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EVOLUTION OF RIBOSOMAL DNA: FIFTY MILLION YEARS OF RECORDED HISTORY IN THE FROG GENUS *RANA*

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Abstract.—Evolution of nuclear ribosomal DNA (rDNA) arrays of frogs of the genus *Rana* was examined among 32 species that last shared a common ancestor approximately 50 million years ago. Extensive variation in restriction sites exists within the transcribed and nontranscribed rDNA spacer regions among the species, whereas rDNA coding regions exhibit comparatively little interspecific variation in restriction sites. The most parsimonious phylogenetic hypothesis for the evolution of the group was constructed based on variation in restriction sites and internal spacer lengths among the 32 species of *Rana* and one species of *Pyxicephalus* (examined for outgroup comparison). This analysis suggests that *R. sylvatica* of North America is more closely related to the *R. temporaria* group of Eurasia than to other North American *Rana*. The hypothesized phylogeny also supports the monophyly of the *R. boylii* group, the *R. catesbeiana* group, the *R. palmipes* group, the *R. tarahumarae* group, and the *R. pipiens* complex. Furthermore, the restriction site data provide information about the evolution within and among these species groups. This demonstrates that restriction site mapping of rDNA arrays provides a useful molecular technique for the examination of historical evolutionary questions across considerable periods of time.

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The vast record of evolutionary history contained within DNA sequences of extant organisms remains largely unexplored. Most evolutionary studies of DNA have concerned the genomes of mitochondria and chloroplasts, rather than the nuclear genome, because of the relative simplicity and ease of isolation of these sequences (e.g., Avise et al., 1979; Gordon et al., 1982; Palmer and Zamir, 1982; Avise and Lansman, 1983; Bowman et al., 1983; Brown, 1983; Ferris et al., 1983; Lansman et al., 1983; Palmer et al., 1983; Cann et al., 1984; Clegg et al., 1984; Spolsky and Uzzell, 1984). As noted by Brown et al. (1979), Kessler and Avise (1984), and Lansman et al. (1981), the rapid divergence of mitochondrial DNA (mtDNA) coupled with its maternal inheritance make mtDNA a useful tool for examination of evolutionary problems on the level of intraspecific populations and closely related species. However, the attribute of rapid divergence places a limit on the time scale across which mtDNA can provide useful information. Chloroplast DNA (cpDNA) is evolutionarily more conservative than is mtDNA (Curtis and Clegg, 1984), but studies of cpDNA obviously are limited to problems concerning plants. Therefore, studies of DNA that deal with phylogenetic questions across a broad evolutionary timescale among animals must involve relatively conservative nuclear DNA sequences. To date, few such studies have been attempted.

Because nuclear genomes are many orders of magnitude larger than the genomes of organelles, it is not possible to analyze the entire nuclear component by restriction-fragment analysis at one time. However, small genomic segments can be analyzed using cloned DNA segments as hybridization probes in genomic Southern experiments. Nuclear ribosomal DNA (rDNA) arrays should be especially useful for evolutionary studies because: 1) the rDNA sequence is mid to highly repetitive (Britten and Kohne, 1968), so that multiple copies are present and ease of hybridization analysis is insured; 2) the rDNA repeat length is within a range that can be examined by restriction-fragment analysis; 3) rDNA contains both slowly evolving regions (the 18S, 5.8S, and 28S rRNA genes) and more rapidly evolving regions (the transcribed and nontranscribed spacers), so that information from various levels of evolutionary history can be recovered (Appels and Dvořák, 1982*b*); and 4) rDNA evolves in a concerted

fashion (Dover and Coen, 1981), so the rDNA of single individuals usually is representative of the species. Although the potential evolutionary information contained in conservative nuclear DNA such as rDNA arrays is immense, phylogenetic applications of restriction-fragment analyses of rDNA have been rare and confined to a few groups of closely related organisms (Appels and Dvořák, 1982*a*; Wilson et al., 1984; Sytsma and Schaal, 1985).

Here we present information on the utility and limitations of restriction-site analysis of rDNA as applied to questions of evolutionary history in a group of vertebrates. We have chosen as an example a Holarctic and Neotropical assemblage of frogs of the genus *Rana*. The genus *Rana* is older than are most orders of placental mammals; some species within the genus have not shared a common ancestor in over 100 million years (Wallace et al., 1973). The genus contains close to 300 species (Frost, 1985), among which morphological changes are for the most part conservative. Because of the great age of the genus, its numerous contained species, its worldwide distribution, and the limited morphological divergence among the species, phylogenetic relationships within *Rana* are poorly understood. Immunological studies have defined a Holarctic and Neotropical subgroup within *Rana* (Wallace et al., 1973; Case, 1978), but immunological data concerning relationships within this subgroup remain controversial (Post and Uzzell, 1981; Farris et al., 1980, 1983). Paleontological, biogeographical, and immunological data suggest that Holarctic and Neotropical *Rana* first diversified about 50 million years ago in the mid-Eocene when a land connection between eastern North America and western Eurasia was disrupted (Case, 1978). Therefore, we analyzed restriction sites within the rDNA arrays of this group of *Rana* in order to provide information on the phylogenetic utility of slowly and rapidly evolving portions of rDNA through 50 million years, and to resolve some of the conflicts surrounding the evolution of these frogs.

