CHAPTER 5

Molecular Systematics of Amphibians

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I. INTRODUCTION

IN the past 30 years the rapid development and steady improvement of molecular methods have had and still are having a paramount impact on almost all fields of biology. Population genetics, evolutionary biology, and phylogenetic systematics are disciplines that particularly have benefited from the rise of molecular biology and have faced a tremendous evolution by themselves. Perceptions of fundamental questions in biology concerned with processes and causes of evolution and diversification of life are tightly connected with advances in molecular biology.

Molecules (RNA, DNA, proteins) carry important information that allows one to distinguish taxa, to reconstruct their phylogenetic relationships, to estimate divergence times, and to analyse evolutionary processes. As can be demonstrated by an ever-growing amount of molecular data, molecular approaches which supplement traditional nonmolecular methods are a pivotal part of the toolkit of evolutionary biologists and systematists. For Amphibia, internet sequence databases (GenBank, EMBL) currently contain about 1.76 million entries of DNA and RNA sequences. DNA sequences in particular, because they bear the code of life, provide the most detailed anatomy possible for any organism — the instructions for how each working part should be assembled and operate (Page and Holmes 1998).

Up to the end of the 1980s, most molecular work in systematics was conducted using proteins. Since then, the development of new DNA-manipulation techniques has promoted a shift from protein-based to DNA-based markers (Schlötterer 2004). The Polymerase Chain Reaction (PCR) (Saiki *et al.* 1985; Mullis *et al.* 1986), developed in the mid-1980s, was a significant milestone in molecular biology. For the first time it became possible to analyse any genomic region without the requirement of isolating large amounts of ultra-pure genomic DNA or to clone DNA segments. Since then many further technological innovations, for example, automated sequencers, have significantly simplified the technical procedures; DNA-sequencing is now a standard and routine method. Dramatic advances in computational technology have allowed processing large datasets and applying the complex mathematical algorithms and sophisticated statistical procedures that are needed to analyse phylogenetic relationships (e.g., Nei and Kumar 2000; Arbogast *et al.* 2002).

Progress in molecular biology has also had a deep impact on the systematics of recent amphibians. Initial molecular investigations in the 1950s and 1960s were concerned with aspects of population genetics and genetic polymorphisms within and among species. Pioneer work was carried out by Guttman and collaborators on serum protein polymorphisms in toads of the genus *Bufo* (reviewed by Guttman 1973). In the following decades protein markers, mostly allozymes, were successfully applied in population genetics for the reconstruction of phylogenetic relationships of closely related species, for the estimation of divergence times in the context of historical biogeography, and for simple taxonomic purposes, e.g., to detect and differentiate species that are morphologically indistinguishable (e.g., Hotz and Uzzell 1982; Nishioka *et al.* 1987; Nishioka and Sumida 1992; Beerli *et al.* 1994, 1996).

As elsewhere in biology, the study of nucleotide sequences has becoming increasingly popular in amphibian systematics since the early 1990s. As for other animal groups, systematists almost exclusively used mitochondrial (mt) DNA sequences. One of the early, more comprehensive studies is that of Hedges and Maxson (1993), who analysed the relationships among higher-ranked amphibian groups ("families") on the basis of a relatively short fragment (333 bp) of the mt 12S ribosomal RNA (rRNA) gene. Shortly thereafter, Hay *et al.* (1995) employed a dataset of about 1 300 bp of mt ribosomal sequences from species representing 28 of the 40 recognized amphibian families. More recent studies have gone further in analysing complete mt genomes (e.g., Zardorya *et al.* 2003; Mueller *et al.* 2004; Zhang *et al.* 2005). The number of investigations using mt DNA sequences to infer phylogenetic relationships has continued to increase rapidly, but nowadays, nuclear genes, such as the recombination-activating gene 1 (RAG1), play an increasing role in systematic studies on amphibians (e.g., Hoegg *et al.* 2004; San Mauro *et al.* 2005). Shortly before this chapter went to print, a phylogeny of the living amphibians based on a combined analyis of mitochondrial and 2 300 bp of nuclear genes from 522 species representing all families and subfamilies within the Amphibia, except Protohynobiinae, was analysed on a parallel computer cluster, which resulted in the most comprehensive hypothesis on the phylogenetic relationships within the Amphibia available to the present time (Frost *et al.* 2006).

In the present chapter the molecular methods and statistical procedures that are commonly applied in systematics are surveyed and their advantages and disadvantages are

discussed. The molecules that are most frequently used for systematic investigations are also described. In the second part of the chapter, new hypotheses on the phylogeny of major amphibian groups, based on molecular data, are presented. Since this chapter is primarily dedicated to amphibian systematics, the molecular methods that are considered relatively unimportant for systematics, e.g., DNA flow cytometry, are addressed only cursorily. It is far beyond the scope of this chapter to go into details concerning population genetics and evolutionary biology, fields that deserve chapters on their own.

A note on the nomenclature used in this chapter is in order. The frequently used term "Lissamphibia" was coined by Haeckel (1866) for frogs and salamanders, but not for caecilians. For this reason, Dubois (2004) felt that this term might not be a proper choice to denominate all extant amphibians. Accordingly in the chapter reference is made simply to living or extant amphibians, and the formal term "Lissamphibia" not employed. This procedure has also been followed by Frost *et al.* (2006) who restricted the term "Amphibia" to the group formed by caecilians, salamanders and frogs. Contrary to usage otherwise in systematic literature, including elsewhere in the Amphibian Biology series, Dubois (2004) is also followed in preferring the term Urodela Duméril, 1806 as the correct scientific name for salamanders, and considering the name Caudata Scopoli, 1777 as not a valid amphibian taxon.

II. MOLECULES

A. Proteins

Proteins are natural polymer molecules composed of amino acids that are joined together by covalent peptide bonds to form polypeptides (Fig. 1). There are 20 different essential amino acids in native proteins, each composed of a central carbon atom (the alpha carbon) to which are attached a hydrogen atom, a carboxyl group (COO^-), an amino group (NH_3^+), and a unique side chain or R-group. The side chains determine the chemical properties of a protein, such as its ability to bind ligands and catalyse biochemical reactions. They also direct the folding of the polypeptide chain and stabilize its final conformation. While most side chains are neutral, those of five of the amino acids are either basic and thus positively charged (lysine, arginine, and histidine), or acidic and negatively charged (aspartic acid and glutamic acid). Charged side chains are responsible for the net charge of a protein and thus for its migration under the influence of an electrical field. This property of proteins is the basis for a molecular technique called protein electrophoresis (see Section on that topic).

The genetically determined linear arrangement (sequence of amino acids in a protein) is called "primary structure". Uncharged amino acids are either non-polar and hydrophobic or polar. They can become hydrogen-bonded, resulting in a "secondary structure", which is defined as the local spatial arrangement of the main-chain atoms of a polypeptide chain segment. Three common secondary structures can be distinguished: alpha-helix, alpha-sheet, and turns. Alpha-helices and alpha-structures are generally the most thermodynamically stable conformations of regular secondary structures. Depending on the primary and secondary structure the molecule usually undergoes additional folding resulting in its tertiary structure. The "tertiary structure" of a protein molecule, or of a subunit of a protein molecule, is defined as the arrangement of all its atoms in space without regard

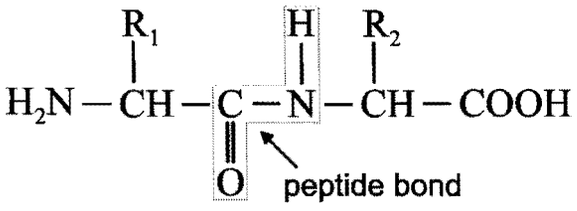


Fig. 1. Primary structure of a polypeptide chain.

to its relationships with neighbouring molecules or subunits. Proteins consisting of more than one polypeptide build a "quarternary structure", which is defined as the arrangement of subunits in space and the ensemble of intersubunit contacts and interactions, without regard to the internal geometry of the subunits. Many proteins consist of more than one polypeptide chain (subunit). Haemoglobin, for example, contains four polypeptide chains held together noncovalently in a specific conformation as required for its function. The stability of a protein molecule depends on such factors as hydrogen bonding, van der Waals forces, ionic bonds, disulfide bridges, and/or hydrophobic interactions.

Proteins can be classified according to their biological function. Some, like actin and collagen, make up the physical structure of cells and tissues. Other proteins act in transportation and storage processes (e.g., haemoglobin and myoglobin), in regulatory processes (e.g., hormones), or in the immune system (e.g., immunoglobulins). A large group of enzymes (e.g., transferases and dehydrogenases) catalyze chemical reactions in the organism. When studying enzymic variation with protein electrophoresis, it is useful to distinguish between *Allozymes*, which are variants of polypeptides encoded by different alleles of the same locus and *Isozymes*, which include functionally similar forms of enzymes, whether encoded by the same or by different gene loci.

Although nucleic acid sequences have replaced proteins as the main source of molecular data, particularly since the invention of the polymerase chain reaction (PCR), proteins still play an important role in systematics, not only for the identification and separation of species, but also for the reconstruction of phylogenetic relationships. For the latter purpose, the amino acid sequences of proteins provide important information but in the case of closely related species electrophoretic data are still suitable.

B. Nucleic Acids

Nucleic acids reveal genetic differences with the highest possible resolution and thus provide direct and exact information on genetic variability within and between taxa (von Haeseler *et al.* 1993). Deoxyribonucleic acid (DNA) occurs in the nucleus (nDNA) and in organelles such as chloroplasts (cpDNA) and mitochondria (mtDNA). It consists of four chemically different nucleotides. Each nucleotide contains a phosphate group, a sugar (deoxyribose), and one of four bases: adenine (A), thymine (T), cytosine (C), and guanine (G). The bases can be grouped into purines (adenine and guanine), and pyrimidines (cytosine and thymine). In ribonucleic acid (RNA) deoxyribose is replaced by ribose and thymine by uracil (U).

1. Nuclear DNA

The size of the nuclear genome is indicated by the C-value, which is a measure of the amount of DNA per haploid genome. Compared to other vertebrate taxa the size of amphibian genomes is relatively large and can vary greatly among species (Gregory 2005). For example, C-values of 4.69 pg and 13.95 pg were found in the gymnophionan species *Geotrypetes seraphini* and *Siphonops annulatus*, respectively (Beçak *et al.* 1970; Olmo *et al.* 1970; Olmo 1973). In *Rana ridibunda*, a member of the western Palearctic water-frog group, C-values ranged between 7.5 pg and 9 pg (Mazin and Borkin 1979; Borkin *et al.* 1987; Vinogradov and Borkin 1993); this is more than twice the size of the *Homo sapiens* genome. More information on the size of animal genomes is available at the Animal Genome Size Database (http://www.genomesize.com/amphibians.htm).

Eukaryotic nDNA can be differentiated into coding and non-coding sequences (Fig. 2). Coding DNA comprises protein-coding genes (single-copy genes, multigene families), and RNA coding genes that produce transfer RNAs (tRNA), ribosomal RNAs (rRNA) or small nuclear RNAs (snRNA). Protein-coding genes (Fig. 3) usually consist of a coding region flanked by various regulatory elements (promotors, enhancers) that play an important role in gene expression. Protein-coding genes are transcribed into messenger RNAs (mRNA), which are in turn translated into the amino acid sequences of proteins.

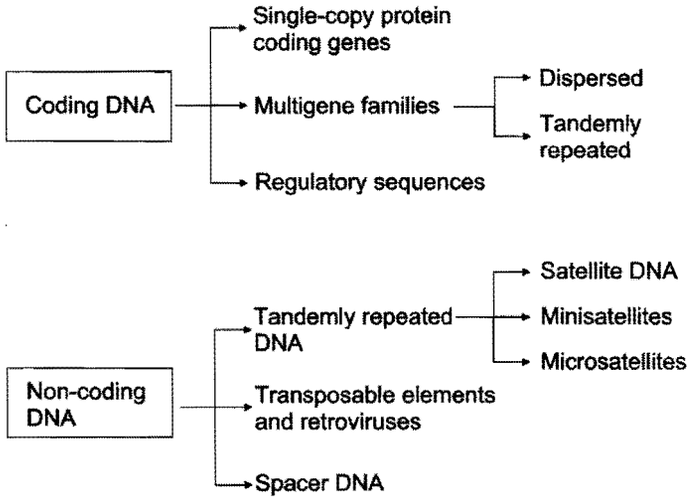


Fig. 2. A diagramatic survey of the eukaryote genome. Noncoding regions within genes, such as introns, are not considered separately. After Page and Holmes (1998).

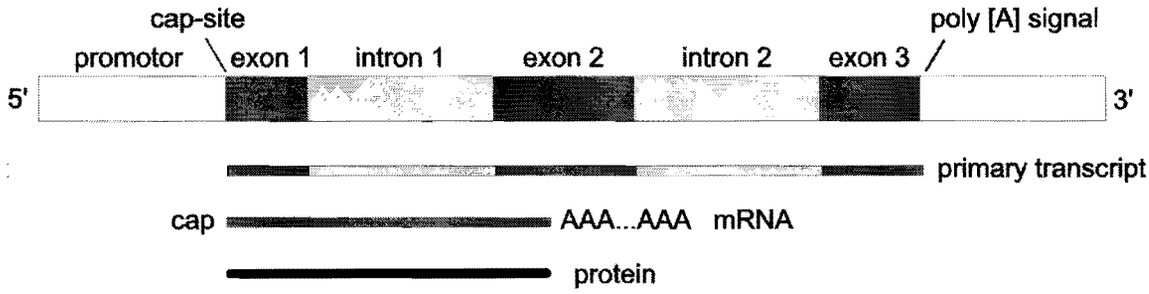


Fig. 3. Structure and transcription of globin genes. The introns are removed by splicing from the primary transcript. Adapted from Karlsson and Nienhuis (1985).

Beside coding regions (exons), most eukaryotic protein-coding genes also contain introns that are excised from the coding regions during protein synthesis. The number, size, and organization of introns are gene-specific and species-specific. In closely related western Palearctic water-frog species, for example, the size of intron I of the serum albumin gene differs in about 540 nucleotides between *Rana lessonae* and central European *R. ridibunda* (unpublished results).

Ribosomal RNA-coding genes occur as multiple tandem repeats that are separated by intergenic spacers (IGS). Together, these rRNA genes plus an IGS constitute an rDNA repeat unit (Fig. 4). The copy number of repeat units can vary between species and individuals, and is positively correlated with the size of the genome (Prokopowich *et al.* 2000).

The coding region comprises the genes for the small subunit rRNA (SSU), the 5.8S rRNA, and the large subunit rRNA (LSU); these are separated from each other by two spacer sequences, i.e., the SSU gene is separated from the 5.8S rRNA gene by the first spacer (termed "internal transcribed spacer 1" or ITS1); the second spacer (ITS2) separates

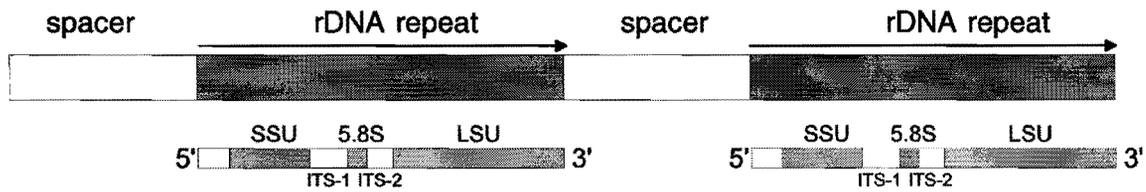


Fig. 4. Two repeat units of eukaryotic rDNA. The arrows indicate the direction of transcription. After Wellauer *et al.* (1976) and Reeder (1984).

the 5.8S rRNA gene and the LSU gene (e.g., Sollner-Webb and Tower 1986; Moore and Steitz 2002; Fromont-Racine *et al.* 2003). Genes and spacers are transcribed as a single rRNA precursor, which is subsequently cleaved by a series of nucleolar events leading to the mature SSU rRNA, 5.8S rRNA, and LSU rRNA.

