH-1<sup>a</sup>), (MDH-2<sup>c</sup>), (PEP(LA)-1<sup>a</sup>), (PEP(LA)-2<sup>c</sup>), (PGM<sup>a</sup>), (PGK-1<sup>a</sup>), (SOD-2<sup>a</sup>), (TPI<sup>a</sup>).

2. Alleles found in the *R. tarahumarae* group but not in the *R. pipiens* complex (includes autapomor-

phies of species within the group as well as synapomorphies and symplesiomorphies of the group). Brackets indicate alleles not present in all species within the *R. tarahumarae* group: [AP-1<sup>a</sup>], [AP-1<sup>a</sup>], [AP-2<sup>a</sup>], [AP-2<sup>d</sup>], [ACO<sup>e</sup>], [ACO<sup>f</sup>], [ADA<sup>g</sup>], ALD<sup>g</sup>, CAT<sup>h</sup>, G-6-PD<sup>g</sup>, [GPI<sup>i</sup>], [GPI<sup>j</sup>], [ $\beta$ -GSD<sup>k</sup>], [ $\beta$ -GSD<sup>l</sup>],  $\beta$ -GUR<sup>m</sup>, [GOT-1<sup>n</sup>], [G-3-PD<sup>o</sup>], [G-3-PD<sup>o</sup>], [ $\alpha$ -GPD<sup>p</sup>], [ $\alpha$ -GPD<sup>q</sup>], [GPT<sup>r</sup>], [GPT<sup>r</sup>], [HK<sup>s</sup>], [HK<sup>s</sup>], IDH-1<sup>a</sup>, IDH-2<sup>b</sup>, [LDH-1<sup>a</sup>], [MDH-1<sup>c</sup>], [MDH-2<sup>a</sup>], [MDH-2<sup>d</sup>], [ME-1<sup>e</sup>], [ME-1<sup>e</sup>], ME-2<sup>f</sup>, [MPI<sup>g</sup>], [MPI<sup>h</sup>], [ $\alpha$ -MAN<sup>i</sup>], [ $\alpha$ -MAN<sup>j</sup>], [PEP(LA)-1<sup>k</sup>], [PEP(LA)-2<sup>l</sup>], PEP(LGG)<sup>m</sup>, [PEP(LP)<sup>n</sup>], [PEP(LP)<sup>o</sup>], [6-PGD<sup>p</sup>], [6-PGD<sup>q</sup>], PGAM<sup>r</sup>, PK-1<sup>a</sup>, PK-2<sup>b</sup>, [PFK<sup>c</sup>], [PFK<sup>d</sup>], [PGK-2<sup>e</sup>], [PGK-2<sup>f</sup>], [PGK-2<sup>g</sup>], [SOD-1<sup>h</sup>], [SOD-1<sup>i</sup>].

3. Synapomorphies and symplesiomorphies of the *R. pipiens* complex: (AP-1<sup>e</sup>), (AP-2<sup>c</sup>), (ALD<sup>d</sup>), (CAT<sup>e</sup>), (GPI<sup>f</sup>), ( $\beta$ -GUR<sup>g</sup>), (G-3-PD<sup>h</sup>),  $\alpha$ -GPD<sup>i</sup>, (GPT<sup>j</sup>), (IDH-1<sup>k</sup>), IDH-2<sup>a</sup>, (ME-1<sup>l</sup>), (ME-2<sup>d</sup>), (MPI<sup>m</sup>),  $\alpha$ -MAN<sup>n</sup>, (PEP(LGG)<sup>o</sup>), (PEP(LP)<sup>p</sup>), PGM<sup>q</sup>, PGAM<sup>r</sup>, (PK-1<sup>d</sup>), PK-2<sup>b</sup>, (PFK<sup>b</sup>), (SOD-1<sup>b</sup>).

4. Synapomorphies of the Alpha division: ACO<sup>d</sup> (see 28), (AK<sup>d</sup>), G-6-PD<sup>a</sup>, (HK<sup>k</sup>), 6-PGD<sup>d</sup>, PGK-2<sup>b</sup>.

5. Synapomorphies of the *R. areolata* species group: GPI<sup>d</sup>,  $\beta$ -GSD<sup>g</sup>, GPT<sup>c</sup>, MPI<sup>l</sup>, PEP(LA)-1<sup>b</sup>, SOD-1<sup>g</sup>.