Materials and Methods

Tissue samples were obtained from 32 species of Holarctic *Rana* (see Appendix). The majority of the New World species of *Rana* were examined, as well as representatives of the Old World species groups. Although one of these species groups (the *R. ridibunda* group, as represented by *R. ridibunda* in our analysis) has been identified as an outgroup to the other Holarctic and Neotropical *Rana* (Post and Uzzell, 1981), we also included a more distantly related ranid (*Pyxicephalus adspersus*) in our analyses for further outgroup clarification.

Liver, muscle, or blood samples were removed from recently killed (live in the case of blood) individuals and stored at −80°C. Blood samples were diluted in STE (0.1 M NaCl, 0.05 M tris, 0.001 M EDTA; pH 7.5) to avoid problems with coagulation. Approximately 1 g of tissue was pulverized to fine powder in liquid nitrogen using a prechilled mortar and pestle. This powder was suspended in STE (approximately 1:20 ratio of tissue : buffer). Cells were lysed by the addition of 20% sodium dodecyl sulfate in water (0.05 ml/ml) and proteins were digested with 100 U/ml proteinase K (from *Tritirachium album*) for 2 hr at 55°C. After digestion, the preparation was extracted twice with an equal volume of a 25:25:1 solution of phenol : chloroform : isoamyl alcohol. Samples were centrifuged for 3 min at 7,000 *g* during each extraction to facilitate separation of the layers. The supernatant was then extracted twice with chloroform, again with 3 min centrifugation at 7,000 *g*. DNA was precipitated from the supernatant by the addition of a 1/10 volume of 2M NaCl and two volumes of 95% ethanol. Precipitated DNA was recovered by centrifuging for 10 sec at 7,000 *g*, and then was dissolved in 0.001 M tris, 0.0001 M EDTA (pH 7.2). This solution was stored at 4°C until use. Nitrogen-powdered skeletal muscle yielded approximately 50 μg of high molecular weight DNA per 100 mg of tissue; the yield from liver was approximately 150 μg DNA/100 mg tissue.

Sixteen restriction endonucleases (New England Biolabs) were used to digest 0.5–1.0 μg of DNA in 50 μl reactions following the manufacturer's specifications. Enzymes used (and their recognition sequences) were *Apa* I (GGGCC/C), *BamH* I (G/GATCC), *Bcl* I (T/GATCA), *Bgl* II (A/GATCT), *BstE* II (G/GTNACC), *Dra* I (TTT/AAA), *EcoR*

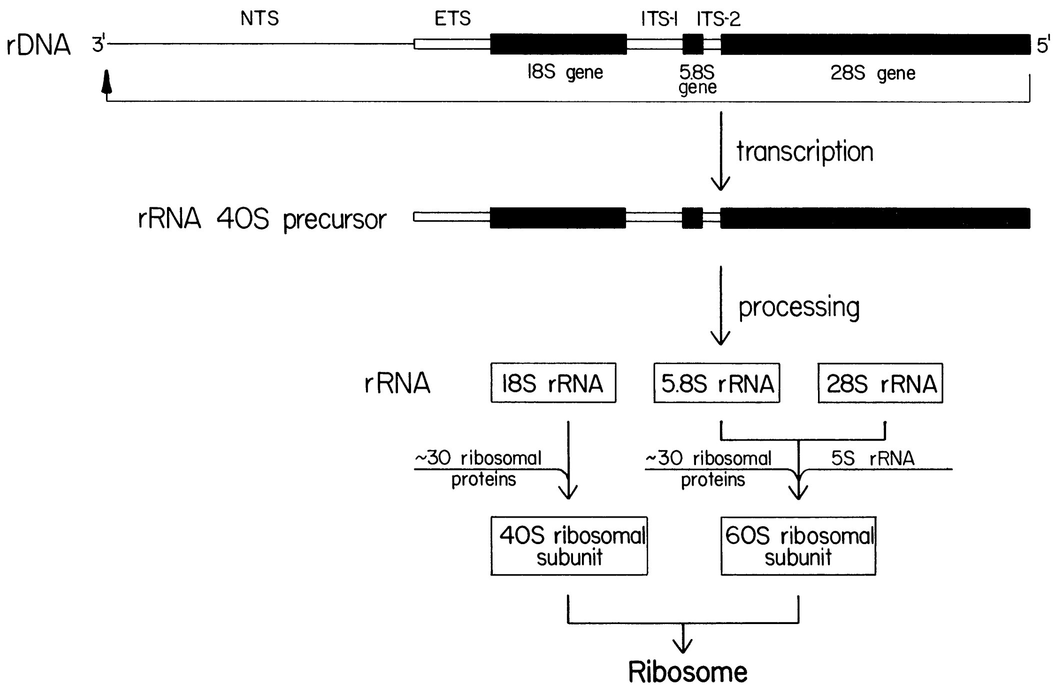

FIG. 1. Structure and function of the ribosomal DNA repeat. Abbreviations are as follows: NTS: nontranscribed spacer; ETS: external transcribed spacer; ITS: internal transcribed spacer.

I (G/AATTC), *Hind* III (A/AGCTT), *Kpn* I (GGTAC/C), *Nco* I (C/CATGG), *Pst* I (CTGCA/G), *Pvu* II (CAG/CTG), *Sac* I (GAGCT/C), *Stu* I (AGG/CCT), *Xba* I (T/CTAGA), and *Xmn* I (GAANN/NNTTC). DNA was digested for 4 to 6 hr.