The internal transcribed spacers are thought to be involved in processing events during the maturation of rRNAs (Musters *et al.* 1990; Liu and Schardl 1994; Michot *et al.* 1999). Secondary structures resulting from RNA strand folding seem to play an important role in this process. As shown for ITS2, the secondary structure is highly preserved in vertebrates (Joseph *et al.* 1999) despite dramatic sequence variation. Nucleotide and length polymorphisms may occur not only among ITS regions of distantly related taxa but also among those of closely related species (e.g., Furlong and Maden 1983), which make alignments of ITS sequences difficult. Structural constraints dictated by secondary structure models may help to optimize alignments by, for example, recognizing highly conserved paired motifs and identifying complementary base substitutions (Gottschling and Plötner 2004 and literature cited therein).

Intergenic spacers (IGS) contain several distinct repeated-sequence elements that greatly influence IGS length and heterogeneity (reviewed by Weider *et al.* 2005). In the IGS of *Xenopus laevis*, for example, transcription terminators, duplicated spacer promotors, enhancer elements, and several repeats, were detected (Caudy and Pikaard 2002).

Non-coding DNA constitutes about 97% of the vertebrate genome. Beside introns and spacer sequences, it also contains repetitive sequences such as transposable elements, retroviruses, and tandemly arranged DNA (Fig. 2).

Transposable elements (transposons) are short sequences of DNA (<1 kb) that have the ability to make additional copies of themselves and/or to move to new locations in the genome (transposition). They constitute a significant proportion of the moderately repetitive DNA. Two kind of transposons differ in their mechanism of transposition: (1) retrotransposons (class I transposons) transpose indirectly by reverse transcription of an RNA intermediate (Kazazian and Goodier 2002 and literature cited therein); (2) DNA transposons (class II transposons) are DNA-mediated and transpose themselves directly via translation products (transposases) that are encoded by transposons. A family of interspersed repetitive elements with sequence similarity to the transposase of *hAT* DNA transposons was detected in genomes of eight western Palearctic water-frog taxa and in the brown frog *Rana dalmatina*, but not in *Xenopus laevis* or *Salamandra salamandra* (Casola *et al.* 2004).

While transposable elements are dispersed, tandemly repeated DNA is concentrated at one or only few positions in the genome. Tandem repeats can be classified according to the repeat length into satellite DNA, minisatellites, and microsatellites (Table 1). Satellite DNA consists of very short non-transcribed sequences (<100 bp) that are present in many thousands of copies in the genome. The sequence motifs are often very simple, e.g., ATATAT . . . or ATCATCATC . . . Concentrations of highly repetitive satellite DNA are located in the heterochromatin regions adjacent to the chromosome centromeres and in the telomeres.

Minisatellites or VNTR loci (variable number tandem repeats) consist of a G-rich core motif up to 100 nucleotides long (e.g., GGAGGTGGGCAGGAGG) arranged in up to 1 000 repeats (e.g., Jeffreys *et al.* 1985; Janning and Knust 2004). It is assumed that the length polymorphisms of minisatellites arise from unequal crossing over or from gene conversion (Jeffreys *et al.* 1999).

Table 1. Definition of tandem repeats. From Janning and Knust (2004).

Typ	Repeats per locus	Number of loci	Repeat length [bp]
Satellite DNA	10^3–10^7	1–2/chromosome	1–several thousands
Minisatellites	1–1000	Several thousands/genome	9–100
Microsatellites	1–100	Up to 10^5/genome	1–6

Microsatellites, also called short-tandem repeats (STR), are segments of tandemly repeated sequences with a short repeat length. The core motif typically is composed of a maximum of six nucleotides arranged in up to 100 copies (Janning and Knust 2004). Microsatellite loci are believed to be subject to a mutational change following the slippage model of duplication or deletion of repeat units. DNA slippage occurs when DNA strands mispair during replication and recombination so that short stretches of sequence slip against each other, creating loops of DNA which, when repaired, result in a loss or gain of units (Page and Holmes 1998).

At present, the analysis of nDNA is only in its initial phase. In the future, nuclear markers will be increasingly used for systematic purposes. Beside structural genes, regulatory elements and even noncoding sequences are expected to provide important information for reconstructions of evolutionary relationships of living amphibians.

2. *Mitochondrial DNA*

The mitochondrial (mt) genome comprises a covalently closed circular duplex-histone-free chromosome (Fig. 5), present in one or more copies in every mitochondrion (Wilson *et al.* 1985; Moritz *et al.* 1987; Wolstenholme 1992; Simon *et al.* 1994; Boore 1999; Pereira 2000; William *et al.* 2004). The mt genome is maternally and cytoplasmically inherited. Until recently it was thought that mtDNA does not undergo recombination. Current studies, however, showed that mtDNA recombination might be a common phenomenon (Ladoukakis and Zouros 2001; Rokas *et al.* 2003; Kraytsberg *et al.* 2004; Tsaousis *et al.* 2005).

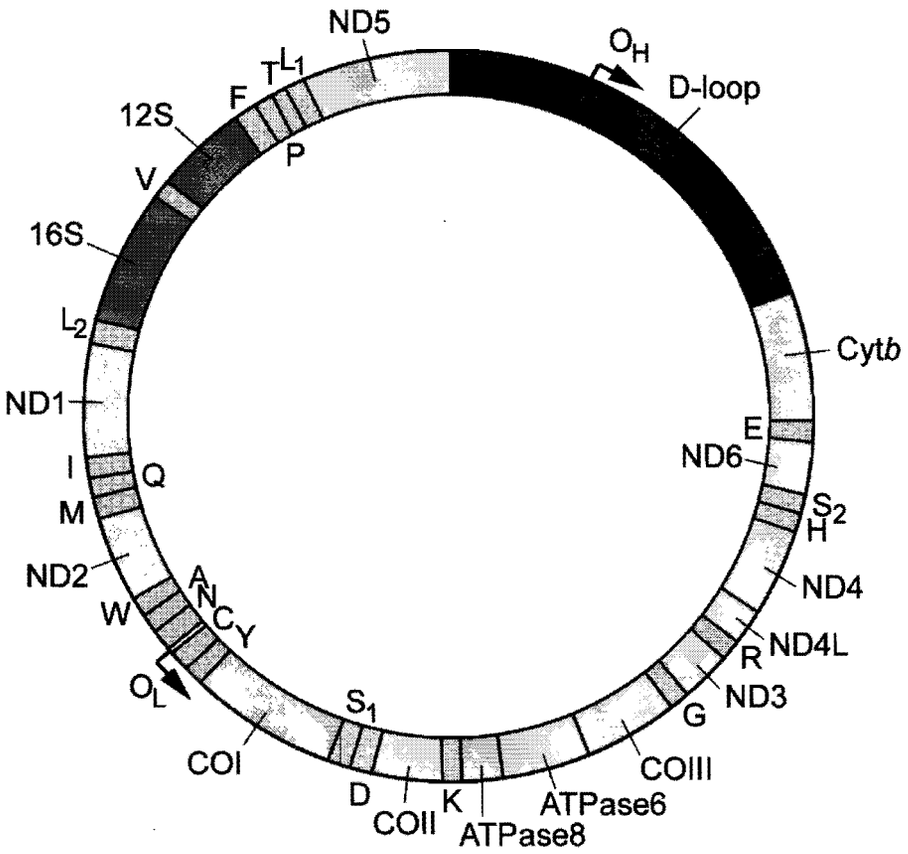


Fig. 5. The mitochondrial genome of *Buergeria buergeri*. All protein coding genes are located on the H strand, except ND6, which is located on the L strand. O_H and O_L represent the replication origins of H-strands and L-strands, respectively. Transfer RNA genes are designated by the standard one-letter amino acid code. L1, L2, S1, and S2 denote $tRNA^{Leu(CUN)}$, $tRNA^{Leu(UUR)}$, $tRNA^{Ser(UCN)}$, and $tRNA^{Ser(AGY)}$. Genes are abbreviated as in Table 3. After Sano *et al.* (2004).

Beside nematodes, mussels, fish, and mammals, direct evidence for mtDNA recombination has been presented for ranids (Tsaousis *et al.* 2005). For recombination it is necessary that some individuals be heteroplasmic, i.e., they carry more than one type of mtDNA molecules. Heteroplasmic individuals are common among vertebrates and have also been found in different amphibian species, for example in hybridogenetic water frogs (Monnerot *et al.* 1984) and in toads (Goebel 1996; Goebel *et al.* 1999; Pauly *et al.* 2004). Whether recombination is indeed involved in mtDNA replication and repair and whether most animal genomes have the necessary enzymes for this process as claimed by Rokas *et al.* (2003) needs further investigation.

In amphibians, especially in the Urodela, a substantial size variability of mt genomes has been recognized with values ranging from about 14.4 to 22.2 kb (Table 2). Size polymorphisms of mtDNA also occur among conspecific individuals, for example, in frogs of the genus *Rana* (Monnerot *et al.* 1984; Lee and Park 1991; Sumida *et al.* 2001a) and in newts of the *Triturus cristatus* complex (Wallis 1987).

Table 2. Sizes and GC contents of amphibian mt genomes. Accession No. from GenBank (http://www.ncbi.nlm.nih.gov/). *identified with RFLP.

Taxon/Species	Size [bp]	GC content [%]	Accession No.	Reference
GYMNOPHIONA				
Caeciliidae				
Gegeneophis ramaswamii	15,897	33	NC_006301	San Mauro *et al.* (2004)
Ichthyophiidae				
Ichthyophis bannanicus	15,983	39	NC_006404	Zhang *et al.* (2005)
Ichthyophis glutinosus	15,986	36	NC_006302	San Mauro *et al.* (2004)
Rhinatrematidae				
Rhinatrema bivittatum	16,422	42	NC_006303	San Mauro *et al.* (2004)
Scolecomorphidae				
Scolecomorphus vittatus	15,973	34	NC_006304	San Mauro *et al.* (2004)
Typhlonectidae				
Typhlonectes natans	17,005	45	NC_002471	Zardoya and Meyer (2000)
Uraeotyphlidae				
Uraeotyphlus cf. *oxyurus*	16,432	37	NC_006305	San Mauro *et al.* (2004)
URODELA				
Ambystomatidae				
Ambystoma andersoni	16,370	33	NC_006888	Samuels *et al.* (2005)
Ambystoma californiense	16,374	34	NC_006890	Samuels *et al.* (2005)
Ambystoma dumerilii	16,370	33	NC_006889	Samuels *et al.* (2005)
Ambystoma laterale	16,367	34	NC_006330	Mueller *et al.* (2004)
Ambystoma mexicanum	16,369	33	NC_005797	Arnason *et al.* (2004)
Ambystoma tigrinum	16,375	33	NC_006887	Samuels *et al.* (2005)
Cryptobranchidae				
Andrias davidianus	16,503	35	NC_004926	Zhang *et al.* (2003a)
Andrias japonicus	16,298	35	NC_007446	Okamoto *et al.* (unpubl.)
Hynobiidae				
Ranodon sibiricus	16,418	32	NC_004021	Zhang *et al.* (2003b)
Plethodonthidae				
Aneides flavipunctatus	20,197	33	NC_006327	Mueller *et al.* (2004)
Aneides hardii	22,184	35	NC_006338	Mueller *et al.* (2004)
Batrachoseps attenuatus	17,559	35	NC_006340	Mueller *et al.* (2004)
Batrachoseps wrightorum	19,789	35	NC_006333	Mueller *et al.* (2004)
Bolitoglossa sp. n.	21,657	36	NC_006346	Mueller *et al.* (2004)
Desmognathus fuscus	16,628	30	NC_006339	Mueller *et al.* (2004)
Desmognathus wrighti	16,578	33	NC_006337	Mueller *et al.* (2004)
Ensatina eschscholtzii	22,186	32	NC_006328	Mueller *et al.* (2004)
Eurycea bislineata	17,184	35	NC_006329	Mueller *et al.* (2004)
Gyrinophilus porphyriticus	16,778	37	NC_006341	Mueller *et al.* (2004)
Hemidactylium scutatum	17,421	36	NC_006342	Mueller *et al.* (2004)
Hydromantes brunus	17,220	30	NC_006345	Mueller *et al.* (2004)
Oedipina poelzi	16,731	36	NC_006326	Mueller *et al.* (2004)
Phaeognathus hubrichti	16,294	33	NC_006344	Mueller *et al.* (2004)
Plethodon cinereus	20,001	36	NC_006343	Mueller *et al.* (2004)
Plethodon elongatus	18,767	36	NC_006335	Mueller *et al.* (2004)
Plethodon petraeus	19,235	36	NC_006334	Mueller *et al.* (2004)
Pseudotriton ruber	16,661	36	NC_006332	Mueller *et al.* (2004)

Table 2 — *continued*

Taxon/Species	Size [bp]	GC content [%]	Accession No.	Reference
URODELA *(continued)*				
Plethodonthidae *(continued)*				
Rhyacotriton variegatus	21,606	31	NC_006331	Mueller *et al.* (2004)
Stereochilus marginatus	19,631	39	NC_006325	Mueller *et al.* (2004)
Thorius sp. n.	19,097	38	NC_006336	Mueller *et al.* (2004)
Salamandridae				
Lyciasalamandra atifi	16,650	38	NC_002756	Zardoya *et al.* (2003)
Paramesotriton hongkongensis	16,285	39	NC_006407	Zhang *et al.* (2005)
ANURA				
Bombinatoridae				
Bombina bombina	17,575	40	NC_006402	Zhang *et al.* (unpubl.)
Bombina orientalis	17,847	42	NC_006689	San Mauro *et al.* (2004)
Bufonidae				
Bufo melanosticus	16,328	40	NC_005794	Arnason *et al.* (2004)
Discoglossidae				
Alytes obstetricans	17,490	43	NC_006688	San Mauro *et al.* (2004)
Discoglossus galganoi	17,014	43	NC_006690	San Mauro *et al.* (2004)
Hylidae				
Hyla chinensis	18,180	40	NC_006403	Zhang *et al.* (2005)
*Pseudacris crucifer**	? 23,000	–	–	Kessler and Avise (1985)
Microhylidae				
Kaloula pulchra	16,818	40	NC_006405	Zhang *et al.* (2005)
Microhyla heymonsi	16,707	38	NC_006406	Zhang *et al.* (2005
Pipidae				
Silurana tropicalis	17,610	42	NC_006839	Macey *et al.* (unpubl.)
Xenopus laevis	17,553	36	NC_001573	Roe *et al.* (1985)
Ranidae				
Fejervarya limnocharis	17,717	41	NC_005055	Liu *et al.* (2005)
Limnonectes fujianensis	17,654	42	NC_007440	Nie *et al.* (unpubl.)
*Rana esculenta**	18,700–19,700	–	–	Monnerot *et al.* (1984)
Rana nigromaculata	17,804	43	NC_002805	Sumida *et al.* (2001)
Rhacophoridae				
Buergeria buergeri	19,959	39	NC_008975	Sano *et al.* (2004)
Polypedates megacephalus	16,473	39	NC_006408	Zhang *et al.* (2005)
Rhacophorus schlegelii	21,359	37	NC_007178	Sano *et al.* (2005)

The mt genome typically contains two ribosomal RNA coding genes (12S rRNA, 16S rRNA), 22 transfer RNA (tRNA) coding genes, 13 protein coding genes, and a control region (CR) (Table 2, Fig. 5). Eight tRNAs and one mRNA are encoded on the light (L) strand, and 14 tRNAs, 12 mRNAs, and two rRNAs are encoded on the heavy (H) strand, as shown, for example, for the mt genomes of *Buergeria buergeri* (Sano *et al.* 2004), *Rana nigromaculata* (Sumida *et al.* 2001b) and various *Ambystoma* species (Samuels *et al.* 2005).

The arrangement of genes is widely conserved in vertebrate mt genomes, i.e., all 37 genes are arranged in the same order (Boore 1999; Pereira 2000). Variations of the "universal" gene order have been found in such different amphibians as lungless salamanders (Plethodontidae) (Mueller and Boore 2005) and the frog species *Rana catesbeiana* (Yoneyama 1987), *R. porosa* (Sumida *et al.* 2000), *R. nigromaculata* (Sumida *et al.* 2001b), *Buergeria buergeri* (Sano *et al.* 2004), *Fejervarya limnocharis* (Macey *et al.* 1997; Liu *et al.* 2005), and *Polypedates megacephalus* (Zhang *et al.* 2005). All rearrangement-mediating duplications include either the origin of light-strand replication and the nearby tRNA genes or the regions flanking the origin of heavy-strand replication (Mueller and Boore 2005). Beside tRNA genes arranged around the control region, the protein-coding gene ND5 was also affected by a translocation event in some frog species (Fig. 6). In the mt genome of *Polypedates megacephalus* the ATP8 and ND5 genes are absent; instead a noncoding sequence 853 nucleotides long has replaced the ATP8 gene at its original position.