6. Synapomorphies of the *R. montezumae* species group: AP-1<sup>c</sup>, CAT<sup>h</sup>, (F-1,6-DP<sup>p</sup>) (see 16, 39),  $\beta$ -GSD<sup>a</sup> (see 31), LDH-2<sup>p</sup>, (ME-1<sup>e</sup>) (see 38, 39), (ME-2<sup>b</sup>), (MPI<sup>h</sup>), PEP(LA)-1<sup>a</sup>, PEP(LA)-2<sup>e</sup>, SOD-2<sup>c</sup>.

7. Synapomorphies that unite *R. montezumae*, *R. megapoda*, and *R. dunni*: ADA<sup>a</sup>, GPT<sup>g</sup>, LDH-1<sup>g</sup>, (PEP(LP)<sup>h</sup>).

8. Synapomorphies that unite *R. montezumae* and *R. megapoda*: F-1,6-DP<sup>p</sup>, GOT-1<sup>b</sup>.

9. Synapomorphies of the Beta division (ACO<sup>c</sup>), G-6-PD<sup>b</sup>, (6-PGD<sup>b</sup>).

10. Synapomorphy of the *R. pipiens* species group: PGK-2<sup>a</sup>.

11. Synapomorphies that unite *R. pipiens* and *R. blairi*: ADA<sup>b</sup>,  $\beta$ -GSD<sup>d</sup>, 6-PGD<sup>a</sup>.

12. Synapomorphies of the *R. berlandieri* species group: (ADA<sup>d</sup>), ( $\beta$ -GSD<sup>a</sup>), (HK<sup>c</sup>) (see 26), MDH-2<sup>a</sup>, (PEP(LP)<sup>h</sup>), (PGK-2<sup>g</sup>).

13. Synapomorphies that unite *R. magnaocularis* and the lowland form: AP-1<sup>b</sup>, GPI<sup>h</sup>, 6-PGD<sup>p</sup>.

14. Synapomorphy that unites all of the species in the *R. berlandieri* species group exclusive of *R. magnaocularis* and the lowland form: (G-3-PD<sup>h</sup>).

15. Synapomorphies that unite the Mexican Plateau and coastal species of the *R. berlandieri* species group: (LDH-1<sup>h</sup>), (6-PGD<sup>b</sup>).

16. Synapomorphies that unite the Mexican Plateau species of the *R. berlandieri* species group: F-1,6-DP<sup>c</sup> (see 6, 39),  $\beta$ -GSD<sup>h</sup>, HK<sup>d</sup>.

17. Synapomorphies that unite the Chapala form and the Xochimilco form: GPI<sup>f</sup>, G-3-PD<sup>h</sup>, IDH-1<sup>b</sup>.

18. Synapomorphies that unite the coastal species of the *R. berlandieri* species group: ACO<sup>b</sup>, (CAT<sup>d</sup>), (PEP(LP)<sup>h</sup>).

19. Synapomorphy that unites *R. berlandieri* and the Papagayo form: PK-1<sup>c</sup>.

20. Synapomorphies that unite *R. forreri*, the Colima form, and the Arcelia form: (G-3-PD<sup>m</sup>), ME-2<sup>c</sup> (see 35).

21. Synapomorphies that unite *R. forreri* and the Colima form: GPT<sup>d</sup>, 6-PGD<sup>g</sup>.

22. Autapomorphies of *R. montezumae*: AP-2<sup>b</sup>, GPI<sup>g</sup>, PEP(LP)<sup>f</sup>.

23. Autapomorphies of *R. megapoda*: HK<sup>h</sup>, ME-1<sup>c</sup>, ME-2<sup>a</sup>, MPI<sup>b</sup>.

24. Autapomorphies of *R. dunni*: F-1,6-DP<sup>a</sup>, MPI<sup>d</sup>, PEP(LGG)<sup>e</sup>.

25. Autapomorphies of *R. chiricahuensis*: ADA<sup>c</sup>, ADA<sup>h</sup>,  $\beta$ -GSD<sup>c</sup>, GOT-1<sup>p</sup>, G-3-PD<sup>g</sup>, LDH-1<sup>f</sup>,  $\alpha$ -MAN<sup>a</sup>.

26. Autapomorphies of *R. areolata*: ADA<sup>p</sup>, CAT<sup>a</sup>, G-3-PD<sup>h</sup>, HK<sup>c</sup> (see 12), ME-1<sup>b</sup> (see 35), SOD-2<sup>h</sup>, TPI<sup>b</sup>.

27. Autapomorphies of *R. palustris*: ADA<sup>p</sup>, ADA<sup>m</sup>, AK<sup>a</sup>, F-1,6-DP<sup>d</sup>, GOT-1<sup>c</sup>, IDH-1<sup>d</sup>, LDH-1<sup>b</sup>, ME-2<sup>c</sup>, PEP(LA)-2<sup>a</sup>, PEP(LGG)<sup>e</sup>, PK-1<sup>b</sup>, TPI<sup>f</sup>.