Restricted DNA was electrophoresed at approximately 5 V/cm for 15 hr in 0.8% agarose gels (buffer system: 0.04 M tris, 0.02 M sodium acetate, 0.018 M sodium chloride, 0.001 M EDTA; pH 8.0). Lambda C1857 phage DNA cut with *Hind* III was included on each gel as a standard. After electrophoresis, DNA was stained with ethidium bromide and then viewed and photographed under 302 nm UV light. DNA in the agarose gels was denatured in 1.5 M NaCl, 0.5 N NaOH for 1 hr, and then neutralized in 3 M NaCl, 0.5 M tris for 1.5 hr. DNA fragments were transferred to nitrocellulose paper (Schleicher and Schuell BA85) following the method of Southern (1975). Southern blots were probed with two radioactively labeled clones that contained the mouse 18S gene (p 2546) and the mouse 28S gene (p I19); these segments had been subcloned by Norman Arnheim from the λgtWES clones described by Arnheim (1979) and Tiemeier et al. (1977). After hybridization, filters were washed and exposed to X-ray film for 12–48 hr at −80°C. Hybridization conditions and washes were identical to those described by Sytsma and Schall (1985). After autoradiography, DNA fragments were sized by fitting migration distances to a least-squares regression line of lambda C1857 phage DNA-*Hind* III fragment migration distances (excluding the 23,130 base-pair and 125 base-pair fragments), using the algorithm of Schaffer and Sederoff (1981). Mapping of sites was accomplished by double digestions, using as reference points the *EcoR* I sites that are uniformly present in vertebrates near the 5′ ends of the 18S and 28S genes (Cortadas and Pavon, 1982).

Phylogenetic trees were produced following the principle that parallel loss of a restriction site is much more likely than parallel gain of the site (Templeton, 1983*a*, 1983*b*). This method has been shown to be an efficient estimator of phylogeny for restriction map data (DeBry and Slade, 1985). However, our method differed from that de-

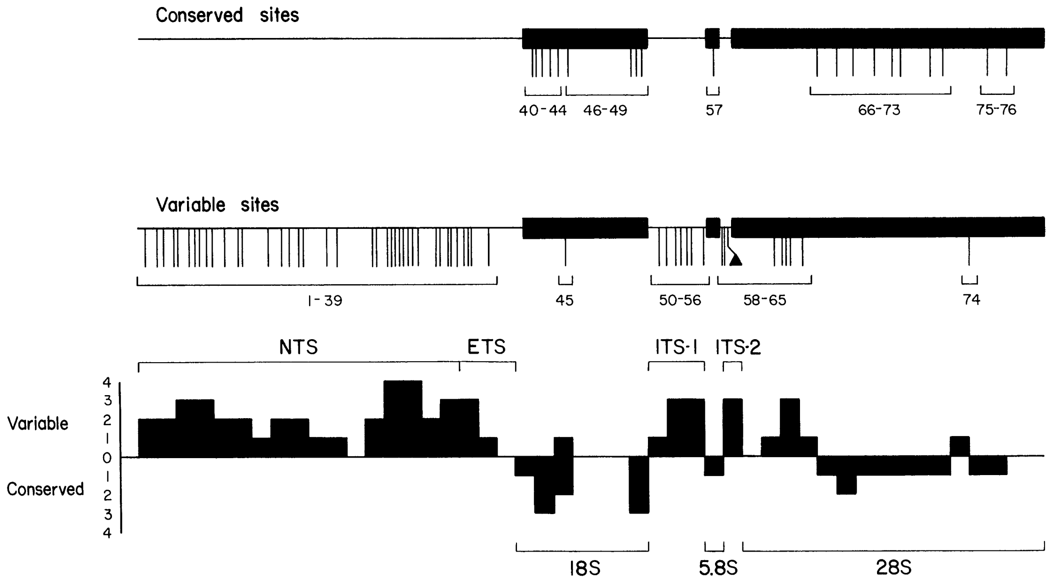

FIG. 2. Variable versus conserved restriction sites and length changes (triangle) in the rDNA repeat among 32 species of *Rana*. The graph at the bottom of the figure displays the number of variable sites (above the axis) and conserved sites (below the axis) for each 250-base-pair segment of the repeat. Site numbers correspond to numbers in Table 1 and Figure 3. Sites present only in *Pyxicephalus adspersus* are not illustrated.

scribed by DeBry and Slade (1985) in that we included an outgroup to differentiate between ancestral and derived restriction sites. Sites present in both ingroup and outgroup were considered symplesiomorphic; sites present or absent in only part of the ingroup were considered synapomorphic (Wiley, 1981).

RESULTS

The genes that code for 18S, 5.8S, and 28S rRNAs are found in tandemly repeated arrays within the nucleolar organizer region of eukaryotic genomes. The rDNA of the anuran *Xenopus laevis* has been studied extensively and the repeat unit sequenced in its entirety. The structure and role of a single repeat from the rDNA array of *Xenopus laevis* is diagrammed in Figure 1 (Boseley et al., 1979; Hall and Maden, 1980; Salim and Maden, 1981; and Ware et al., 1983). The coding regions are separated by internal transcribed spacers (ITS-1 and ITS-2); adjacent repeats are separated by a nontranscribed spacer (NTS). In addition, an external transcribed spacer (ETS) is positioned between the NTS and the 18S gene sequence (Fig. 1). This basic structure of rDNA arrays is highly conserved throughout eukaryotes (Goldman et al., 1983).