Several mechanisms, such as inversion, transposition, intramolecular recombination, tandem and nontandem dublication, imprecise initiation and termination of replication, slipped-strand mispairing, deletion, and remolding of tRNA genes have been invoked to

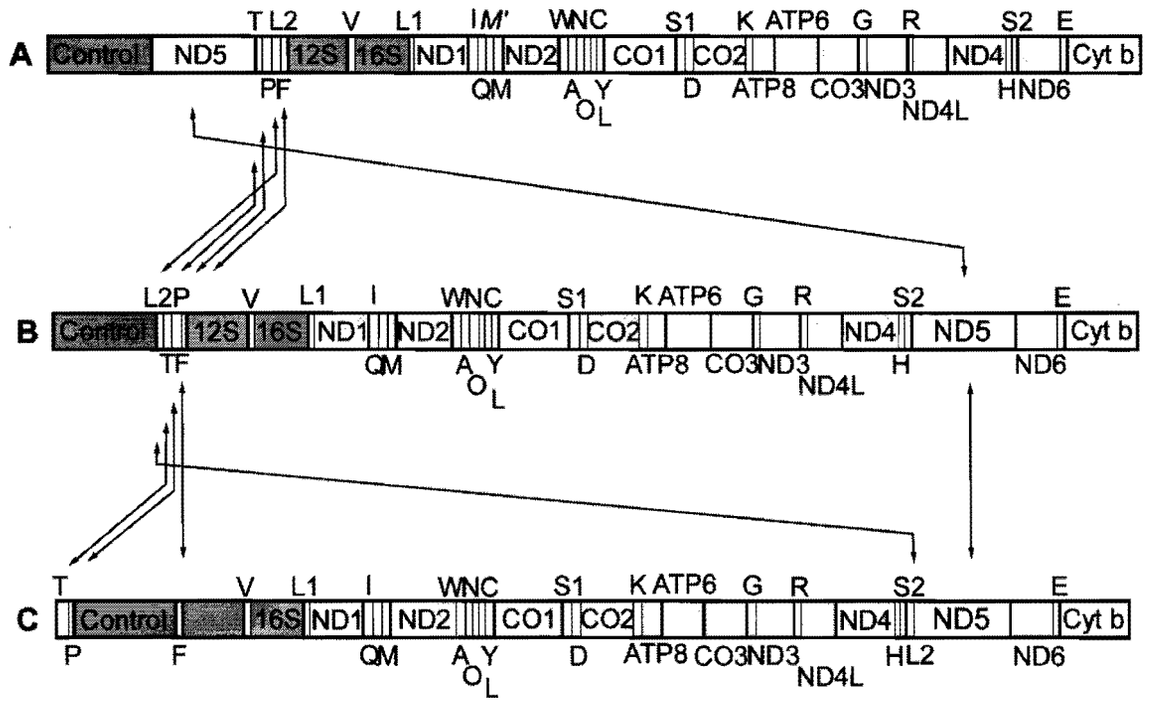


Fig. 6. Comparison of gene arrangements in the mt genomes of (A) *Fejervarya limnocharis*, (B) *Rana nigromaculata*, and (C) *Xenopus laevis*, *Typhlonectes natans*, *Ranadon sibiricus*, *Lyciasalamandra atifi*, and *Andrias davidianus*. Arrows indicate the rearranged homologous genes. *M'* indicates an extra copy of $tRNA^{Met}$ in *Fejervarya limnocharis* (adapted from Liu *et al.* 2005).

explain such gene rearrangements (Stanton *et al.* 1994; Pereira 2000; Rawlings *et al.* 2003; Mueller and Boore 2005). Sumida *et al.* (2001b) postulated that in the mt genome of *Rana nigromaculata* the gene rearrangement occurred by a tandem duplication of a region comprising the $tRNA^{Leu\ (CUN)}$ gene to the $tRNA^{Phe}$ gene followed by multiple deletions of redundant genes (Fig. 7).

For lungless salamanders there is evidence that the rearrangement was mediated by duplication of parts of the mt genome, including the presence of pseudogenes and additional, presumably functional, copies of duplicated genes.

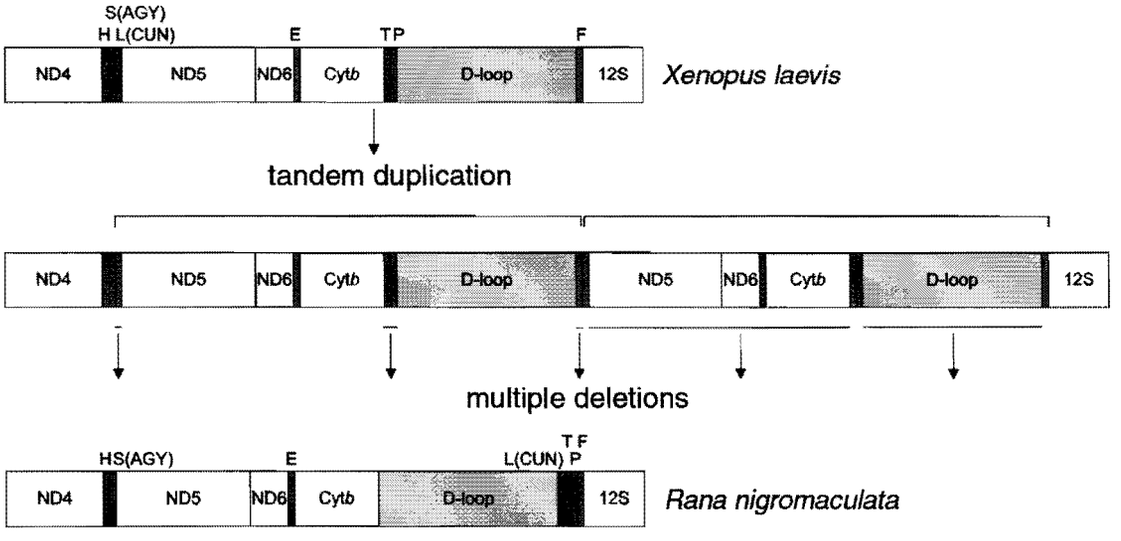


Fig. 7. Tandem duplication of a gene region followed by deletion of redundant genes as a possible mechanism of gene rearrangment in the mt genome of *Rana nigromaculata*. Adapted from Sumida *et al.* (2001b).

Base composition is one of the major sources of variation of mtDNA genomes. It can be observed that the AT/GC content is rather variable between taxa (Table 2), probably because of the tendency to avoid G at the third codon position of protein-coding genes (Saccone *et al.* 2002). In amphibians the GC content of the mt genome varies considerably with extremes between 30% (*Hydromantes brunus*) and 45% (*Typhlonectes natans*). The biases towards AT-ending codons may be caused by factors that impinge on rates of DNA damage (Martin 1995) and the relative availability of each nucleotide in the cellular medium of the mitochondrion (Xia *et al.* 1996). Alternatively, selection may also be involved in the high AT content of mtDNA because AT-rich genomes may replicate more quickly than GC-rich ones and thus, have a selective advantage (Ballard 2000; Denver *et al.* 2000).

The mt protein-coding genes (Table 3) code for subunits of enzymes functioning in the electron-transport chain where carbohydrates and fats are oxidized to generate carbon dioxide, water and ATP. Compared to the standard genetic code of nuclear genes the mt genetic code shows a few differences. Current evidence suggests as many as 27 codon reassignments in mt genes, although several specific changes seem to have recurred frequently (Knight *et al.* 2001a,b). For example, the codon TGA is not a termination (stop) codon but codes for tryptophane. By contrast, codons AGA and AGG are termination codons instead of arginine codons. In *Ambystoma* mt genes, however, the AGG stop codon was not observed (Samuels *et al.* 2005). Incomplete stop codons are common for several mt genes as ND genes and CO genes (Liu *et al.* 2005). In such cases the stop coding sequence is completed by polyadenylation of the terminating T nucleotide (Gissi and Pesole 2003), i.e., two A's are added via a polymerase catalyzed reaction.

The pattern of codon usage is markedly nonrandom. As already mentioned above there is a general deficiency of codons ending in G (Brown 1985; Moritz *et al.* 1987). Reading frame overlaps of protein-coding genes (ATP8 and ATP6; ND4L and ND4) and between a protein-coding gene (ND1) and a tRNA gene ($tRNA^{Ile}$) were found in the mt genomes of *Rana nigromaculata* (Sumida *et al.* 2001b) and *Fejervarya limnocharis* (Liu *et al.* 2005).

Mitochondrial tRNA genes produce a single strand of RNA that is later folded to form a secondary structure composed of self-paired helical stems and unpaired loops (Fig. 8). Two tandemly repeated $tRNA^{Met}$ genes were found in the mt genome of the rice frog, *Fejervarya limnocharis*. The two copies, located between the $tRNA^{Gln}$ gene and the ND2 gene, had a similarity of 74.6% (Liu *et al.* 2005).

The control region lacks structural genes but contains sequences that initiate replication and transcription. In most vertebrate mt genomes the control region is located between the genes for $tRNA^{Pro}$ and $tRNA^{Phe}$. It contains a displacement loop (D-loop) structure that plays a role in mtDNA replication. Each mtDNA strand has its own replication origin. The replication origin of the H-strand (O_H) is located in the D-loop region. The L-strand origin of replication (O_L) is located between the tRNA genes for asparagine and cysteine in the so called WANCY cluster of tRNAs (those for tryptophane, alanine, asparagine, cysteine, and tyrosine; Fig. 5). The O_L is suggested to form a stem-loop secondary structure (Fig. 9).

The control region possesses short-sequence elements that are conserved among most vertebrates studied so far. Control regions of many amphibians, especially frogs, also contain repetitive elements at the 5'-end, the 3'-end, or both. Highly repetitive sequences consisting of tandemly arranged 16–17 bp repeat units were detected in the CR of the eastern Palearctic water-frog species *Rana nigromaculata* and *R. porosa*. The total length of repetitive sequences varied between 0.6 kb and 1.2 kb and caused extensive size variation in the mtDNA of these species (Sumida *et al.* 2000, 2001a). In the CR of *R. nigromaculata* the repetitive segment had a length of 757 bp (Table 3). Beside 40 copies of a 16 bp sequence, it consisted of three copies of a 22 bp sequence, and three copies of a 17 bp sequence (Sumida *et al.* 2001b). Repetitive sequences in the 5'-end of the CR have also been reported for other amphibian species as, for example, *Xenopus laevis* (Wong *et al.* 1983; Roe *et al.* 1985), *Rhacophorus taipeianus* (Yang *et al.* 1994) and *Rana catesbeiana* (Yoneyama 1987).

Table 3. Features of the mtDNA of *Buergeria buergeri* (Sano *et al.* 2004) and *Rana nigromaculata* (Sumida *et al.* 2001b).

	Buergeria buergeri				*Rana nigromaculata*			
			Codon				Codon	
Gene	Size [bp]	Strand (sense)	Start	Stop	Size[bp]	Strand (sense)	Start	Stop
D-loop	4 576	–			2 425	H		
TAS	14	–			14	H		
O_H	64	–			64	H		
CSB-1	28	–			28	H		
CSB-2	18	–			18	H		
CSB-3	15	–			19	H		
Tandem repeat	785/2 235	–			757	H		
tRNA-Leu (CUN)	72	H			72	H		
tRNA-Thr	70	H			70	H		
tRNA-Pro	69	L			69	L		
tRNA-Phe	68	H			70	H		
12S-rRNA	927	H			933	H		
tRNA-Val	69	H			69	H		
16S rRNA	1 574	H			1 588	H		
tRNA-Leu (UUR)	74	H			74	H		
ND1	964	H	ATT	T	973	H	ATG	T
tRNA-Ile	71	H			71	H		
tRNA-Gln	71	L			71	L		
tRNA-Met	70	H			69	H		
ND2	1 038	H	ATT	TAG	1 038	H	ATT	TAG
tRNA-Trp	71	H			70	H		
tRNA-Ala	70	L			70	L		
tRNA-Asn	73	L			73	L		
O_L	29	H			31	H		
tRNA-Cys	64	L			67	L		
tRNA-Tyr	67	L			67	L		
COI	1 551	H	ATA	AGG	1 539	H	ATA	TAA
tRNA-Ser (UCN)	71	L			71	L		
tRNA-Asp	68	H			69	H		
COII	687	H	ATG	AGA	688	H	ATG	T
tRNA-Lys	72	H			69	H		
ATP8	165	H	ATG	TAA	165	H	ATG	TAG
ATP6	682	H	ATG	T	682	H	ATG	T
COIII	784	H	ATG	T	784	H	ATG	T
tRNA-Gly	69	H			69	H		
ND3	343	H	ATA	T	340	H	ATG	T
tRNA-Arg	69	H			69	H		
ND4L	285	H	ATG	TAA	285	H	ATG	TAA
ND4	1 366	H	ATG	T	1 360	H	ATG	T
tRNA-His	69	H			69	H		
tRNA-Ser (AGY)	67	H			67	H		
ND5	1 791	H	ATG	AGA	1 795	H	ATA	T
ND6	492	L	ATG	AGG	501	L	ATG	AGA
tRNA-Glu	68	L			69	L		
Cyt*b*	1 170	H	ATG	TAA	1 143	H	ATG	TAA

TAS: termination associated sequence; O_H and O_L: replication origins of the H- and L-strands; CSB: conserved sequence block; ND1–6, ND4L: subunits 1–6 and 4L of nicotineamide adenine dinucleotide dehydrogenase; ATP6 and 8: subunits 6 and 8 of adenosine triphosphatase; COI–III: subunits I–III of cytochrome c oxidase; Cyt*b*: cytochrome *b* apoenzyme.

In mt genomes, except for the CR, noncoding sequences as spacing introns or intergenic spacers (IGS) are usually few or absent. In all mt genomes of salamanders studied so far, IGS's have been detected. For example, all ambystomatids have a basic IGS of 240 bp between the $tRNA^{Thr}$ gene and the $tRNA^{Pro}$ gene (Shaffer and McKnight 1996; McKnight and Shaffer 1997; Arnason *et al.* 2004; Samuels *et al.* 2005; Zhang *et al.* 2005). In the IGS of five *Ambystoma* species inserts have been detected that vary from 87bp to 444 bp (Uzzell, unpubl.).

Noncoding sequences were also found in the mt genome of the rice frog (*Fejervarya limnocharis*): (1) small sequences of 15 and 38 nucleotides flank the $tRNA^{Leu(CUN)}$ gene, (2) a 25-base sequence is located between the ND5 and the $tRNA^{Thr}$ genes, and (3) a 34-nucleotide sequence is located between the $tRNA^{Ser(AGY)}$ gene and the ND6 gene (Liu *et al.* 2005; Fig. 10).

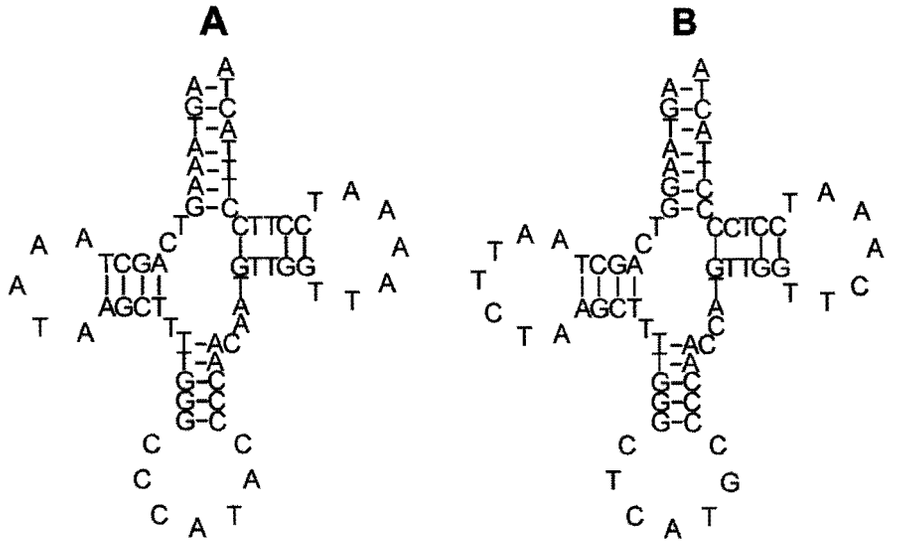


Fig. 8. Putative secondary structures of $tRNA^{Met}$ of (A) *Rana nigromaculata* (Sumida *et al.* 2001a) and (B) *Fejervarya limnocharis* (Liu *et al.* 2005).

