Phylogenetic Relationships of the Pipid Frogs *Xenopus* and *Silurana*: An Integration of Ribosomal DNA and Morphology

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Relationships of the pipid frog genus *Silurana* (= *Xenopus tropicalis* group of some authors) are of particular interest to developmental and molecular biologists because of the purported ancestral (i.e., unduplicated) karyotype of *S. tropicalis* relative to the genus *Xenopus*. Although most previous studies have assumed that *Silurana* is the sister group of *Xenopus*, recent morphological work suggests that *Silurana* is more closely related both to the South American genus *Pipa* and to the African genera *Hymenochirus* and *Pseudohymenochirus* than it is to *Xenopus*. We examined 1,486 bp of relatively variable regions of the ribosomal DNA array (including portions of the 18S and 28S genes, as well as part of an internal transcribed spacer) in *Hymenochirus, Silurana,* and *Xenopus,* as well as the outgroup genus *Spea,* in order to test the alternative hypotheses of relationships for *Silurana.* Maximum-parsimony analysis using bootstrapping and an analysis using Lake’s method of invariants both significantly support the sister-group relationship between *Xenopus* and *Silurana* rather than the relationship suggested by morphology. Analysis of the combined morphological/molecular data matrix also significantly supports the *Xenopus-Silurana* relationship. Although our results are not inconsistent with the recognition of the genus *Silurana* to accommodate the species formerly called *X. tropicalis* and *X. epitropicalis,* the proposed relationships do not require the recognition of this genus in order to render *Xenopus* monophyletic.

Introduction

Frogs in the family Pipidae (especially the genus *Xenopus*) are among the most studied nonmammalian vertebrates. Species of *Xenopus* are especially important to developmental and molecular biologists because of their ease of maintenance, their easily manipulated reproductive system, and their relatively large and numerous ova (Dawid and Sargent 1988). However, investigations of the molecular biology of *Xenopus* often are hindered because almost all of the species in the genus are polyploid, so that most genes have functional or potentially functional paralogs. Only *X. tropicalis* has an unduplicated genome, so this species often is thought to represent the ancestral diploid condition for the genus (Tymowska and Fischberg 1982).

Immunological studies (Bisbee et al. 1977) first suggested that the genus *Xenopus* consists of two distinct species groups: (1) *X. tropicalis* and its sister species *X. epitropicalis* in the *X. tropicalis* group and (2) the remaining species in the *X. laevis* group. This division subsequently was supported by sperm protein patterns (Mann et al. 1982), karyological data (Tymowska and Fischberg 1982), globin patterns (Bürki and Fischberg 1985), and DNA content (Thiébault and Fischberg 1977). However,

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Table 1
Primers Used to Sequence the 18S-28S rDNA EcoRI/EcoRI Cloned Region Shown in Figure 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Positiona</th>
<th>Strandb</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8c</td>
<td>3494-3470</td>
<td>S</td>
<td>5'-GTGCGTTTCGAAGTGCATGATCAA-3'</td>
</tr>
<tr>
<td>5.8d</td>
<td>3470-3494</td>
<td>C</td>
<td>5'-TTGATCATGCAGGTTGGCGACGAC-3'</td>
</tr>
<tr>
<td>18h</td>
<td>2594-2624</td>
<td>C</td>
<td>5'AGGAATTTCCAGTAAGTGCAGGTCATAAAGCT-3'</td>
</tr>
<tr>
<td>28g</td>
<td>2180-2166</td>
<td>S</td>
<td>5'CTGCCCTTCCAAAGC-3'</td>
</tr>
<tr>
<td>28i</td>
<td>1704-1690</td>
<td>S</td>
<td>5'CCGGCCATCCATTTC-3'</td>
</tr>
<tr>
<td>28j</td>
<td>1529-1515</td>
<td>S</td>
<td>5'CCATTCCTGCTACC-3'</td>
</tr>
<tr>
<td>28k</td>
<td>1268-1254</td>
<td>S</td>
<td>5'CGATTGTCAGAACC-3'</td>
</tr>
<tr>
<td>28l</td>
<td>971-957</td>
<td>S</td>
<td>5'GGTCCGTGTTCAGAACC-3'</td>
</tr>
<tr>
<td>28p</td>
<td>207-193</td>
<td>S</td>
<td>5'CGAACAGAAGCAGCTATGAC-3'</td>
</tr>
<tr>
<td>28u</td>
<td>90-70</td>
<td>S/C</td>
<td>5'GTTACTGGGGGAAATCTGGT-3'</td>
</tr>
<tr>
<td>m13F</td>
<td>NA</td>
<td>S/C</td>
<td>5'GTTTTCCCAGTCACGAC-3'</td>
</tr>
<tr>
<td>m13R</td>
<td>NA</td>
<td>S/C</td>
<td>5'GTTTTCCCAGTCACGAC-3'</td>
</tr>
</tbody>
</table>

a In X. laevis rDNA sequences.