The rDNA arrays of the 33 species studied were mapped with respect to the restriction sites listed in Table 1. These maps were figured in detail by Hillis (1985). Twenty restriction sites that are conserved throughout the 33 species of *Rana* and 56 restriction sites that are variable within *Rana* are shown in Figure 2. All of the sites that have been conserved throughout the history of these species are located within the regions that code for 18S, 5.8S, or 28S rRNA. In contrast, only seven of 56 sites that vary among these *Rana* are found within a gene sequence; six of these sites are in the 28S gene, and five of those six are clustered within the first 1,000 base pairs (from the 3′ end) of the gene.

All of the restriction sites that are conserved throughout *Rana* in the 18S, 5.8S, and 28S genes are also found in *Xenopus laevis* (Hall and Maden, 1980; Salim and Maden, 1981; Ware et al., 1983), with the exception of the *Xmn* I site in the 18S gene and one of the *Sac* I sites in the 28S gene. In the *Xenopus* sequence, the corresponding sites are each one nucleotide different from the appropriate recognition sequence. In ad-

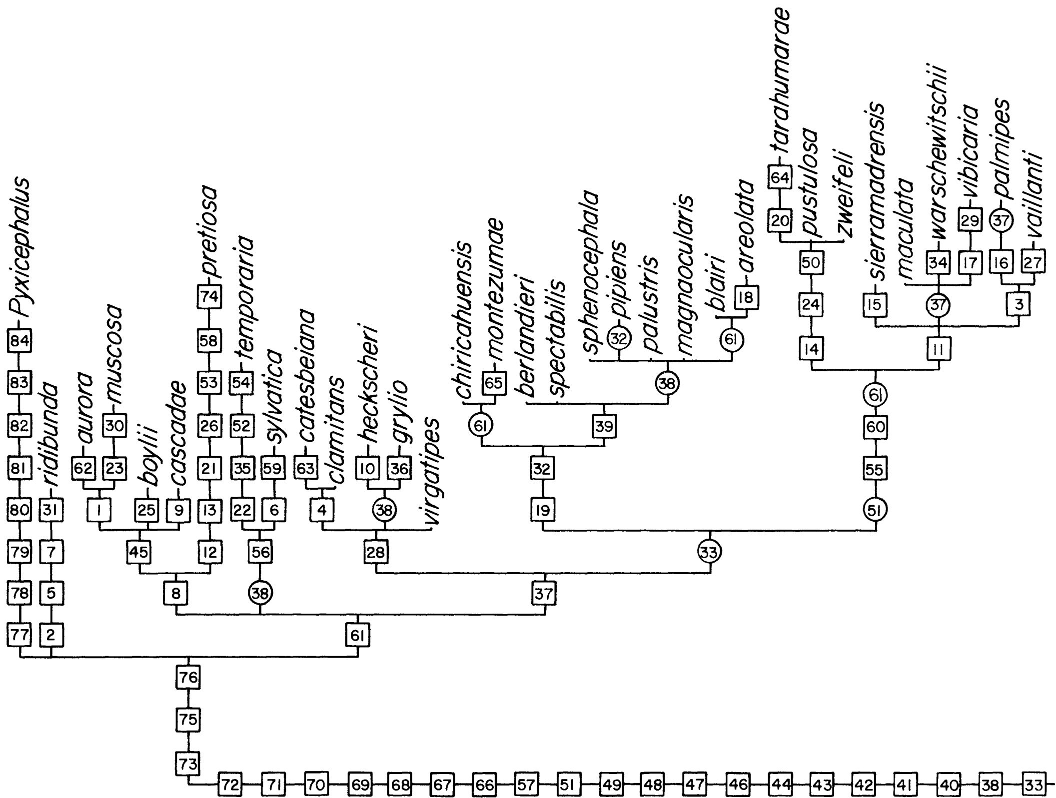

FIG. 3. Phylogenetic tree of 32 species of *Rana* based on variation in rDNA restriction sites. The squares represent site gains, and the circles represent site losses. The site numbers refer to Figure 2 and Table 1; sites 77–84 are those unique to *P. adspersus.*

dition, there is an *Xmn* I site in the 18S gene of *Xenopus* that is absent in *Rana.* There are also two *Xba* I sites near the 3′ end of the 18S gene in *Xenopus,* whereas we mapped only a single *Xba* I site at that location. However, this may be a result of the loss of the *Xba* I-*Xba* I fragment during electrophoresis because of its small size.

The rRNA repeat is approximately 12,000 base pairs long in all of the species of *Rana* that we examined, with only minor length heterogeneity within individuals. The rDNA repeat of nine species of *Rana* was found to be 200 base pairs longer than the repeat of the remaining species; this 200-base-pair insertion is located between the conserved *Bcl* I site in the 5.8S gene (site 57 in Fig. 2) and the first conserved site in the 28S gene, a *BamH* I site (site 66 in Fig. 2). Therefore, we hypothesize that this insertion is located in the ITS-2 sequence between the 5.8S and 28S genes (Fig. 2). In addition, a deletion and an insertion with respect to the *Rana* map were found in the *Pyxicephalus* rDNA repeat; the deletion (200 base pairs) was located between the 18S and 5.8S genes (ITS-1) and the insertion (600 base pairs) was located in the NTS.

The restriction-site data were used to construct a phylogenetic tree for the 33 species (Fig. 3). Site gains and losses that have occurred in the rDNA repeat over the past 50 million years in these species are shown along each of the branches of the tree in Figure 3.