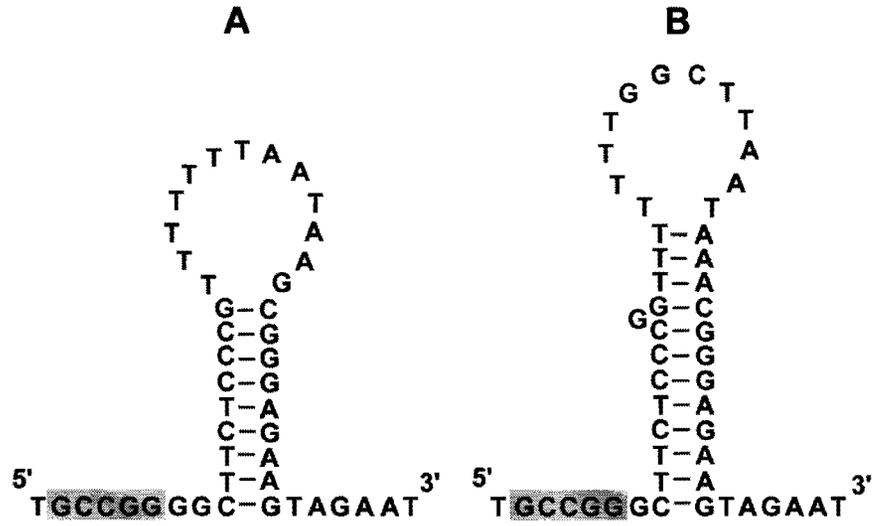


Fig. 9. Proposed secondary structure for the L-strand origin of mtDNA replication of (A) *Rana nigromaculata* and (B) *Xenopus laevis*. The pentanucleotides predicted to be involved with the transition of RNA to DNA are boxed. After Sumida *et al.* (2001a) and Roe *et al.* (1985), respectively.

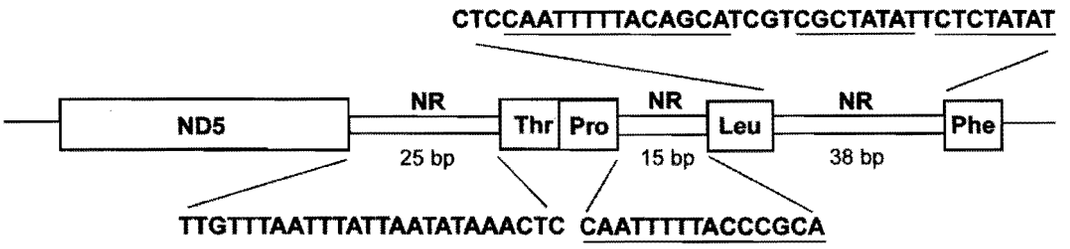


Fig. 10. Diagrammic representation of noncoding regions (NR) in *Fejervarya limnocharis*. ND5: NADH dehydrogenese subunit V; Thr, Pro, Leu, Phe: genes for $tRNA^{Thr}$, $tRNA^{Pro}$, $tRNA^{Leu(CUN)}$ and $tRNA^{Phe}$, respectively. Repeated sequence motifs are underlined. From Liu *et al.* (2005).

Mitochondrial DNA has been used extensively in the last three decades to infer the systematics of living organisms and even of fossils. Compared to nuclear DNA, mtDNA has a fourfold lower effective population size (N_e) and therefore tends to coalesce and become monophyletic relatively quickly (Moore 1995), which in turn makes it a reliable tool for estimating relationships among closely related and even incipient species (Wiens and Penkrot 2002). On the other hand, substitutions become saturated more quickly in mt genes, which diminishes the resolution of deeper phylogenetic splits. That the substitution rate varies among different functional parts of the mt genome also must be considered when using mtDNA for estimating phylogeny.

In vertebrates the control region usually represents the most rapidly changing sequence. As a rare exception Samuels *et al.* (2005) found only a relatively low level of nucleotide variation in the CR of five mole-salamander species (*Ambystoma*) comparable with that of the slowly evolving 12S rRNA and 16S rRNA genes. Beside the CR, different protein-coding genes (e.g., NADH dehydrogenease subunit genes) evolve relatively rapidly (Moritz *et al.* 1987; Saccone *et al.* 1999). Rapidly-evolving sequences probably are better suited to genetic analyses of population networks and of phylogenetic relationships between closely related species, whereas more slowly evolving sequences (e.g., COI, COIII, tRNA sequences, 12S rDNA, 16S rDNA) are generally useful for phylogenetic reconstructions of taxa above the species level.

Unequal substitution rates occur not only between different genes, but also within a single gene as a result of structural and functional constraints. For example, the rate of evolution of mt rRNA genes varies considerably along the length of the molecule (Hillis and Dixon 1991; Simon 1991). Highly conserved nucleotide sites are associated with sites of ribosomal protein attachment, messenger RNA processing, tRNA attachment, and core helices (Noller *et al.* 1990). In protein-coding genes many third and some first codon positions evolve at higher rates than do many first and all second positions, as noted above (Nei 1987; Irwin *et al.* 1991). This is caused by the fact that many (but not all) substitutions at the third and some at the first position are silent (synonymous), i.e., they do not replace amino acids and thus they are less constrained. In vertebrate mt genomes, transitions (A→G, C→T) greatly outnumber transversions (A→C, A→T, G→C, G→T). The predominance of transitions is greatest in comparisons of closely related sequences, and it decreases as sequences diverge, until no bias is detectable (Moritz *et al.* 1987).

In their comprehensive survey of 143 phylogenetic studies that used mtDNA, Funk and Omland (2003) found a surprisingly large number of cases in which conspecific mt sequences appear not as a monophylum (e.g., in 21% of all investigated amphibian species). A fundamental problem that may lead to incorrect phylogenies is that the rate of mtDNA evolution is not constant, even in closely related taxa (Saccone *et al.* 1999). Furthermore, incomplete lineage sorting (i.e., the survival of parental mt lineages in each daughter species) may affect mitochondrial phylogenies, although stochastic lineage sorting is expected to progress more rapidly for mt alleles because of their lower N_e-value of mtDNA. Incomplete lineage sorting occurs especially when closely related species are investigated. It may have major effects on phylogenies in the case of rapidly radiating taxa, in which succeeding speciation events occur before sorting is completed (Funk and Omland 2003).

The "correctness" of phylogenetic hypotheses based on mtDNA sequences may also be influenced by introgressive hybridization which is difficult to distinguish from incomplete lineage sorting. Introgressive hybridization is often connected to species radiations and/or the colonization of new environments (reviewed by Seehausen 2004). Hence, it can be observed especially among closely related species that hybridize in zones of secondary contact. Fertile hybrids may transmit one parental genome into the gene pool of the other parental species via backcrosses of hybrid × parental species (horizontal genome transfer). Introgression of complete parental mt genomes was detected, for example, in European water frogs (Spolsky and Uzzell 1984, 1986; Plötner 1998), in newts of the genus *Triturus* (Wallis and Arntzen 1989; Babik *et al.* 2003), and in *Bombina* species (Szymura *et al.* 2000).

Because horizontal transfer of mt genomes over species boundaries is relatively common (e.g., Gilbert 2003; Schliewen and Klee 2004) it can be assumed that introgressed mtDNA does not significantly influence individual fitness.

Despite these problems and difficulties mtDNA has proven to be a valuable and powerful tool that can be successfully applied to the analysis of the systematics of living amphibians. At an increasing scale, entire mt genomes are sequenced to exhaust their full information contents. Beside sequence comparisons, more complex features such as secondary structures of ribosomal genes or specific gene arrangements may also be useful for setting up or verifying phylogenetic hypotheses.

III. MOLECULAR MARKERS AND METHODS OF INVESTIGATION

Depending on the molecules used for systematic investigations three conceptually different classes of molecular markers can be distinguished: protein variants, DNA repeat variation, and DNA sequence polymorphism (Schlötterer 2004). Undoubtedly, DNA sequences provide the highest information content of all molecular markers. The choice of genetic markers and the method of analysis depends on the particular question and requires basic information about the structure, function, and evolution of the markers. For example, if the phylogenetic relationships of younger, closely related species are to be analysed, one should not use conserved or slowly-evolving characters. In the following section a brief survey of the main methods and their applications in systematic studies is provided.

A. Detection of Protein Polymorphisms

1. Protein Electrophoresis

In general terms electrophoresis is the movement of charged particles under the influence of an electrical field. The electrophoretic mobility of a protein depends on two main factors determined directly by its primary structure: net charge and size of molecule. The strength of the electrical field and the consistency of the support matrix (gel) also have an effect on protein migration.

The net charge of each protein varies with pH; at low pH the amino groups become positively charged, and at high pH the carboxyl groups become negatively charged. Proteins having a positive net charge (cations) migrate toward the cathode, and negatively charged proteins (anions) migrate toward the anode. At certain pH values most proteins reach an isoelectric point at which the effects of positive and negative charges are equal. In this case proteins do not move in an electrical field because they are attracted to neither the anode nor the cathode.

Following electrophoretic separation of protein samples in a gel matrix the individual proteins are visualized by specific histochemical staining procedures. For enzymes, the stain solutions contain a specific substrate, cofactors, and an oxidized salt (for example, nitro-blue tetrazolium) while non-enzymatic proteins are visualized with such specific reagents as Amido Black or Coomassie Violet.

The recognizable gel bands represent enzyme phenotypes that are also termed "electromorphs" (King and Ohta 1975). For a correct interpretation of the banding pattern one must know something about the number of subunits of the protein (enzyme). For example, a dimeric enzyme shows one band in a homozygous state, but three bands in a heterozygous state (Fig. 11).

Different electromorphs are assumed to reflect changes in the DNA sequence of the encoding gene, i.e., different alleles. On the other hand, not all nucleotide substitutions can be detected by electrophoresis because many do not change the encoded amino acid (e.g., silent substitutions, synonymous substitutions), even when they cause an amino acid substitution; however, there may be no change in the net charge and/or conformation of

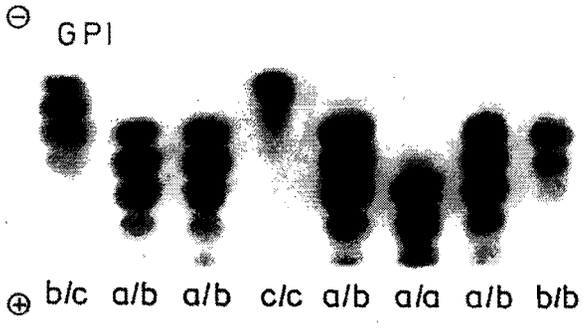


Fig. 11. Electrophoretic pattern of glucosephosphate isomerase (*gpi*) of European water frogs (*Rana esculenta* complex) suggesting the existance of three alleles (a-c). Usually, homozygous a/a and b/b individuals show an intensely stained band and 1–2 anodal subbands that probably result from post-translational modifications. Because of the dimeric structure of *gpi*, heterozygous individuals show a multibanded pattern consisting of three dense bands and 1–2 weaker subbands.

the protein (Fig. 12). Thus, electromorph identity, even within a species, does not always mean identity in nucleotide-base sequence (Allendorf 1977). Apparent identity of electromorphs between species is probably at best a weak indication of nucleotide-sequence similarity: different electromorphs within a species are likely to be more similar to each other genetically than to apparently identical electromorphs of a different species. This insensitivity to genetic variation within species is the main criticism levelled at protein markers. The assumption that similar migration of electromorphs indicates genetic similarity between species may well be misleading. Both within and among species, changes of buffer systems, gel concentrations, and/or the support medium (starch gel, polyacrylamide gel, agarose gel, cellulose acetate gel) may increase the number of detectable electromorphs (alleles). Modifications of the basic technique, for example continuous-buffer electrophoresis, multiphasic electrophoresis, isoelectric focusing, SDS and urea electrophoresis and two-dimensional electrophoresis, offer additional analytical possibilities (reviewed by Ferguson 1980). Even these refinements, however, fail to eliminate all the problems.

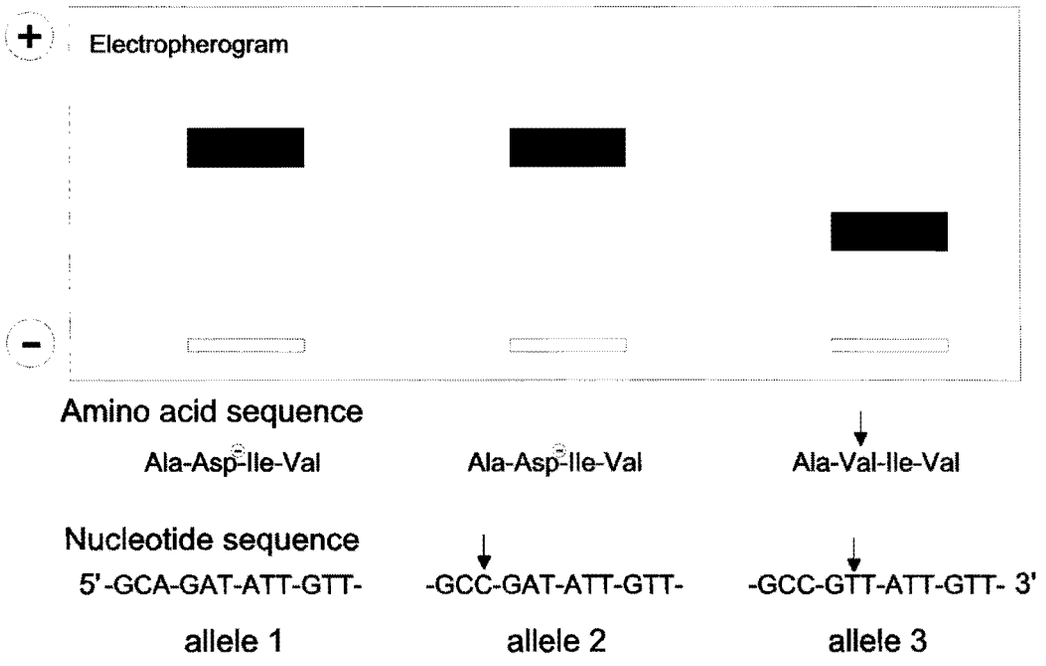


Fig. 12. Relation between the nucleotide sequence of a gene, the amino acid sequence of the encoded protein (primary structure) and the electrophoretic mobility of this protein. While the A→C tranversion in codon 1 does not influence the primary structure of the encoded protein and remains undetected in electrophoresis, the transversion T→A in codon 2 results in an amino acid substitution (asp→val) that changes the net charge of the protein and thus, its electrophoretic mobility.

Several factors may complicate the interpretation of protein-banding patterns in a gel (Murphy *et al.* 1996). Alleles with reduced or no expression of a protein product (so called null alleles) may cause apparent heterozygote deficiencies because heterozygotes for null alleles are scored as homozygotes. Misinterpretations of banding patterns may also occur if isozymes encoded by different loci show identical electrophoretic mobilities. Such isoloci can often be separated by changing the buffer systems. Furthermore, differences in electrophoretic mobility may result from artefacts in the gel, or from post-translational modifications of the proteins, such as denaturation, deamination, phosphorylation, sulphatation, oxidation, reduction, addition of other molecules, aggregation, and cleavage of polypeptides (Ferguson 1980). So-called secondary isozymes or subbands (Richardson *et al.* 1986) are a common phenomenon for starch gels (Fig. 11).

Up to the end of the 1980s, protein electrophoresis was the most widely used method in molecular systematics. Today, it plays an important role for the determination of species boundaries, the recognition of species hybrids, and the analysis of hybrid zones (see below). A large number of electrophoretic studies have been published that address such questions in amphibians (e.g., Uzzell and Berger 1975; Hotz and Uzzell 1982; Günther and Plötner 1994; Donnellan *et al.* 1999; Kozak and Montanucci 2001; Veith *et al.* 2002). Protein electrophoretic data have also been used for phylogenetic reconstructions of closely related species (e.g., Beerli *et al.* 1996). Since the development of DNA sequencing, however, this aspect has become less significant.