28. Autapomorphies of *R. pipiens*: ACO<sup>d</sup> (see 4),  $\beta$ -GUR<sup>g</sup>, HK<sup>l</sup>, IDH-1<sup>e</sup>, MPI<sup>g</sup>, MPI<sup>p</sup>, PEP(LA)-1<sup>d</sup> (see 34), PGK-1<sup>c</sup>.

29. Autapomorphies of *R. blairi*: AP-1<sup>f</sup>, AP-2<sup>e</sup>, HK<sup>a</sup>, PEP(LP)<sup>a</sup>, SOD-1<sup>a</sup>.

30. Autapomorphies of *R. sphenoccephala*: ADA<sup>l</sup>,  $\beta$ -GSD<sup>h</sup>,  $\beta$ -GUR<sup>c</sup>, GOT-1<sup>p</sup>, G-3-PD<sup>d</sup>, HK<sup>l</sup>, LDH-1<sup>d</sup>, MDH-1<sup>f</sup>, ME-1<sup>g</sup>, MPI<sup>p</sup>, MPI<sup>o</sup>, PEP(LP)<sup>e</sup>, PGK-1<sup>b</sup>, SOD-1<sup>c</sup>.

31. Autapomorphies of the lowland form:  $\beta$ -GSD<sup>a</sup> (see 6), GOT-1<sup>a</sup>, HA-2<sup>b</sup>, ME-1<sup>a</sup>.

32. Autapomorphies of *R. magnaocularis*: ACO<sup>a</sup>,  $\beta$ -GUR<sup>d</sup>, G-3-PD<sup>i</sup>, HK<sup>p</sup>, ME-1<sup>q</sup>, MPI<sup>p</sup>, PFK<sup>q</sup>.

33. Autapomorphies of the Atenquique short form: AK<sup>c</sup> (see 36), CAT<sup>h</sup>,  $\beta$ -GSD<sup>b</sup>,  $\beta$ -GUR<sup>a</sup>, PFK<sup>d</sup>, PGK-2<sup>i</sup>.

34. Autapomorphies of the Chapala form: HK<sup>c</sup>, MPI<sup>a</sup>, PEP(LA)-1<sup>d</sup> (see 28), PEP(LP)<sup>d</sup>, PGK-1<sup>d</sup>, PGK-2<sup>e</sup>.

35. Autapomorphies of the Xochimilco form: ALD<sup>b</sup>, CAT<sup>g</sup>, GOT-1<sup>d</sup>, ME-1<sup>h</sup> (see 26), ME-2<sup>c</sup> (see 20), PGK-2<sup>c</sup>, SOD-1<sup>d</sup>.

36. Autapomorphies of the Hidalgo form: AK<sup>c</sup> (see 33), CAT<sup>c</sup>, LDH-1<sup>e</sup>, MDH-1<sup>b</sup>, ME-1<sup>b</sup>, MPI<sup>p</sup>, PGK-1<sup>f</sup>, SOD-1<sup>f</sup>.

37. Autapomorphies of *R. berlandieri*: ADA<sup>h</sup>, ALD<sup>a</sup>, GPI<sup>e</sup>,  $\beta$ -GUR<sup>b</sup>, G-3-PD<sup>a</sup>, MDH-1<sup>a</sup>, PEP(LGG)<sup>b</sup>, PGK-1<sup>a</sup>, PGK-2<sup>d</sup>.

38. Autapomorphies of the Papagayo form: GPI<sup>b</sup>, GOT-1<sup>b</sup>, G-3-PD<sup>c</sup>, MDH-1<sup>d</sup>, ME-1<sup>e</sup> (see 6, 39), MPI<sup>p</sup>, MPI<sup>q</sup>, PEP(LA)-2<sup>b</sup>, PEP(LP)<sup>e</sup>, PFK<sup>l</sup>, PGK-1<sup>q</sup>, SOD-1<sup>e</sup>.

39. Autapomorphies of the Arcelia form: CAT<sup>f</sup>, F-1,6-DP<sup>c</sup> (see 6, 16), FUM<sup>b</sup>, GPT<sup>b</sup>, ME-1<sup>e</sup> (see 6, 38).

40. Autapomorphies of the Colima form: ADA<sup>g</sup>, PEP(LA)-2<sup>p</sup>.

41. Autapomorphies of *R. forreri*: GOT-1<sup>a</sup>, G-3-PD<sup>e</sup>, PFK<sup>l</sup>.