Twenty-one restriction sites are informative about the phylogeny of these species (i.e., have been gained or lost in more than one species). Of these, only two are in coding regions (one each in the 18S gene and the 28S gene); these represent only 7.4% of the sites in coding regions. Three of the four sites in the ETS, five of the ten sites in ITS-1 and ITS-2, and 11 of the 35 sites in the NTS

TABLE 1. Presence (+) or absence (−) of restriction sites within the rDNA arrays of 33 species of ranid frogs. Site numbers refer to Figure 2. Number codes for species are as follows: 1) *Pyxicephalus adspersus*; 2) *R. ridibunda*; 3) *R. temporaria*; 4) *R. sylvatica*; 5) *R. aurora*; 6) *R. boylii*; 7) *R. cascadae*; 8) *R. muscosa*; 9) *R. pretiosa*; 10) *R. catesbeiana*; 11) *R. clamitans*; 12) *R. grylio*; 13) *R. heckscheri*; 14) *R. virgatipes*; 15) *R. areolata*; 16) *R. berlandieri*; 17) *R. blairi*; 18) *R. chiricahuensis*; 19) *R. magnaocularis*; 20) *R. montezumae*; 21) *R. palustris*; 22) *R. pipiens*; 23) *R. spectabilis*; 24) *R. sphenocephala*; 25) *R. pustulosa*; 26) *R. tarahumarae*; 27) *R. zweifeli*; 28) *R. maculata*; 29) *R. palmipes*; 30) *R. sierramadrensis*; 31) *R. vaillanti*; 32) *R. vibicaria*; and 33) *R. warschewitschii*. Sites unique to *P. adspersus* are not listed.

Species	Restriction site or insertion																								
	1. *Pst* I	2. *BstE* II	3. *Pvu* II	4. *Bcl* I	5. *Pvu* II	6. *Xmn* I	7. *Bgl* II	8. *Pvu* II	9. *Nco* I	10. *Bcl* I	11. *Stu* I	12. *Nco* I	13. *Pvu* II	14. *Pvu* II	15. *Nco* I	16. *Pst* I	17. *Bcl* I	18. *Pst* I	19. *Nco* I	20. *Hind* III	21. *Bgl* II	22. *Xmn* I	23. *Sac* I	24. *Xmn* I	25. *BstE* II
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
4	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
7	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
9	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−
10	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
13	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
17	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
18	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
21	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
22	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
23	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
24	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
25	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−
26	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−
27	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−
28	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
30	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−
31	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
32	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
33	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 1. Extended.

Species	Restriction site or insertion																								
	26. *EcoR* I	27. *Xmn* I	28. *BstE* II	29. *Xmn* I	30. *Stu* I	31. *BstE* II	32. *Hind* III	33. *Kpn* I	34. *EcoR* I	35. *Hind* III	36. *Kpn* I	37. *Sac* I	38. *BamH* I	39. *Pst* I	40. *Xba* I	41. *Nco* I	42. *BstE* II	43. *Xmn* I	44. *Dra* I	45. *BamH* I	46. *Pst* I	47. *BstE* II	48. *EcoR* I	49. *Xba* I	50. *BamH* I
1	−	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−
2	−	−	−	−	−	+	−	+	−	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−
3	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	+	+	+	+	−	+	+	+	+	−
4	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	+	+	−	+	+	+	+	−
5	−	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−
6	−	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−
7	−	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−
8	−	−	−	−	+	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−
9	+	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−
10	−	−	+	−	−	−	−	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
11	−	−	+	−	−	−	−	+	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
12	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+	+	−	+	+	+	+	−
13	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	+	+	+	−	+	+	+	+	−
14	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
15	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	−	+	+	+	+	−
16	−	−	−	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+	+	+	−
17	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	−	+	+	+	+	−
18	−	−	−	−	−	−	+	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
19	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	−	+	+	+	+	−
20	−	−	−	−	−	−	+	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
21	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	−	+	+	+	+	−
22	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+	+	−	+	+	+	+	−
23	−	−	−	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+	+	+	−
24	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	+	+	−	+	+	+	+	−
25	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	+
26	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	+
27	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	+
28	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−
29	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−
30	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
31	−	+	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
32	−	−	−	+	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−
33	−	−	−	−	−	−	−	−	+	−	−	−	+	−	+	+	+	+	+	−	+	+	+	+	−

TABLE 1. Extended.

Species	51. *Kpn* I	52. *Bcl* I	53. *Sac* I	54. *Xba* I	55. *Kpn* I	56. *Pvu* II	57. *Bcl* I	58. *Xmn* I	59. *BstE* II	60. +200 bp	61. *Stu* I	62. *Sac* I	63. *Hind* III	64. *Hind* III	65. *Hind* III	66. *BamH* I	67. *Dra* I	68. *Nco* I	69. *Bgl* II	70. *Xmn* I	71. *Sac* I	72. *Pvu* II	73. *Xmn* I	74. *Pst* I	75. *Sac* I	76. *EcoR* I
	Restriction site or insertion																									
1	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
2	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
3	+	+	−	+	−	+	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
4	+	−	−	−	−	+	+	−	+	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
5	+	−	−	−	−	−	+	−	−	−	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+
6	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
7	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
8	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
9	+	−	+	−	−	−	+	+	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
10	+	−	−	−	−	−	+	−	−	−	+	−	+	−	−	+	+	+	+	+	+	+	+	−	+	+
11	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
12	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
13	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
14	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
15	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
16	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
17	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
18	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
19	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
20	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	+
21	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
22	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
23	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
24	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
25	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
26	−	−	−	−	+	−	+	−	−	+	−	−	−	+	−	+	+	+	+	+	+	+	+	−	+	+
27	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
28	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
29	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
30	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
31	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
32	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+
33	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	+	+

TABLE 2. Currently recognized species groups of New World *Rana* (Frost, 1985; Hillis and Frost, 1985; Moler, 1985).