2. Immunological Methods

Comparative immunological techniques were frequently used for phylogenetic inference until DNA-based methods became available. Although immunological techniques are no longer widely used in systematic studies, their principles are explained here because they have provided interesting hypotheses that challenged current systems and initiated extensive research into phylogenetics.

In both qualitative and quantitative immunological methods, antibodies are produced to an antigen. The degree of reactivity between the antibodies and the antigens can be measured using direct and indirect properties of the antigen-antibody binding process. When a foreign antigen (a protein) is injected repeatedly into an unrelated animal (typically a rabbit), the animal produces a spectrum of antibodies that have different binding affinities and specificities to the antigen. After the immunization period, the animal is bled and the resultant serum contains the antibodies. The degree of antibody-binding affinity and specificity can vary with immunization protocol from low-affinity and narrow-specificity (low-diversity) antibodies that recognize only major antigen sites to very high-affinity and broad-specificity antibodies capable of detecting single amino acid replacements (Maxson and Maxson 1990).

Antibodies raised against antigens from one species can be used to measure relative cross-reactivities of the corresponding antigen from another species. When the antiserum is exposed to the original antigen (the homologous antigen), an antigen-antibody reaction occurs, and a precipitate is produced. If heterologous antigens are used, i.e., antigens from related species, the immunological cross reaction can be measured relative to that obtained with the homologous antiserum. It is generally assumed that the stronger the immunological reaction observed, the more similar the proteins.

A variety of immunological techniques has been developed, for example precipitin and immunodiffusion tests, immunoelectrophoresis, radioimmunoassay, and complement fixation tests. In particular, the micro-complement fixation or MC'F method (Champion *et al.* 1974) became popular for systematic research in vertebrates; examples of its use in amphibians were published by Case and Wake (1977), Uzzell (1978), Maxson (1977, 1984), Maxson *et al.* (1979), Ebendahl and Uzzell (1982) and Busack *et al.* (1988). The general principle of MC'F is to measure the extent of antigen-antibody reaction using the property of antigen-antibody complexes to bind different serum proteins subsumed under the term

"complement" (Fig. 13). The greater the number of antigen-antibody complexes, the more complement is bound. In an heterologous antigen-antibody-reaction a higher concentration of antiserum is needed to fix the same amount of complement that is fixed in an homologous reaction. Unbound (free) complement is able to lyse sensitized erythrocytes, i.e., red-blood cells (typically from sheep) that are exposed to antibodies directed against these cells. After lysis, the released haemoglobin can be measured with a spectrophotometer. The concentration of haemoglobin is inversely proportional to the amount of complement that is fixed in the experimental antigen-antibody complex.

By working with very dilute concentrations of antigen (typically serum albumin) and antisera, only antibodies with a high affinity will be bound. Antigenic sites with amino-acid replacements do not bind antibodies and are excluded from the antigen-antibody reaction. The amount of bound antigen, however, is not affected because there are enough antigenic sites that bind the antigen. The amount of excluded antibodies is proportional

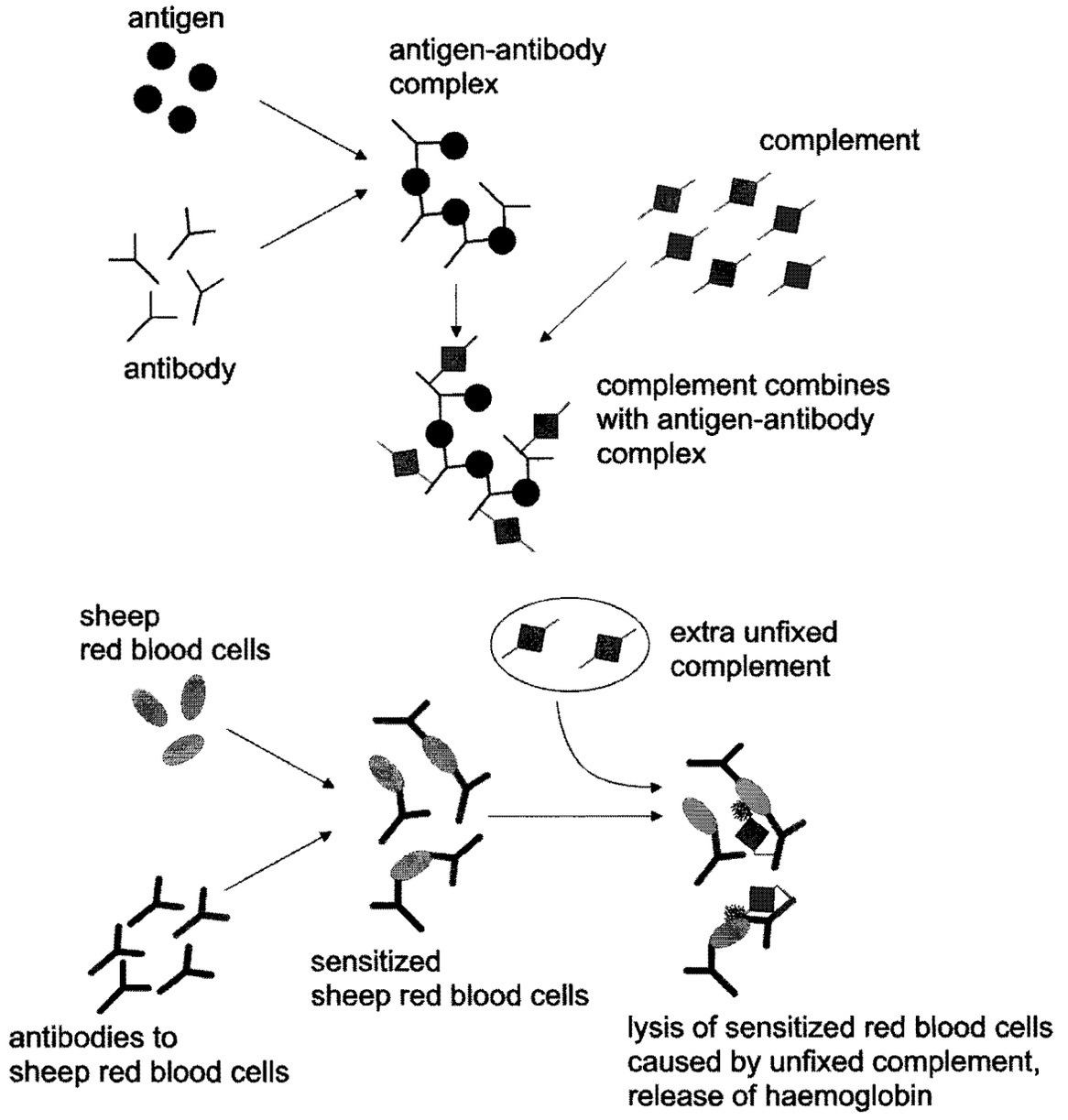


Fig. 13. Principle of complement fixation test (adapted from Ferguson 1980).

to the amount of unfixed complement because complement only binds to bound antibodies. The amount of extra antiserum needed to obtain an equivalent fixation of complement allows an estimation of the number of excluded antigenic sites. The factor by which the antiserum concentration needs to be raised for a particular antigen to produce the same complement fixation as the homologous antigen is an index of dissimilarity (D_I), which can be used to calculate the immunological distance (ID) between two antigens by the formula: $ID = 100 \times \log (D_I)$.

MC'F is a rapid method that provides an indirect measurement of sequence differences between homologous proteins. In contrast to other immunological techniques, the MC'F method allows estimating the number of amino acid replacements in homologous proteins because of a linear relationship between ID and the number of amino acid substitutions, suggesting that the antigenic effects of amino-acid substitutions are approximately equal and additive. For serum albumin protein, one unit of ID equals approximately one amino-acid substitution. It is not possible, however, to indicate specific amino-acid replacements.

B. DNA-Based Methods

Compared to proteins, DNA has several advantages (Dowling *et al.* 1996): (1) DNA can be prepared from small amounts of tissue and is relatively stable, even in non-cryogenically stored tissues, (2) the methods are, for the most part, general to any type of DNA, (3) the genotype rather than the phenotype is assayed, and (4) DNA sequences can be relatively easily generated and the number of sequences that can be compared directly is not limited. Sequence variation can be examined by direct sequencing, via PCR, or by electrophoretically comparing DNA segments or fragments to look for variation in their number, size, and conformation. Compared to nucleotide sequencing the analysis of DNA segments or fragments offers less genetic information. It is, however, still a powerful and cost-effective alternative when large numbers of individuals or loci or large parts of a genome are being screened (Dowling *et al.* 1996). Differences among individuals in the number and/or pattern of DNA fragments can arise through changes in the amount of DNA, the structure of the DNA, or the number and distribution of restriction sites or, in the case of DNA segments obtained by PCR-based methods, in the number and distribution of primer-binding sites.

The principles of the most frequently used methods, their advantages and disadvantages, and their applicability to different problems in molecular systematics are reviewed below.

1. Analysis of Restriction-Fragment-Length Polymorphisms (RFLP)

DNA can be cleaved into fragments by special enzymes, so called restriction endonucleases. These enzymes are isolated from bacteria and the name of the source bacterium is used to form the enzyme name (Table 4). The enzymes cut DNA at specific recognition sequences (restriction sites), typically 4–6 bp long. As shown in Table 4, the recognition sites are palindromic, i.e., the order of the bases in a segment of one DNA strand is reverse to that in the complementary strand. Base substitutions or small indels can create or eliminate restriction sites for a particular enzyme, thereby altering the number and size of fragments detected by this enzyme alone. The variations in fragment pattern are referred to as restriction-fragment-length polymorphisms (RFLPs).

The DNA fragments produced by endonucleases can be separated on agarose gels according to their size. After visualization of the fragments by staining with ethidium bromide or by end-labelling with radioactive nucleotides or by transfer hybridization, the banding patterns can be compared. In amphibians and other vertebrates, RFLP analysis of mtDNA and nDNA was used for population genetic and phylogenetic studies (e.g., Spolsky and Uzzell 1986; Lee and Park 1991). Today RFLP is applied only in combination with PCR (see section on PCR-based methods) to solve special systematic problems, e.g., for species identification.

Table 4. Recognition sequences and cleavage sites (↓↑) of three restriction enzymes. N: Any of A, T, C, G. More restriction enzymes are stored in the database REBASE (http://rebase.neb.com; Roberts and Macelis 1993).

Enzyme	Recognition sequence	Microorganism Source
*Eco*RI	5'-G↓AATTC-3' 3'-CTTAA↑G-5'	*Escherichia coli*
*Hae*III	5'-GG↓CC-3' 3'-CC↑GG-5'	*Haemophilus aegyptius*
*Hinf*I	5'-G↓ANTC-3' 3'-CTNA↑G-5'	*Haemophilus influenzae*

2. *DNA-Fingerprinting (Minisatellite Analysis)*

Similar to RFLP analysis, DNA-fingerprinting (Jeffreys *et al.* 1985) involves the digestion of genomic DNA with restriction enzymes (Table 4). After separation (usually by sub-marine electrophoresis in agarose gels) the DNA fragments are transferred to nylon membranes according to a technique first described by Southern (1975) and then hybridized with either single-locus or multilocus oligonucleotide probes, for example $(GATA)_4$ (for further details see Kirby 1990; Dowling *et al.* 1996). The resulting banding patterns can be used for identification and differentiation of individuals, populations, and species (e.g., Plötner *et al.* 1994; Scribner *et al.* 1994; Fig. 14). Compared to PCR-based methods, DNA-Fingerprinting is relatively time consuming and cost-intensive which makes this method unattractive for systematic studies.

3. *PCR-Based Methods*

A. PCR-RFLP

The PCR-RFLP combines the principles of PCR and RFLP. After amplification the DNA segment is treated with endonucleases. In the presence of appropriate restriction sites the segment is cut into different parts (sub-segments) that can be separated in an agarose gel. After staining with ethidium bromide the resulting banding patterns can be used for species identification. For example, in mt ND2 sequences of European *Rana ridibunda*, an AluI restriction site (AG↓CT), not found in any of other western Palearctic water-frog species examined (Plötner, unpubl. results), was detected at position 634. If this method is applied to mtDNA, however, the possibility of horizontal genome transfer by hybridization, which may lead to mistakes in species determination, has to be considered. For known sequences, the choice of restriction enzymes can be made using online tools such as WEB CUTTER (www.medkem.gu.se/cutter).

B. ANALYSIS OF MICROSATELLITES

Microsatellites are among the most powerful and practical markers for surveying genetic diversity among and within populations or among closely related species. Before microsatellites can be used in molecular genetic studies, however, they need to be isolated from the genome. For this purpose it is necessary to follow a relatively simple procedure, in principal a combination of DNA digestion and cloning (reviewed by Avise 1994; Bruford *et al.* 1996). Whole genomic DNA is digested with a combination of four-base cutting-restriction enzymes such as *Alu*I, *Hae*III or *Rsa*I. This step removes many unique and low-copy-number DNA sequences from the size fraction to be collected for cloning and produces a large number of DNA fragments of mostly repetitive DNA of which microsatellites are a subclass. Usually the fragments with lengths between 300 and 500 bp are selected for cloning; these are just long enough to contain microsatellites together with their flanking regions, which later are needed as primer-binding sites. These fragments are cloned into vectors followed by hybridization with labelled simple-sequence polymer DNA such as poly(CA) or poly(GA), motifs that occur frequently in microsatellites, in order to identify those recombinants likely to contain microsatellite sequences. This hybridization

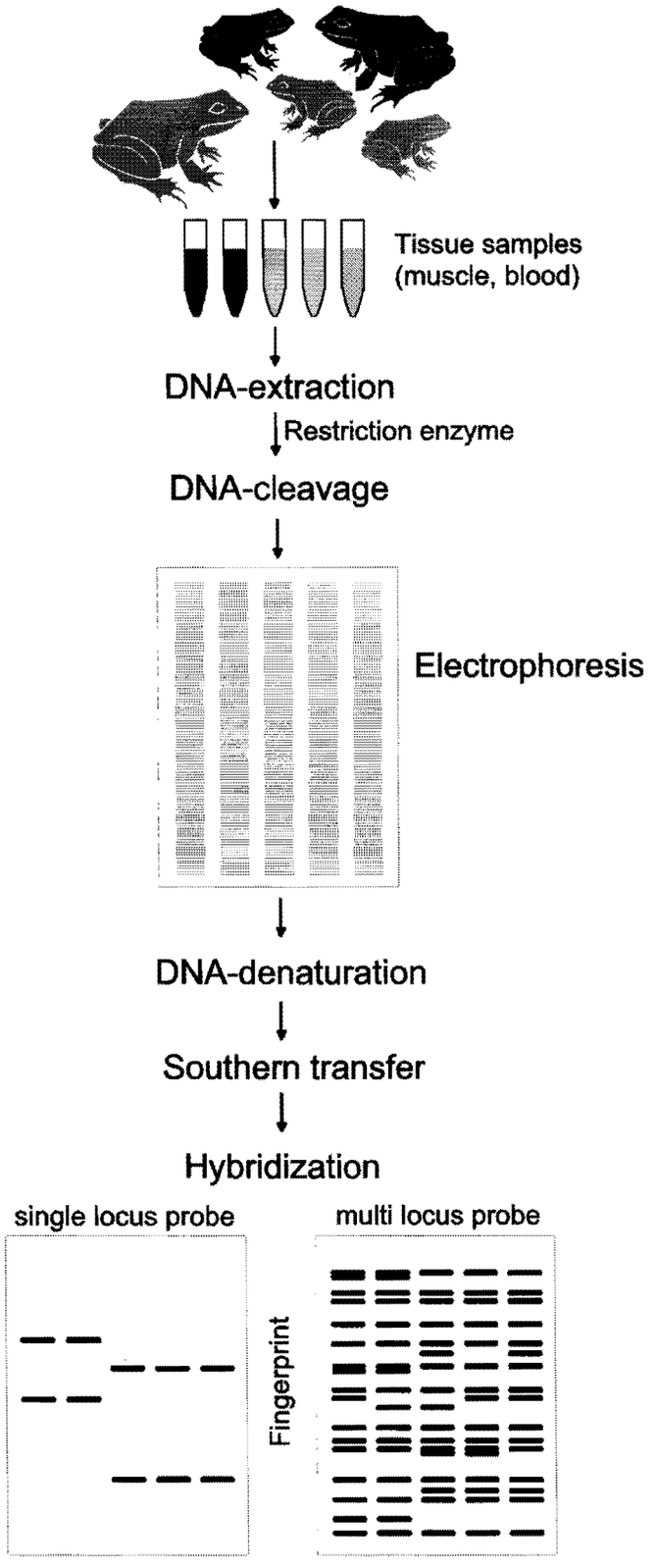


Fig. 14. Principle of DNA-fingerprinting.

step is necessary because only about 0.5–2% of all clones contain microsatellite DNA. It has been stated that even after this labelling, only a third of all positively labelled clones contain microsatellite loci that can be used for later analyses (Bruford *et al.* 1996). Owing to the wastage of consumables needed to successfully screen a genome for a number of microsatellite loci suitable for further investigation of genetic diversity, microsatellite studies are relatively expensive. Once a set of microsatellites is available, which includes successful design of primers that match the flanking sequence regions, allelic variation can simply and effectively be detected by electrophoresis after PCR amplification; PCR segments are electrophoretically separated by size, typically using acrylamide gels in a sequencer. The separated segments are marked by use of fluorescently or radioactively-labelled primers.