Cannatella and Trueb (1988a, 1988b) suggested that the hypothesized relationship between the two species groups was biased by a priori assumptions of the monophyly of the genus Xenopus. They conducted a detailed phylogenetic study of morphological characters of the entire family Pipidae and presented a phylogenetic hypothesis of intergeneric relationships. These authors concluded that the *X. tropicalis* group is more closely related to the genera *Hymenochirus, Pipa,* and *Pseudhymenochirus* than it is to the *X. laevis* group. Consequently, they resurrected the genus *Silurana* to accommodate the species in the former *X. tropicalis* group. Their proposed relationships contradict the idea that the karyotype of *S. tropicalis* is an ancestral for the genus *Xenopus.*

To test these competing hypotheses of the relationships of *Silurana* (= the *X. tropicalis* group), we studied nuclear ribosomal DNA (rDNA) sequences of the relevant taxa. Analyses of rDNA have been used in systematic studies to examine phylogenetic relationships at many levels, from closely related taxa to the earliest branches of life.

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rDNA and *Xenopus* Relationships

(e.g., see Fox et al. 1980; Küntzel and Köchel 1981; Wilson et al. 1984; Lane et al. 1985; Hillis and Davis 1986, 1987; Cedergren et al. 1988; Hillis and Dixon 1989; Larson and Wilson 1989). Study of such a wide spectrum of time is possible because the rDNA transcription units of eukaryotes are composed of highly conserved genes separated by more rapidly evolving transcribed spacers, and adjacent transcription units are separated by very rapidly evolving nontranscribed spacers (Appels and Dvořák 1982). Moreover, there are numerous divergent domains within the 28S gene that exhibit a broad spectrum of rates of divergence and can be used to examine phylogenetic relationships among genera and families of amphibians (Hassouna et al. 1984; Hillis and Davis 1987; Larson and Wilson 1989). We combined our study of rDNA with a reexamination of the morphological data reported by Cannatella and Trueb (1988a), because best estimates of phylogeny are based on consideration of total evidence from all sources (Hillis 1987; Kluge 1989).

**Material and Methods**

High-molecular-weight genomic DNA was extracted from frozen muscle and liver samples from *Hymenochirus curtipes*, *Silurana tropicalis*, and *Spea multiplicata* as described by Hillis and Davis (1986). Samples of DNA were digested with EcoRI, and the cleaved DNA was used to construct subgenomic libraries in the bacteriophage vector Lambda ZAP II (Stratagene). Approximately 100,000 plaques/species were screened through hybridization of nylon filter lifts at high-stringency conditions (65°C). The probe used in filter hybridizations was prepared from the cloned 28S rDNA gene of *Rana catesbeiana* (pE2528; Hillis and Davis 1987), which was radioactively labeled with α-32P-dATP (Rigby et al. 1977). Positive plaques were selected and purified, and the inserts were subcloned into Bluescript plasmids (Stratagene). Plasmid DNA was isolated by cesium chloride centrifugation (Sambrook et al. 1989) and sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1977) as modified by Tabor and Richardson (1987). Samples were run on 55-cm, 6% acrylamide gels with a constant temperature of 50°C at 2,500 V. Gels were visualized by autoradiography after 24–72 h of exposure on Kodak X-OMAT film. Primers used are shown in table 1, and their approximate positions in the rDNA repeat are shown in figure 1.

The DNA sequences were aligned with the homologous rDNA sequences of *Xenopus laevis* (Hall and Maden 1980; Salim and Maden 1981; Ware et al. 1983) by using the alignment subroutines of the IBI/Pustell sequence analysis software described by Pustell and Kafatos (1982, 1984, 1986). Gaps were introduced manually into the sequences to increase their aligned similarity. The full data set (used in maximum-parsimony analysis) consisted of 1,486 nucleotide (nt) positions, some of which were scored as deletions in one or more taxa (fig. 2). For Lake's (1987) method of invariants, positions with deletions or ambiguities in one or more taxa were deleted from the analysis. Regions in which positional homology was ambiguous are presented within brackets in figure 2 and were ignored in a subset of the phylogenetic analyses.