Group	Included Species
I. *R. catesbeiana* group	*R. catesbeiana, R. clamitans, R. grylio, R. heckscheri, R. okaloosae, R. septentrionalis,* and *R. virgatipes*
II. *R. pipiens* complex	Incertae sedis: *R. onca* and *R. fisheri*
A. *R. areolata* group	*R. areolata* and *R. palustris*
B. *R. berlandieri* group	*R. berlandieri, R. forreri, R. magnaocularis, R. miadis, R. neovolcanica, R. spectabilis, R. taylori, R. tlaloci,* and *R. yavapaiensis*
C. *R. montezumae* group	*R. chiricahuensis, R. dunni, R. megapoda,* and *R. montezumae*
D. *R. pipiens* group	*R. blairi, R. pipiens,* and *R. sphenocephala*
III. *R. palmipes* group	*R. maculata, R. palmipes, R. sierramadrensis, R. vaillanti, R. vibicaria,* and *R. warschewitschii*
IV. *R. tarahumarae* group	*R. johni, R. pueblae, R. pustulosa, R. tarahumarae,* and *R. zweifeli*
V. *R. boylii* group	*R. aurora, R. boylii, R. cascadae, R. muscosa,* and *R. pretiosa*
VI. Incertae sedis	*R. sylvatica*

are phylogenetically informative. Although the spacer regions are much more variable than the coding regions, they are conserved enough to be informative about the phylogeny of *Rana* over the 50-million-year history of the group. In contrast, the coding regions are too conserved to provide much phylogenetic information at this level.

DISCUSSION

Phylogeny.—The rDNA data provide an independent test of previous phylogenetic hypotheses about Holarctic and Neotropical *Rana* (Table 2). These data substantiate some previous suggestions as well as address some prominent controversies. For instance, the relationship of *R. sylvatica* to other Holarctic *Rana* has been the subject of several immunological studies without any clear consensus of conclusions (Wallace et al., 1973; Case, 1978; Farris et al., 1980, 1983; Post and Uzzell, 1981). Wallace et al. (1973) and Case (1978) suggested that *R. sylvatica* was related to the eastern North American and Neotropical *Rana,* whereas Farris et al. (1980) concluded that available evidence best supported placement of *R. sylvatica* with western North American (*R. boylii* group) and Palearctic (*R. temporaria* group) species. Post and Uzzell (1981) collected additional immunological data that they believed partly supported Case's (1978) conclusions concerning the relationships of *R. sylvatica,* although Farris et al. (1983) reached different conclusions from the same data.

The rDNA restriction-site data suggest that *R. sylvatica* is related to the *R. temporaria* group of Eurasia (Fig. 3). This is not a new suggestion; on the contrary, the relationship of *R. sylvatica* and *R. temporaria* was accepted widely before the immunological data became available (Cope, 1875; Boulenger, 1920; Darlington, 1957; Savage, 1961). Several morphological characters appear to unite *R. sylvatica* and *R. temporaria,* or both of these species together with the *R. boylii* group (Chantell, 1970; Farris et al., 1980). If one considers the rDNA data, the morphological data, and the equivocal nature of the immunological data, the best-supported position is that *R. sylvatica* is related to the *R. temporaria* group and that these species together are related to the *R. boylii* group. However, the evidence is not overwhelming, and additional data are needed to address the question adequately.

The monophyly of the *R. boylii* group (Table 2), as defined by Case (1978), also has been questioned (Farris et al., 1980, 1983). In this case, the rDNA data support Case's (1978) contention that *R. aurora, R. boylii, R. cascadae, R. muscosa,* and *R. pretiosa* form a monophyletic group, although only one site is restricted to these species (Fig. 3). An additional site, recognized by *BamH* I and the only variable site within the 18S gene, is found in all of the species in the *R. boylii* group except *R. pretiosa* (Fig. 3). Another site unites the species *R. aurora* and *R. muscosa.*

The *Rana catesbeiana* group (Table 2) has been recognized based upon immunological data (Wallace et al., 1973). In addition, sev-