Because microsatellites are codominant markers, analysis of the banding patterns is similar to that in allozymes with the difference that the number of alleles and the degree of heterozygosity usually is much higher in microsatellites. Beside their high level of allelic variation, microsatellites have two further properties that make them ideal markers for population genetic and systematic studies: (1) the number of motif repeats at a given locus usually undergoes no selection pressure, which means that microsatellites undergo neutral evolution, and (2) because of their Mendelian and codominant inheritance they allow distinguishing between heterozygotes and homozygotes. If an individual is heterozygous for two microsatellite alleles, then two differently migrating bands will be detected on the gel (Fig. 15).

Apparently there is an upper limit on number of microsatellite alleles indicated by the fact that the degree of interspecific variability is

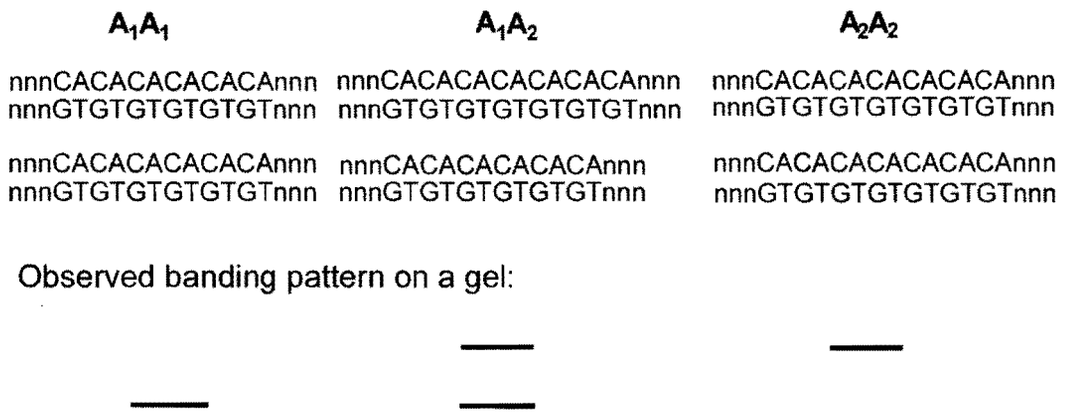


Fig. 15. Relation between the size of microsatellite alleles (A_1, A_2) and the corresponding banding pattern seen on the gel.

lower than would be expected from the degree of heterogeneity observed within populations (Page and Holmes 1998). This constraint clearly limits the value of microsatellites for the inference of genetic relationships to closely related species or to conspecific populations. It has been noted that microsatellite loci suffer from homoplasy problems (Schlötterer 1998) and that a large number of loci (>10) should be analysed when phylogenetic relationships or genetic distances are addressed. In regard to phylogenetic studies, the highly polymorphic nature of microsatellites may cause some bias towards increased heterozygosity levels. So called "null alleles", which do not contain a tandem repeat and account for the apparent noninheritance of parental alleles to the offspring, pose another problem to analyses of microsatellites (Callen *et al.* 1993). If null alleles are present at a given locus, it is not possible to discriminate between homozygotes and heterozygotes with a null allele. Furthermore, microsatellite alleles that have identical positions on a gel may differ in their nucleotide sequence and thus represent different alleles (unpubl. results). This problem has been ignored in most microsatellite studies.

A disadvantage from a practical perspective is that microsatellites must be developed anew for each species or group of closely related species because the sequences of flanking regions, which are the binding sites for the PCR primers, are relatively plastic. On the other hand the applicability of microsatellites developed for a particular species to other, closely related species has been proven in principal (e.g., Hotz *et al.* 2001). The range of taxa to which a certain set of primers can be applied may even be expanded by the use of degenerated primers (e.g., Moore *et al.* 1991; Schlötterer *et al.* 1991).

Microsatellite data have been extensively applied in evolutionary genetics, ecology, population studies, and conservation biology. In this framework questions of cohort and individual identification in large-scale experimental designs, parentage tests and mating system analysis, short-lineage determination, reconstruction of colonization history, estimation of dispersal rates and amounts of genetic exchange between populations have been addressed (e.g., Litt and Luty 1989; Tautz 1989; Weber and May 1989; Bruford and Wayne 1993; Queller *et al.* 1993; Schlötterer and Pemberton 1994; Tautz and Schlötterer 1994; Jarne and Lagoda 1996; Goldstein and Pollock 1997; Schlötterer 1998; Goldstein and Schlötterer 1999; Hedrick 1999; Krenz *et al.* 1999; Jehle and Arntzen 2002). Microsatellites have also been used to infer population structure and its spatial or temporal fluctuations in amphibians, such as various anurans (e.g., Estaoup *et al.* 2001; Zeisset and Beebee 2001; Funk *et al.* 2005; Kraaijeveld-Smit *et al.* 2005) or salamanders (e.g., Murphy *et al.* 2000; Jehle *et al.* 2005; Spear *et al.* 2005). Phylogenetic or systematic aspects, however, played only a minor role in most of these studies.

C. RAPD AND AFLP ANALYSIS

The random amplification of polymorphic DNA (RAPD), developed by Welsh and McClelland (1990) and Williams *et al.* (1990), is a simple, fast and inexpensive method of studying DNA variation. The RAPD technique is designed to detect sequence changes within priming sites, i.e., base substitutions within either priming site will affect the efficiency of amplification and thus the profile of segments (Fig. 16). Unlike standard PCR,

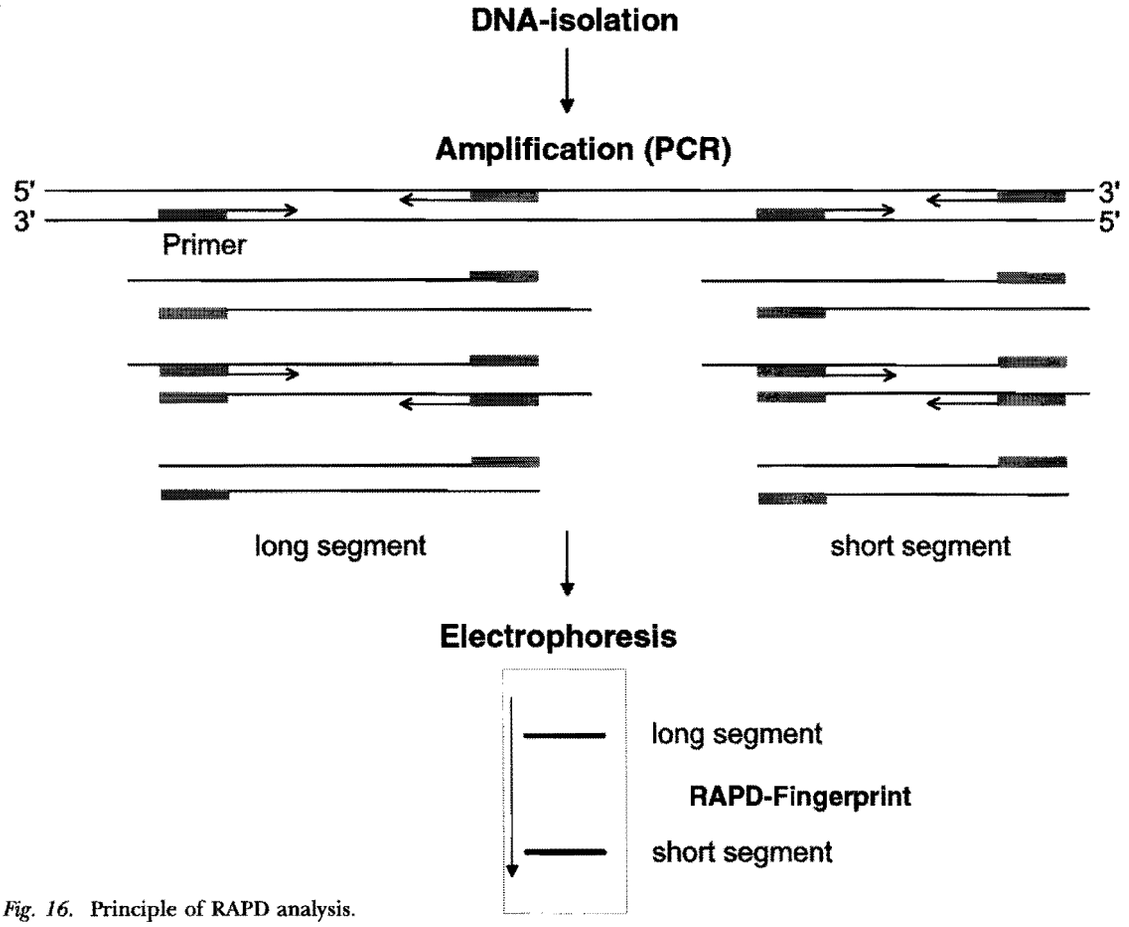


Fig. 16. Principle of RAPD analysis.

only a single random oligonucleotide primer is needed and no prior knowledge of the genome to be analysed is required. Originally, short primers (8–10 nucleotides long) were used, but longer "semi-random" primers can also be used.

When the primer is short, there is a high probability that the genome contains several primer binding sites that are close to one another and in an inverted orientation. The PCR technique scans a genome for these inverted repeats. The amplified products (intervening DNA segments of various lengths) are identified by agarose or acrylamid gel electrophoresis. Modifications of RAPD analysis have been described as Arbitrarily Primed (AP) PCR and DNA Amplification Fingerprinting (DAF) (reviewed by Hadrys *et al.* 1992).

In principle, it is assumed that RAPD markers follow Mendelian inheritance and that alleles from different loci do not comigrate, i.e., they have different positions on a gel (Lynch and Milligan 1994). In the past, RAPD markers were mainly used to estimate genetic differences within and among populations, to recognize "cryptic" species, to clarify species-specificity of individuals or populations, and to identify hybrids and life-cycle stages, e.g., eggs and larvae (Masters 1995; Kimberling *et al.* 1996; Zeisset and Beebee 1998; Snell *et al.* 2005). Today, RAPD analysis plays only a minor role in systematics because its analytical power is not competitive with that of other molecular methods. The principle limitations

of RAPD analysis concern its reproducibility because PCR conditions (e.g., temperature profile and thus even the type of thermal cycler, Mg^{2+} concentration, type of polymerase, quality and concentration of template DNA) may seriously affect the results. The ability to amplify a specific segment will also be affected by large insertions or deletions between priming sites (Dowling *et al.* 1996). Furthermore, co-migration of non-homologous segments because of similar size cannot be ruled out, i.e., bands that have identical positions in a gel do not inevitably represent the same locus. On the other hand, even if the bands have the same position in the gel they may differ in the nucleotide composition and thus represent different alleles. Third, in diploid organisms it is not possible to distinguish between homozygous and heterozygous individuals, which introduces problems in statistical analysis (Nei and Kumar 2000).

Amplified-fragment-length polymorphism (AFLP) is a relatively new technique that has recently became popular among zoologists. AFLP is based on the selective amplification of restricted fragments from a total digest of genomic DNA of any origin and complexity (Voss *et al.* 1995) and essentially represents a combination of two older methods, RFLP and RAPD. A selection of papers is now available that explain the practical implementation of AFLP and also provide hints for troubleshooting (e.g., Mueller and Wolfenbarger 1999; Giannasi 2001; Bensch and Åkesson 2005).

In principal, AFLP consists of two main steps: (1) the digestion and ligation step and (2) the selective amplification step (Fig. 17). In the digestion and ligation step whole genomic DNA is digested with two different restriction enzymes. *Eco*R1 and *Mse*1 are

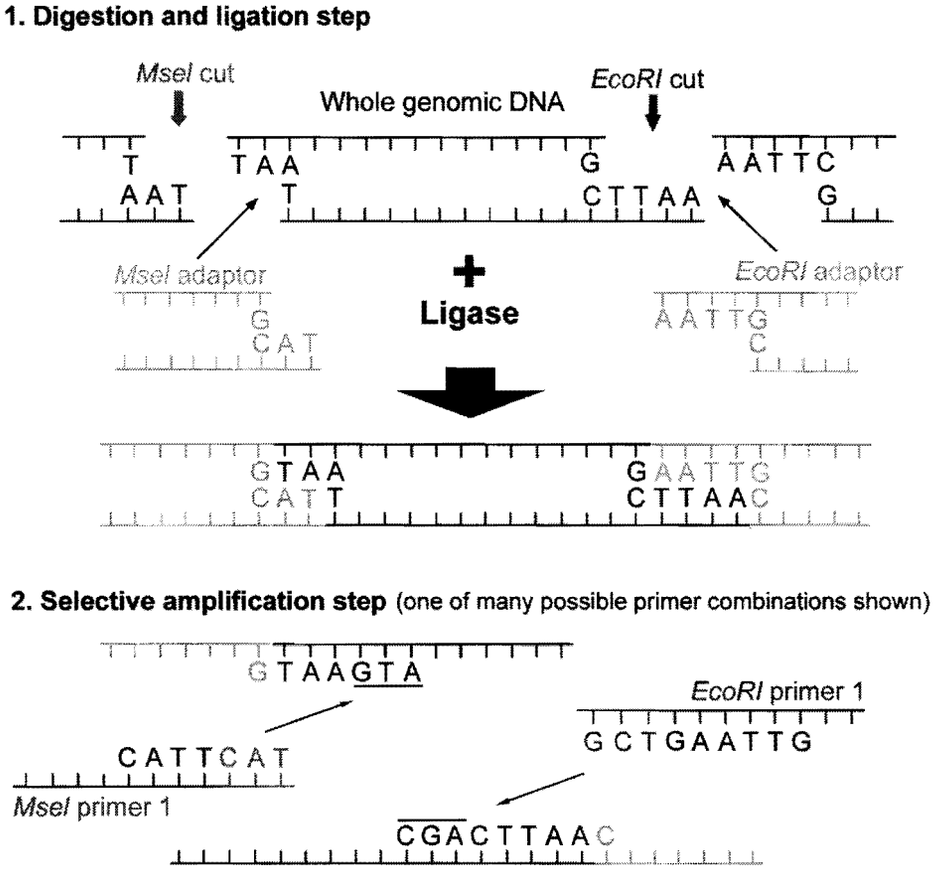


Fig. 17. Principle of AFLP analysis.

commonly used, but other combinations are possible (leading to different yields of fragments). In any case, the restriction enzymes produce a large number of differently sized DNA fragments. Depending on genome size and chosen enzymes, fragments can number in the thousands. AFLP adaptors (very short DNA fragments) are joined to the single-stranded ends of these fragments by use of a DNA ligase. AFLP adaptors are designed so that the ligation of a fragment with an adaptor does not reconstitute a new restriction site. Ligation of adaptor sequences is done in the presence of the restriction enzymes, which immediately re-cleave any fragment-to-fragment pair that may also originate. The ligation step is finished if an adaptor has been added to each end of the produced fragments; now the end sequences of each fragment consist of the adaptor sequence and the remaining part of the restriction sequence and no longer constitute binding sites for restriction enzymes.