Phylogenetic analyses were performed with the "Phylogenetic Analysis Using Parsimony" (PAUP 3.0) software package (Swofford 1990). The *Spea* sequence was used as an outgroup (Lynch 1973; Duellman and Trueb 1986; Cannatella and Trueb 1988a). Confidence limits for branches of the most parsimonious tree were estimated by bootstrap analysis (Felsenstein 1985) with 1,000 iterations (by using the branch-and-bound algorithm of PAUP). The exact binomial test recommended by Holmquist et al. (1988) was used to test the results of Lake's (1987) method of invariants.
Results and Discussion

Data on 1,486 nt positions were obtained for each of the study taxa; 445 of these positions were variable among the species (fig. 2). On the basis of alignment with the published *Xenopus laevis* sequences, 225 nt positions correspond to the 3' end of 18s rRNA, 54 nt positions to the 5' end of the first internal transcribed spacer, and 987 nt positions to the 5' end of 28s rRNA.

The fit of the sequence data to the three possible trees (given *Spea* as the outgroup) is shown in figure 3 (panels a–c). The single most parsimonious tree obtained places *Silurana* as the sister taxon of *Xenopus*; this arrangement is 28 steps shorter than the tree suggested by morphology [(*Silurana*, *Hymenochirus*) *Xenopus*] and 30 steps shorter than the third possibility [(*Hymenochirus*, *Xenopus*) *Silurana*]. We also reanalyzed the rDNA sequences after removing all insertions, deletions, and ambiguous
positions from the data matrix (which reduces the data matrix from 1,486 to 1,276 characters). In this analysis, the Xenopus-Silurana tree is still 18 steps shorter than the Silurana-Hymenochirus (morphological) tree and 22 steps shorter than the third possibility (fig. 3, d-f). The confidence interval on the branch linking Silurana to Xenopus was estimated at >99.9% (on the basis of its presence in all 1,000 bootstrap iterations) in analyses of both the unreduced and reduced data matrices.

Under certain model conditions (trees with long terminal branches separated by comparatively short internal branches), maximum parsimony can lead to spurious results (Felsenstein 1978). Lake (1987) proposed a technique (evolutionary parsimony,
Fig. 3.—Trees obtained from rDNA sequence analysis. a–c, Trees derived from the analysis of all the aligned sequences; d–f, trees derived from the analysis of the rDNA sequences, excluding gaps and ambiguous positions. For each analysis the shortest tree is shown in boldface. Numbers above branches correspond to branch lengths. Total tree lengths when all characters and consistency indices (CI) are used are shown below each tree. Tree lengths and CI when only informative characters are used are given in parentheses.
FIG. 4.—Comparison of the three possible trees linking the four nucleotide sequences, by Lake's (1987) method of invariants. The data matrix consisted of 1,276 (gaps and ambiguities excluded) nucleotide positions. Only the tree uniting *Xenopus* and *Silurana* was significantly supported.

or method of invariants; also see Holmquist et al. 1988) that is reported to be accurate under these conditions [although it is a less powerful method of phylogenetic inference under other conditions (Li et al. 1987; Jin and Nei 1990)]. We applied Lake's method to the reduced rDNA data matrix (because Lake's method does not permit insertions, deletions, or ambiguities). As with the maximum-parsimony analysis, the tree linking *Xenopus* and *Silurana* was significantly favored ($P < 0.05$) in this analysis (fig. 4).

To compare and integrate the morphological and the molecular data, we reanalyzed the morphological data matrix of Cannatella and Trueb (1988a). Our analysis resulted in three equally parsimonious trees, each of 105 steps (one tree corresponded to the tree reported by Cannatella and Trueb 1988a); each of these trees placed *Silurana* as the sister taxon to the *Hymenochirus-Pipa* clade (fig. 5). The different topologies do not concern the relationships of *Silurana*, only the relationships within the genus *Pipa*. The distribution of lengths of all possible trees that is based on the morphological data matrix is strongly skewed to the left (fig. 6), suggesting a strong nonrandom component of interspecific variation (presumably a result of historical relationships; see Fitch 1984). However, three trees that support the alternative relationship of *Silurana* with *Xenopus* are located very close to the short end of the distribution (at 108 steps). The bootstrap analysis on the morphological data did not significantly support the branch linking *Silurana* to *Hymenochirus-Pipa* (fig. 5); this branch was supported in only 82% of the bootstrap iterations.

The results of the combined morphological/rDNA data analysis are shown in figure 7. Again, of the three possible trees, the most parsimonious one links *Silurana* with *Xenopus*, and it is 23 steps shorter than the *Hymenochirus-Silurana* alternative.
FIG. 5.—Consensus trees of the equally parsimonious hypotheses that were obtained from the morphological data of Cannatella and Trueb (1988a). a, Strict consensus tree derived from three trees 105 steps long. Numbers below the internal branches represent their percentage representation in 1,000 iterations of the bootstrap analysis. b, Strict consensus tree derived from three trees 108 steps long.