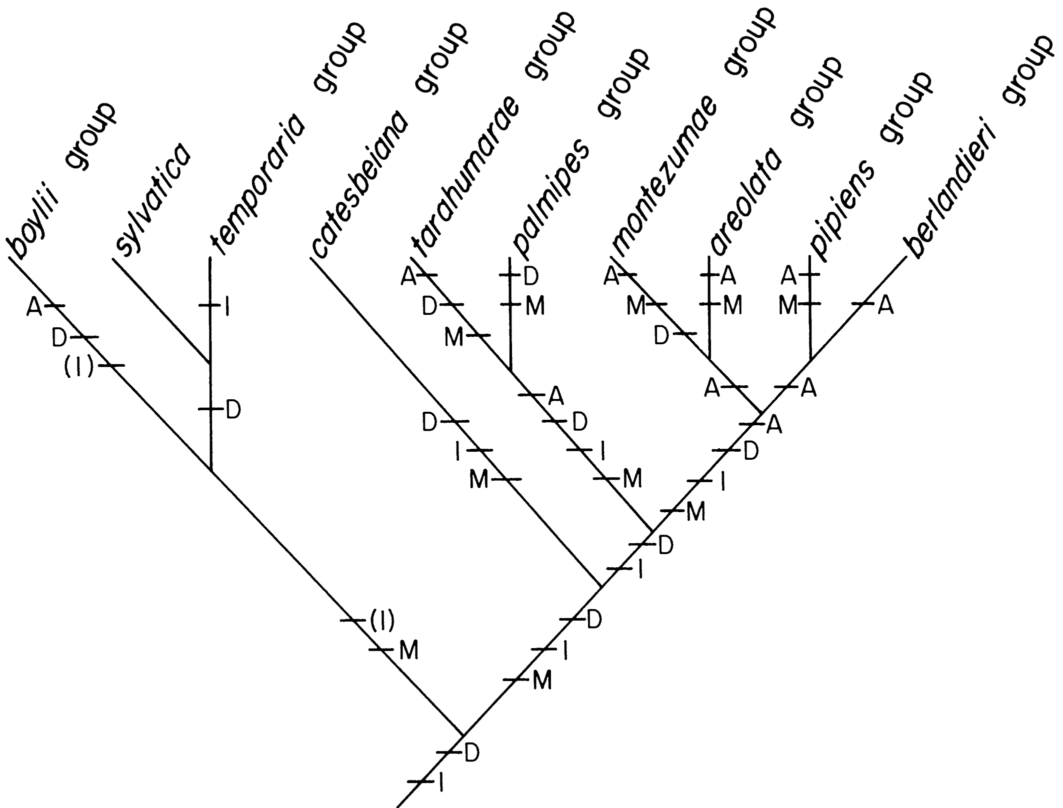

FIG. 4. Congruence of phylogenetic data concerning Holarctic and Neotropical *Rana* from allozymes (A) (Case, 1978; Hillis et al., 1983; Hillis et al., 1984), immunology (I) (Wallace et al., 1973; Case, 1978; Post and Uzzell, 1981), morphology (M) (Chantell, 1970; Webb, 1978; Farris et al., 1980; Hillis et al., 1983; Hillis, 1985), and rDNA (D) (this study). Parentheses indicate disagreement among authors in interpretation of the immunological data.

eral morphological characters define the group, including sexual dimorphism of tympanum size and the presence of post-tympanic folds (Hillis, 1985). The rDNA data also support the monophyly of this group (Fig. 3). Intragroup relationships suggested by the data are the relationship of *R. catesbeiana* and *R. clamitans* and the relationship of *R. heckscheri* and *R. grylio,* although the latter pair is united only by a loss character (Fig. 3). In agreement with immunological data (Case, 1978), the *R. catesbeiana* group is united as the sister group to the primarily Neotropical *R. pipiens* complex, *R. tarahumarae* group, and *R. palmipes* group by a single restriction site in the ETS (Fig. 3).

The classification of the three major groupings within the Neotropical assemblage is strongly supported by the rDNA data (Fig. 3; Table 2). The first dichotomy within this assemblage is between the *R. pipiens* complex and the *R. tarahumarae* plus *R. palmipes* groups. Although the rDNA data do support the recognition of the *R. pipiens* complex, they provide little substantiation for the four species-groups within this complex, defined on the basis of allozymic data by Hillis et al. (1983). The classification of the *R. montezumae* group (represented by *R. chiricahuensis* and *R. montezumae* in this analysis) is supported, but the remaining three groups (the *R. berlandieri* group: *R. berlandieri, R. spectabilis,* and *R. magnaocularis*; the *R. pipiens* group: *R. blairi, R. pipiens,* and *R. sphenocephala*; and the *R. areolata* group: *R. areolata* and *R. palustris*) are not supported. Considering the extent of the allozymic support for these groups and the predominance of losses of

rDNA sites within the *R. pipiens* complex, the groupings within the *R. pipiens* complex in Figure 3 may be mostly the result of convergent losses. This is the most ambiguous part of the phylogeny, because the restriction maps of rDNA arrays are very similar among all members of the *R. pipiens* complex examined (Table 1).

The species in which the rDNA repeat has evolved most rapidly are the tropical *R. tarahumarae* and *R. palmipes* groups (Fig. 3). The monophyly of these two groups and their sister-group relationship also are supported strongly by morphological data (Webb, 1978; Hillis, 1985). Immunological data are available for only one species of the *R. tarahumarae* group and three species of the *R. palmipes* group (Case, 1978). In our opinion, Case's interpretation of the immunological data is inconsistent with the morphological and rDNA data. Case suggested that the *R. palmipes* group was paraphyletic with respect to the *R. tarahumarae* group; this is based on her placement of *R. warschewitschii* (see Case, 1978 fig. 6). However, this arrangement was based on just a few unidirectional albumin comparisons; Case (1978) reported an immunological distance of 30 from both *R. palmipes* and *R. maculata* to *R. tarahumarae,* and a distance of 48 from *R. warschewitschii* to *R. tarahumarae.* If one considers that immunological distances from members of the *R. boylii* group to *R. warschewitschii* range from 66 to 100 (Case, 1978), her data concerning the relationship of the species in the *R. tarahumarae* and *R. palmipes* groups are equivocal. The rDNA of these species, on the other hand, is highly distinctive and provides numerous characters for elucidating their relationships (Fig. 3). The two species-groups are united by a 200-bp insertion within ITS-2, a restriction site gain and loss within ITS-1, and a site loss within the 28S gene. Three additional site gains are restricted to the species in the *R. tarahumarae* group, and another site gain defines the *R. palmipes* group (Fig. 3).