The adaptors, which form the end sequences of the fragments produced, serve as priming sites in the PCR that follows (selective amplification step). In this reaction only a subset of fragments is amplified. Selectivity is achieved by use of primers that extend into the unknown part of the fragment. Usually two amplification steps are performed to minimize artefacts. In the first step, primers are used that extend for a single, randomly chosen base into the unknown fragment. Since these primers bind only to fragments that possess the complimentary nucleotide to the additional primer base, only every forth fragment is amplified. After the first step, generally the number of amplified products is still too large for analysis. For this reason a second amplification step is performed using primers that extend for another two to three bases into the fragment. The number of amplified products is reduced accordingly by factors of 16 and 64, respectively. Because of the high selectivity, primers differing by only a single base lead to a different subset of segments. Ideal extension lengths will vary with genome size. Optimal selection of primers will result in a number of amplified products neither too large nor too small for reproducible analyses.

AFLP-PCR products can be separated and scored with a variety of techniques, from manual scoring on agarose gels to automated genotyping. In most cases, automated polyacrylamide gel electrophoresis or capillary electrophoresis is the method of choice for providing resolution of banding patterns to the level of single nucleotide differences. The separated bands are scored by use of fluorescently dye-labelled primers. For phylogenetic analyses, scores are simply coded as binary characters (i.e., present/absent) in a data matrix containing the information on all bands; special software for computerized analyses is available (e.g., BinThere, developed by N. Garnhart and available through the T. Kocher laboratory http://hcgs.unh.edu).

The reliability of the AFLP method essentially depends on a complete digestion of the DNA because otherwise uncut fragments will be amplified that produce artefacts. To ensure complete digestion, restriction enzymes should be used in excess. They also should not be sensitive to DNA methylation, which potentially may cause incomplete digestion. Low DNA quality may badly affect the reproducibility of the method because heavily degraded DNA will not provide reproducible banding patterns. It has been claimed that slightly degraded DNA was successfully analysed (Mueller and Wolfenbarger 1999). It cannot be completely excluded, however, that the use of even slightly degraded DNA will definitely affect the banding pattern, which in turn will influence the results of phylogenetic reconstructions. To what degree misleading information remains within an acceptable range cannot be stated in general but depends on the questions asked and the organisms chosen. Because AFLP markers are used in particular to infer relationships on a very fine scale, the acceptable error level may indeed be very low. Ultimately, high stringency PCR conditions, such as annealing temperature, are essential to ensure that primers bind only to perfectly matching templates. It will take some tests to assure that the PCR protocols used are sufficiently stringent. If all these prerequisites are fulfilled, however, AFLP has proven a very reliable and reproducible method, with average errors of 0–2%, equivalent to that of microsatellites (Jones *et al.* 1997; Arens *et al.* 1998; Winfield *et al.* 1998; Mueller and Wolfenbarger 1999).

AFLP is able to screen simultaneously many different loci randomly distributed across the whole genome. Even minor genetic differences between the samples examined can be

detected which is a major advantage of this method. From a practical point of view, high time and cost efficiency are also major advantages, as is relatively simple handling.

Compared to co-dominant genetic markers such as microsatellites or allozymes, the major disadvantage of AFLP markers is inability to discriminate between homozygotes and heterozygotes at a locus (e.g., Yan *et al.* 1999). Size homoplasy can be another problem because AFLP does not allow distinguishing between homologous and non-homologous loci that produce fragments of exactly the same length. Size homoplasy increases with the density of amplified products (Vekemans *et al.* 2002; Koopman 2005) and can be detected using a protocol developed by O'Hanlon and Pekall (2000).

Of more general concern is the fact that AFLP is particularly sensitive to varying or low DNA quality and to certain types of mutations, both of which affect the observed banding patterns and thus, potentially pose major problems to later data analyses. Nucleotide exchanges at the restriction sites and insertions or deletions of nucleotides in the fragment, for example, will influence the banding patterns because of the altered lengths of amplified segments. This will probably obscure homologies because it is impossible to trace homologous bands with different lengths among the many bands produced by amplification. Single nucleotide exchanges that occur within a fragment, however, will not influence the banding pattern and therefore cannot be recovered.

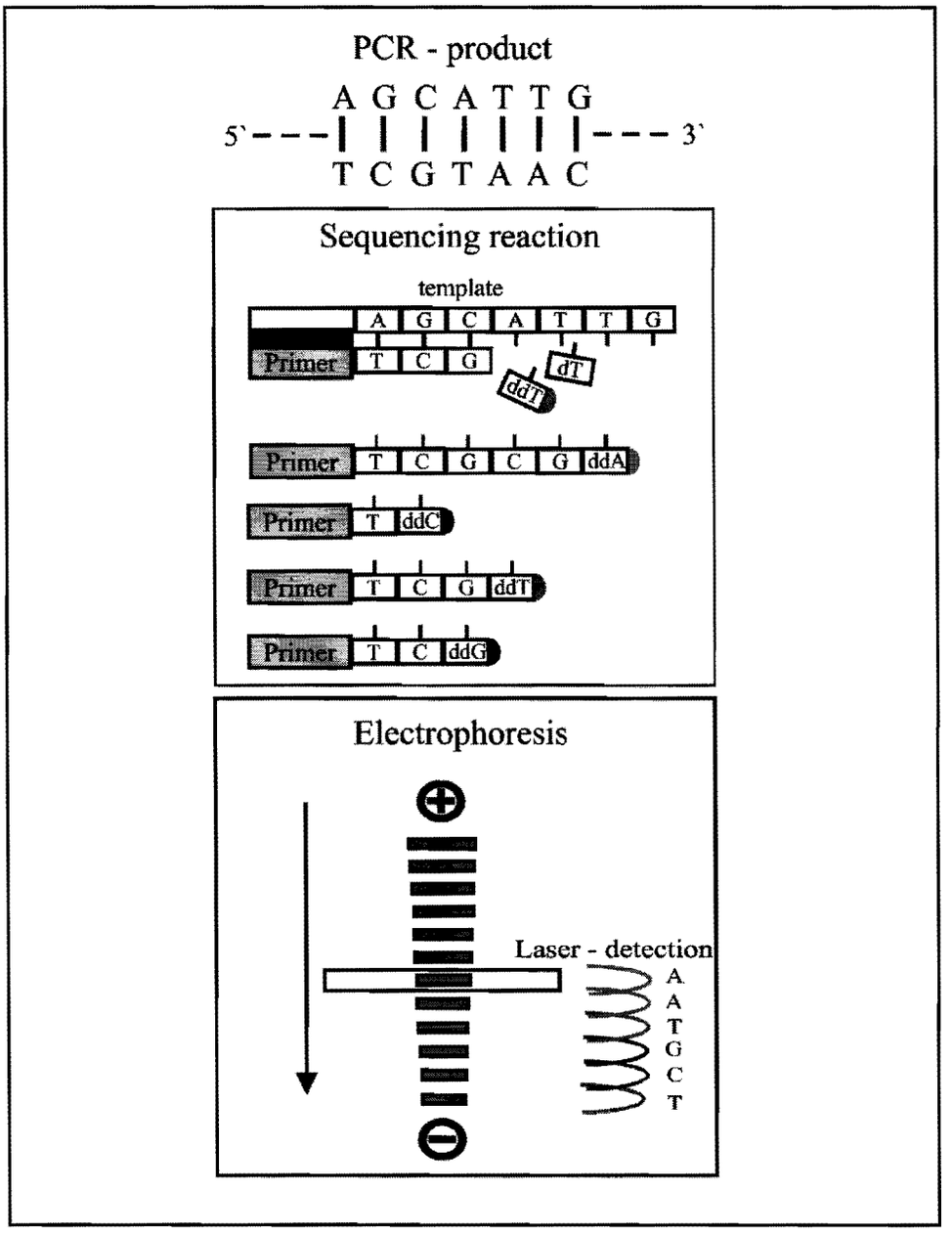
In summary, in many cases AFLP can compete with methods based on co-dominant markers, such as microsatellites and allozymes (Mueller and Wolfenbarger 1999; Campbell *et al.* 2003; Bensch and Åkesson 2005). AFLP markers have proven useful for assessing genetic differences among species, populations, and even individuals (Mueller and Wolfenbarger 1999; Groot *et al.* 2003) and also to infer phylogenetic relationships, especially among closely related taxa (e.g., Albertson *et al.* 1999; Giannasi *et al.* 2001; Parsons and Shaw 2001; Wilding *et al.* 2001; Vekemans *et al.* 2002; Allender *et al.* 2003; Beardsley *et al.* 2003; Creer *et al.* 2004; Sullivan *et al.* 2004; Koopman 2005; Mendelson and Shaw 2005). Bensch and Åkesson (2005) gave a survey of possible applications. Some of these studies provided evidence that AFLP markers are able to resolve relationships on a fine scale even in cases in which other markers failed to produce useful results (e.g., Creer *et al.* 2004; Schliewen and Klee 2004). On higher systematic levels, however, phylogenetic inferences based on similarities of AFLP profiles become problematic because the high variability of AFLP markers reduces similarities between distantly related taxa to the level of chance (Mueller and Wolfenbarger 1999). To date, only a small number of studies have been published in which AFLP data were used to address genetic, systematic, or evolutionary questions in amphibians (e.g., Voss *et al.* 2001; Curtis and Taylor 2003; Kochan *et al.* 2003; Riberon *et al.* 2004).

D. SEQUENCE ANALYSIS

DNA sequences are the main source of information for phylogenetic studies. Over the past three decades sequencing technology has developed rapidly. Today, sequence analysis is one of the most utilized methods in molecular systematics as shown by the rapid increase of DNA sequence data.

The cycle-sequencing method, which is a modification of the traditional sequencing method developed by Sanger *et al.* (1977), forms the basis of automated sequencing. The sequencing reaction consists of three major steps: (1) denaturation of DNA, (2) annealing of primers, and (3) extension of DNA via polymerase. In the denaturation step p, usually at 94°C c, the double-stranded DNA melts open to single-stranded DNA and all enzymatic reactions are stopped. Unlike standard PCR, in which two primers are used, only one is required in the sequencing reaction and therefore, only one strand is copied. In the extension step dinucleotides (dNTP's) are added complementary to the template DNA. The incorporation of a modified base (fluorescent labelled dideoxynucleotides; ddNTP's) stops the extension reaction because a ddNTP contains an H-atom on the 3rd carbon atom instead of an OH-group as in dNTP's. The incorporation of ddNTP's is determined by chance and leads to a mixture of DNA segments that, when sorted by size, differ by a

single nucleotide. After the sequencing reaction the DNA segments are separated by acrylamid gel electrophoresis according to their size, because the electrophoretic mobility decreases with the size of the segments. Because the ddNTP's are fluorescently labelled the colour of the last base of the single segments can be detected when they are passing



Electropherogram

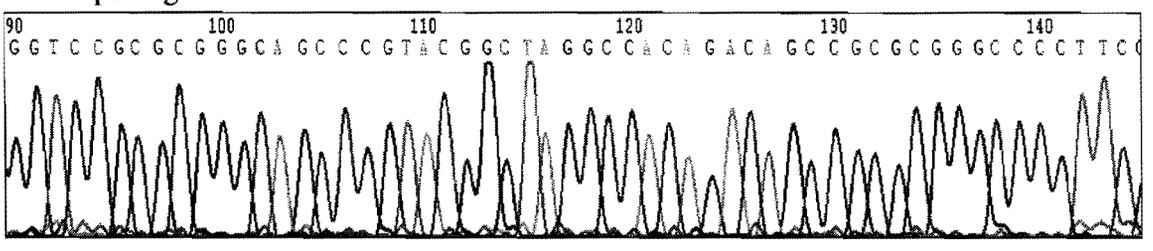


Fig. 18. Principle of cycle sequencing.

a laser beam (Fig. 18). The different ddNTP's (ddATP, ddGTP, ddTTP, ddCTP) have specific dyes, hence the complete sequence can be reconstructed base by base starting with the last base of the shortest segment and ending with the last base of the longest segment.

IV. METHODS OF ANALYSIS OF MOLECULAR DATA

A. Phylogenetic Analysis

The phylogenetic inference approaches described here can be used not only for amphibians but for all organisms. Only a short overview of the most widely-used techniques is included. Recommended, more detailed reviews include: "Molecular Systematics" (Hillis *et al.* 1996), which provides not only laboratory techniques but also describes types of data and analytical methods and "Inferring Phylogenies" (Felsenstein 2004), which is currently the most complete reference on phylogenetic methods. Newcomers to the field may also find "Phylogenetic Trees Made Easy" (Hall 2004) useful.

Using a sample dataset from research on western Palearctic water frogs (Plötner 1998; Plötner *et al.* 2001), several standard phylogenetic analyses are compared. Results from other individual studies may differ, of course, but these comparisons help highlight the strengths and weakness of the several methods described.

It is crucial to understand the programmes that generate phylogenies. Sometimes programmes are run with default settings, often with unfortunate results. Time spent reading the programme manual is certainly worthwhile. Examples of programmes and sources of manuals include PAUP* (Swofford 2002; manual and starter's guide available at *http://paup.scs.fsu.edu*), MrBayes (Ronquist and Huelsenbeck 2003; online manual at *http://mrbayes.scs.fsu.edu*), MEGA (Kumar *et al.* 2004; online manual at *http://www.megasoftware.net*), PHYLIP (Felsenstein 1989, 2006; online manuals at *http://evolution.gs.washington.edu/phylip.html*), MacClade (Maddison and Maddison 2000; *http://macclade.org*), among many others. Joseph Felsenstein maintains an exhaustive list of programmes that are related to phylogenetic analyses (*http://evolution.gs.washington.edu/phylip/software.html*).

1. Tree-Building Methods

One of the frequent first goals when working with multiple species or presumptive species is to visualize their relationships using a tree structure. Typically, one assumes that exchange of genetic material between groups ceased after the ancestral species split into two or more sub-groups. When a tree is constructed on the basis of this assumption then the path-length between the individuals in the dataset reflects the relatedness of the species and their phylogenetic relationships. This assumption forces one to accept that the observed pattern is also true for unsampled individuals from the same species. This poses a problem for widespread species or heterogeneous taxa that are lumped together, for example the genus *Rana*. Another class of problems appear when species are still able to produce rare fertile hybrids that would accommodate horizontal transfer of genetic material or when the speciation events happened quite recently. This problem is discussed in the section: *Phylogenetics versus Population Genetics*.

Typically no fossil data accompany molecular data, except in the rare situation when DNA can be extracted from subfossil bones and there are no reports of ancient amphibian DNA. Several tree-building methods for such data were described in the 1960s (e.g., Fitch and Margoliash 1967). Felsenstein (2004) gave an historical view of the introduction of phylogenetic methods into the fields of taxonomy and systematics. While many different methods exist, the following four groups of methods are most widely used: (1) parsimony-based methods, (2) genetic-distance methods, (3) maximum-likelihood methods, and (4) Bayesian methods.

A. PARSIMONY

The principle of parsimony, minimal use of resources or assumptions, has a long history reaching back to William of Ockham. The application of minimal-change analyses was

used by systematists for classification purposes. In 1969, Kluge and Farris introduced a method to infer phylogenetic trees using DNA sequence data that assumed that all changes between the nucleotides are equally probable (cf. Felsenstein 2004). Several different parsimony methods and improvements exist today. Most commonly used is Fitch parsimony (Fitch 1970) and extensions that allow for different weights among the changes from one nucleotide to another; Felsenstein (2004) provided details.

Parsimony is often the method of choice because it is fast and seems not to require a specific mutation model. It is not model free, however, because it forces the researcher to assume that the data measured at the tips of the tree reflects only minimal changes, which requires an implicit mutation model. With complex "mutations" (for example, development of an additional finger), it is unlikely that multiple lineages gained the same trait independently. With morphological or paleontological datasets it might be easier to establish unique traits than with DNA data. A given nucleotide site has only four possible states and commonly some sites show the same nucleotide on different branches of the phylogeny, indicating parallel mutations or a shared polymorphism in the ancestral species. In exons, the triplets TCA, TCC, TCG, TCT differ only in the third position, and code for the amino acid serine, but only one (TGG) codes for tryptophane. Highly variable sites, such as 3rd codon positions, are therefore often dropped from parsimony analyses. From a statistical viewpoint, however, dropping 3rd positions is not justified because even highly variable sites can contain information: likelihood and Bayesian inference use all information.