FIG. 6.—Distribution of the lengths of all possible trees that is based on morphological data of Cannatella and Trueb (1988a). Arrows indicate the close position of the two alternative hypotheses (see fig. 5) at the short end of the distribution.
In this case, the bootstrap analysis also suggested a confidence interval of >99.9% for the *Xenopus-Silurana* branch of the tree.

Several other analyses were performed to test our results. Maximum-parsimony and bootstrap analyses were conducted on the molecular data excluding the regions of relatively poor alignment (indicated by brackets in fig. 2), as well as on this reduced matrix combined with the morphological data. Lake’s method of invariants also was applied to the molecular data set excluding the sequences of questionable alignment within brackets. In all of these analyses, the *Silurana-Xenopus* tree was significantly supported (P < 0.05 in bootstrap or binomial tests, as appropriate) over the *Silurana-[Hymenochirus-Pipa]* (morphological) tree.

The relationship of *Silurana* to *Hymenochirus* and *Pipa* was supported by one behavioral and eight morphological traits in the analysis by Cannatella and Trueb (1988a). These authors noticed that two of the morphological features (contact of the epicoracoid cartilages and fusion of epicoracoids with the sternum) could have arisen independently in *Silurana* and *Hymenochirus*, providing an equally parsimonious explanation for their evolution. However, the *Silurana-Xenopus* relationship supported by the rDNA data requires a new interpretation for the other six morpho-
logical traits. If the molecular tree is correct, the loss of vomers, fusion of presacral vertebrae I and II, presence of a large sternum, and lack of pyriform muscle are either convergences between *Silurana* and the *Hymenochirus-Pipa* clade or ancestral pipoid features that have reversed within the *X. laevis* group. The presence of anterolateral processes of the prefrontals probably is a convergence between *Silurana* and *Hymenochirus-Pipa*; both *Pseudohymenochirus* and members of the *X. laevis* group lack these processes (Cannatella and Trueb 1988a, 1988b). The complex mating behavior of *Silurana, Hymenochirus*, and *Pipa* also might have arisen twice in pipoids or could be primitive to Pipidae, with secondary loss in the *X. laevis* group. However, four derived morphological characters support the rDNA data. These four synapomorphies for *Silurana-Xenopus* are (1) presence of an elongate zygomatic ramus of the squamosal, (2) presence of an epipubis, (3) presence of a subocular tentacle, and (4) partial fusion of the sartorious and semitendinosus muscles. The palpebral membrane is absent in *Hymenochirus* and *Pipa* and present in *Silurana, Pseudohymenochirus*, and *Xenopus*. Cannatella and Trueb (1988a) considered the presence of a palpebral membrane as the primitive state and considered its reduction or loss as the derived condition for pipids (it is reduced in *Silurana* and *Pseudohymenochirus*). However, the rDNA tree suggests that the presence of a palpebral membrane in *Silurana* is an ancestral condition shared with *Xenopus* and *Pseudohymenochirus* and that the loss of this structure is a convergence between *Hymenochirus* and *Pipa*. The *Silurana-Xenopus* tree is supported by 35 molecular and four morphological characters, whereas the *Silurana-Hymenochirus* tree is supported by seven molecular and nine morphological characters. The relative support for the two trees on the basis of morphology and the rDNA sequences is significantly different, as indicated by a $G$-test ($G = 11.8, P < 0.001$).

Our results do not invalidate the recognition of the genus *Silurana* to accommodate the species *S. tropicalis* and *S. epitropicalis*, because such an arrangement is not inconsistent with the rDNA data. However, on the basis of rDNA data, *Silurana* is phylogenetically closest to *Xenopus*, and the recognition of the genus *Silurana* is not required in order to render *Xenopus* monophyletic. The two species of *Silurana*—*S. tropicalis* and *S. epitropicalis*—have at least two unique morphological synapomorphies: (1) an elliptical tympanic annulus and (2) a particular anatomical relationship of the epicoracoid cartilages to the coracoids (Cannatella and Trueb 1988a). In view of the distinctiveness—on the basis of all sources of data (behavioral, morphological, and molecular)—of the *tropicalis* group, the recognition of *Silurana* is not without merit. However, we found no grounds to recognize the subfamily Siluraniinae, and we therefore suggest that *Silurana* should remain within the subfamily Xenopodinae (which should be restricted to the genera *Silurana* and *Xenopus*). The remaining genera of pipid frogs (*Hymenochirus, Pipa*, and *Pseudohymenochirus*) remain in the subfamily Pipinae, as suggested by Cannatella and Trueb (1988a).

Sequence Availability

These sequences have been deposited in GenBank under accession numbers M32844, M32845, M32846, M32847, M32848, M32849, M32850 M32851, and M32852.

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