Congruence of rDNA, allozymic, morphological, and immunological data sets with regard to the phylogeny of Holarctic and Neotropical *Rana* is summarized in Figure 4. Although the rDNA data are not particularly informative about phylogeny within closely related groups (such as the *R. pipiens* complex, in which allozymic data provide the most extensive information on relationships), they provide clarification of several unresolved or poorly resolved portions of the phylogeny of these species at the intergroup level. Because species of *Rana* are important experimental animals in both laboratory and field situations, this phylogenetic information should be invaluable in comparative studies of the biology of these species.

Advantages and Limitations of the Method.—Restriction-site data on the rDNA of Holarctic and Neotropical *Rana* provide a documentation of the evolution of a diverse group of species over a 50-million-year time span. Most of the phylogenetically informative sites for these species occur within the spacer regions of rDNA; conservation of restriction sites within the coding regions throughout the evolution of these species suggests that the coding regions provide phylogenetic information for a much greater time scale. Perhaps the most restrictive limitation of biochemical techniques on phylogenetic reconstruction has been that most methods are either useful only among relatively closely related species or else provide only distance data that are subject to numerous difficulties in interpretation (Farris, 1981; Farris et al., 1983). Analysis of rDNA provides easily interpretable data that are phylogenetically informative over broad periods of evolutionary time. Phylogenetic analyses of DNA coding regions should be particularly informative about ancient evolutionary history because of the highly conserved nature of these regions throughout the eukaryotes (Goldman et al., 1983).

The primary limitation of this study is related to the use of restriction maps rather than nucleotide sequence data. The restriction sites analyzed involve only about 500 of the 12,000 base pairs in the rDNA repeat of *Rana,* and the only changes that can be detected involve the gain or loss of a recognition sequence at a site and major changes in length of the repeat. Losses of restriction sites can be accomplished by changes at any of the six nucleotides in the recognition sequence, so the acquisition of parallel, nonhomologous losses is relatively likely. However, the relative likelihood of

various convergent events is fairly well understood (Templeton, 1983*a*, 1983*b*; Nei and Li, 1979), so tree-construction algorithms can correct for inherent biases in the data set. Furthermore, the extent of homoplasy within rDNA arrays is limited as a result of their conservative nature. Therefore, restriction-site mapping of these sequences provides a valuable tool for determining evolutionary history across a broad spectrum of time.

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Appendix

Collection Localities of Specimens Examined

R. areolata: 8 km N of Galena, Cherokee Co., KS, USA.
R. aurora: 3.8 km N of Hwy. 199, Kings Valley Rd., Del Norte Co., CA, USA.
R. berlandieri: 10 km S of Río Verde, San Luis Potosí, México.
R. blairi: Lawrence, Douglas Co., KS, USA.
R. boylii: Skaggs Springs, Sonoma Co., CA, USA.
R. cascadae: Tamarack Lake, Trinity Co., CA, USA.
R. catesbeiana: Lawrence, Douglas Co., KS, USA.
R. chiricahuensis: Three Forks, Apache National Forest, Apache Co., AZ, USA.
R. clamitans: 3 km W of Danville, Montgomery Co., MO, USA.
R. grylio: Lake Iamonia, Leon Co., FL, USA.
R. heckscheri: Overflow creek of Ochlockonee River, Gadsden-Leon Cos., FL, USA.
R. maculata: Colonia Rudolfo Figueroa, Oaxaca, México.
R. magnaocularis: 15 km N of Nuri, Arroyo Hondo, Sonora, México.
R. montezumae: Lagunas Zempoala, Morelos, México.
R. muscosa: Below Levitt Lake, Mono Co., CA, USA.
R. palmipes: Misahuallí, Napo Province, Ecuador.
R. palmipes: Neblina Base Camp on Río Mawarinuma, Amazonas Province, Venezuela.
R. palustris: 5 km N of Campbellsburg, Cave River, Washington Co., IN, USA.
R. pipiens: Near Alburg, Grande Isle Co., VT, USA.
R. pretiosa: Dry Creek at Hwy. 56, Lincoln Co., MT, USA.
R. pustulosa: 10 km SW of El Batel, Sinaloa, México.
R. ridibunda: Estany, Barcelona Province, Spain.
R. sierramadrensis: Agua del Obispo, Guerrero, México.
R. spectabilis: La Estanzuela, Hidalgo, México.
R. sphenocephala: 5 km E of Bastrop, Bastrop Co., TX, USA.
R. sylvatica: Tyson Environmental Study Area, St. Louis Co., MO, USA.
R. tarahumarae: 15 km E of Yécora, Sonora, México.
R. tarahumarae: 3 km N of Tapalpa, Jalisco, México.
R. temporaria: 1.8 km NNE of Grand St. Bernard Pass, Valais Canton, Switzerland.
R. vaillanti: 8.9 km NE of Tapanatepec, Oaxaca, México.
R. vaillanti: Tinalandia, Pichincha Province, Ecuador.
R. vibicaria: El Empulme, San José Province, Costa Rica.
Pyxicephalus adspersus: Africa (no other data).