As an example, Figure 19 shows the most parsimonious tree of eight western Palearctic water-frog species (an eastern Palearctic water frog, *Rana nigromaculata,* was used as the outgroup) using mtDNA data set of 1 376 nucleotides from the ND2 and ND3 loci. Using PAUP* (Swofford 2002), the best tree has a score of 1 096 steps. Three hundred and fifty-six sites were parsimony-informative, 819 were constant, and 201 were variable but parsimony-uninformative. In a parsimony analysis only the substitutions that are shared with other species are used, thus ignoring lots of information, for example sites that mark a single species different from all others; such information is used in likelihood or Bayesian methods. It is also evident from the number of changes (1 096) of 356 parsimony-informative sites that several of these must have changed multiple times; the programme MacClade (Maddison and Maddison 2000) allows tracing the changes of each of the sites. Many ambiguous nucleotides are present in this dataset (analyses not shown).

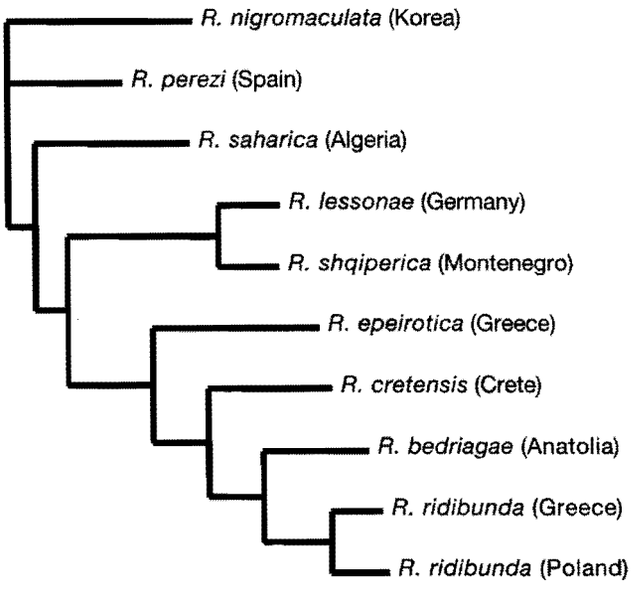


Fig. 19. Maximum parsimony tree of nine water-frog species.

Figure 19 shows branch lengths as relative number of changed sites. Often branch length is not shown in published parsimony trees but for evolutionary questions it certainly is useful to know whether a branch is short or long.

Analyses with simulated datasets, where the true phylogeny contains very long branches that are not associated with sister taxa, have shown that parsimony methods will find the wrong tree with more and more certainty the more data are available; the method is inconsistent. For many datasets the parsimony method works fine, but one cannot judge whether it produces the best answer possible without comparing the results with other methods. Given the statistical shortcomings (deletion of informative data and implicit mutation model) and given the progress computers have made in the last decade, the use of maximum parsimony for small to medium datasets will decline.

The example of water frogs highlights a case where different methods produce different trees, and where it is likely that maximum parsimony fails because of the long-branch attraction.

B. DISTANCE METHODS

Distance methods are typically run in two steps: (1) calculation of all pairwise distances between all individuals, followed by (2) using the distance matrix to generate a tree. This approach is often used outside of phylogenetics to cluster objects. Many distance measures have been proposed, from the most simple distances such as the p-distance, which counts the number of sites at which two sequences differ, to complex model-based distances such as likelihood distances or the LogDet distance (cf. Hillis *et al.* 1996; Felsenstein 2004). The distance measure used is often arbitrarily chosen. Several current programmes allow calculating such complex distances and make simple measures, such as the p-distance, obsolete. Several methods assemble the tree from the distance matrix; the most famous are certainly the neighbour-joining method developed by Saitou and Nei (1987) and the least-square minimization method developed by Fitch and Margoliash (1967). Neighbour-joining builds up a bifurcating tree by repeatedly joining the species pairs with the smallest genetic distance. This method is fast and often results in trees that are close to the best trees when all species have different pairwise distances. With ambiguous data (as in the water-frog example) or with many ties, the neighbour-joining trees sometimes fail because the algorithm does not reconsider earlier decisions. The choice of the distance is very important because different distances can produce different trees. Figure 20 shows two Neighbour-joining trees, one is based on the p-distance and the other on the LogDet distance.

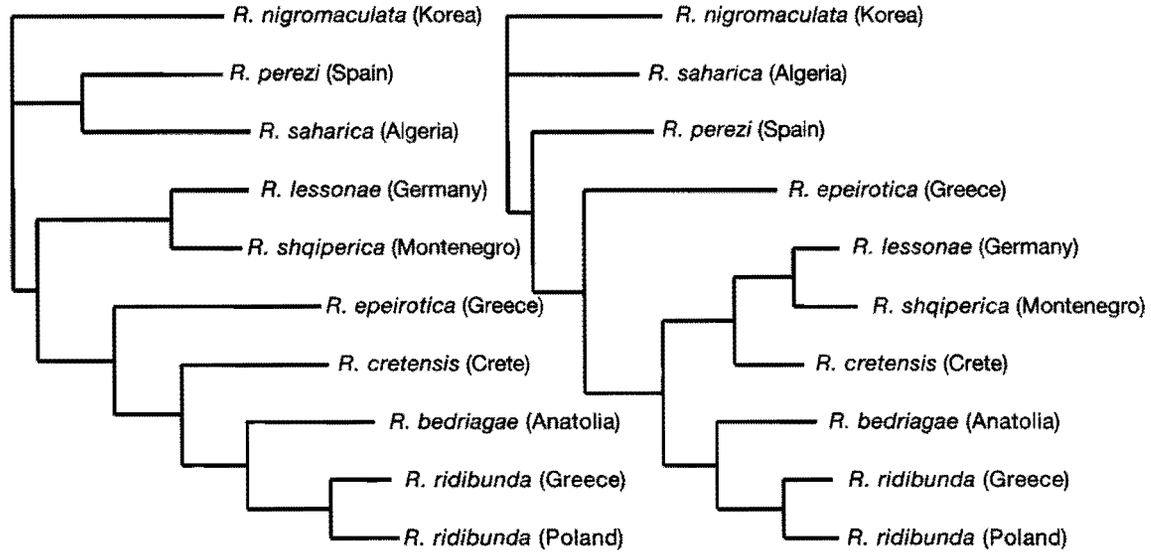


Fig. 20. The phylogeny of nine water-frog species using p-distance (left) and LogDet distance (right), under the Neighbour-joining method.

C. MAXIMUM LIKELIHOOD (ML)

In contrast to maximum parsimony, which minimizes the number of changes on a tree, maximum-likelihood inference needs an explicit model of change, a substitution model, to estimate change along the branches.

The substitution model needs to allow calculation of the probability that one starts on one end of a branch with a certain state, for example an *A*, and ends up on the other end of the branch with another or the same state. These transition probabilities on the trees need to be maximized for the maximum-likelihood tree. For each node one needs to calculate the *conditional probability* for all nucleotides at a site based on the states in the two nodes above it and the given branch length to each of these two nodes. For a DNA substitution model this results minimally in a 4×4 matrix operation for each internal node for each site; as a result, maximum-likelihood estimators are slow, although with the advent of fast computers it is routinely possible to evaluate trees with 100 tips or more. For datasets based on coding sequences one probably should prefer a model that takes into account the amino acids, either through codon-based models or protein-substitution models (cf. Felsenstein 2004). Recently, models that can take secondary protein structure into account have become feasible and will improve phylogenetic inference of taxa that are very divergent on the nucleotide level (Dimmic 2005).

How does one choose the best mutation model? Many researchers are puzzled by this question and resort to *Modeltest* (Posada and Crandall 1998, the programme can be downloaded from *http://darwin.uvigo.es/software/modeltest.html*), which tries a battery of tests from the simple to the more complicated. Likelihood ratio tests or Akaike's information criterion help to figure out the best model. *Modeltest* uses 56 models, but of course there are many more. For example, Huelsenbeck and Ronquist (2005) enumerate 203 possible models. Such tests can be done easily by hand using PAUP* or other programmes. It seems preferable to test starting with the most complicated model that one is willing to accept and then simplify from there. For example, start with the most complicated model available in mainstream programmes: the general time reversible model (GTR). It has six transition parameters, for each of the nucleotide transitions (A to C, A to G, and so on). The full GTR model has five free transition parameters (six total). If two of the five parameters are similar, one can explore the likelihood of the model with four transition parameters, combining the two similar ones in the more complex model. The water-frog data example achieves a considerably higher likelihood with the full GTR model with site variation (Γ) and a proportion of invariant sites (I) than with any other model (hand search and *Modeltest* delivered similar results; data not shown). This model is often referenced as GTR+Γ+I. Figure 21 shows the maximum likelihood tree. The positions of some of the species differ from their position in the parsimony tree (Fig. 19) and the two distance trees (Fig. 20).

D. BAYESIAN INFERENCE (BI)

In contrast to maximum-likelihood tree inference, Bayesian tree inference needs, in addition to the substitution model, a distribution to run the search. This distribution reflects prior belief, what one believed before inspection of the data. The choice of this prior distribution is controversial (cf. Felsenstein 2004; but see Huelsenbeck and Ronquist 2005). The probability of the parameters (based on the prior distribution) and the probability of the data given the parameters (the likelihood) together form the posterior distribution. The Bayesian approach is asymptotically similar to the maximum-likelihood approach when it is assumed that the prior distribution is uninformative. If the data contain information about the phylogeny then the data will overpower the prior distribution and dominate the posterior distribution. With weak data one returns the prior distribution. In contrast to all other methods described, Bayesian inference not only estimates the best tree but also describes the distribution around this best tree and allows the retrieval of clade-probabilities, i.e., how often a clade on the tree was visited during the analysis run. In early Bayesian analyses, researchers unfamiliar with the approximative nature of the computer programmes were not exploring enough topologies and were over-confident in

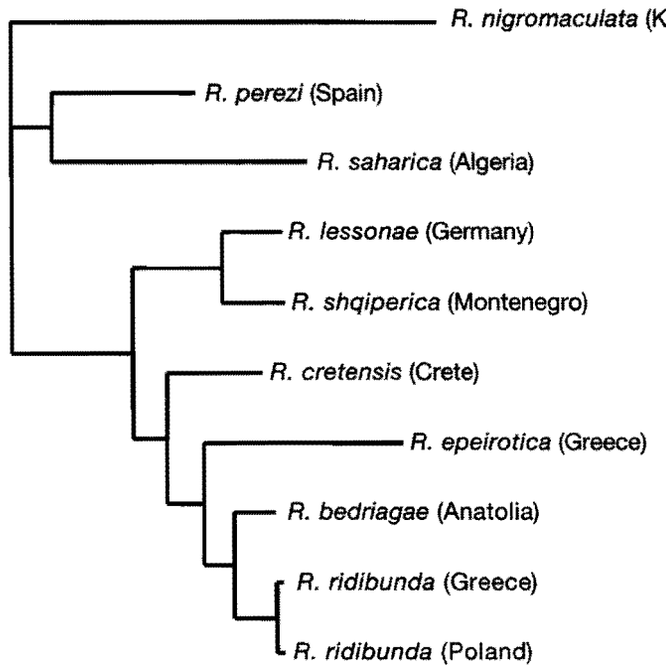


Fig. 21. Maximum-likelihood tree of nine water-frog taxa.

the clade probabilities. This resulted in grave differences from more standard bootstrap estimates. Modern Bayesian programmes inform users whether the method converged and is sampling trees from the posterior distribution or not; only trees sampled from the posterior distribution should be considered for description or analysis. Figure 22 shows the maximum posterior tree of nine water frog taxa.

2. Statistical Methods for Testing the Reliability of a Tree

The water-frog dataset delivers different best topologies (Figs 19–22) depending on the optimality criterion used. The calculation of the likelihood score of all trees that had

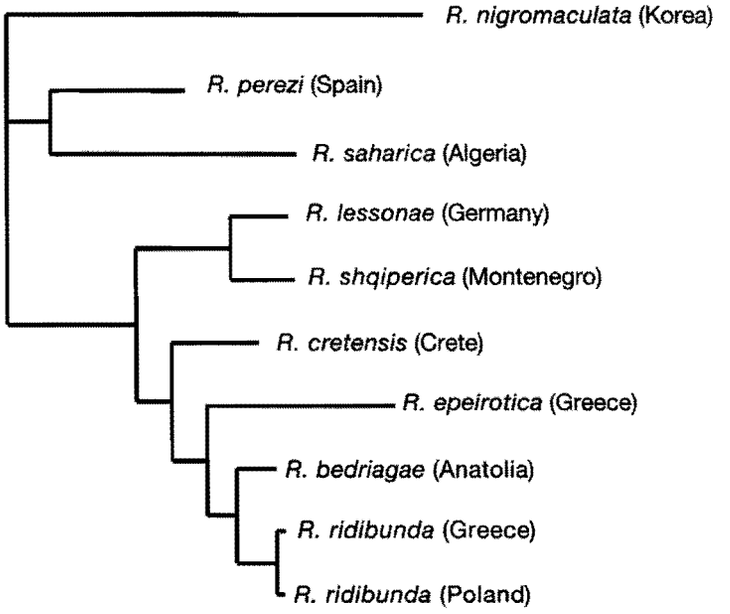


Fig. 22. Maximum-posterior tree of nine water-frog taxa. This is the best tree found during a Bayesian analysis (MrBayes).

a parsimony score between 1 096 (the best score) and 1 106 reveals that several trees that had rather bad scores in the parsimony analyses score well in the likelihood method (Fig. 23). The placement of four taxa, *Rana perezi, R. saharica, R. cretensis,* and *R. epeirotica* is difficult and accounts for all the different topologies in the top 27 trees found by parsimony. Several topologies differ only in the movement of two species between two established clades; *R. cretensis* and *R. epeirotica* show affinities with both the well defined *R. ridibunda/ R. bedriagae* clade and with the *R. lessonae/R. shqiperica* clade. Bootstrap analyses (Fig. 24) show that the available mtDNA data are probably insufficient to make a final judgment on the exact phylogenetic position.

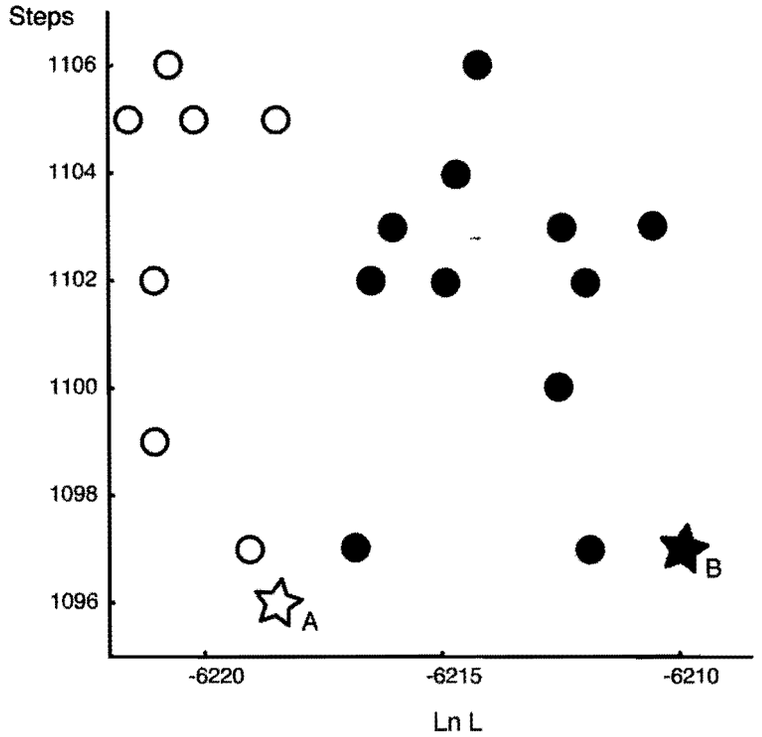


Fig. 23. Comparison of the 27 best trees found using parsimony and their Log-likelihood score. The light star shows the best parsimony tree and the dark star shows the maximum-likelihood tree. All trees with better likelihood scores than the best parsimony tree are shown with dark disks.

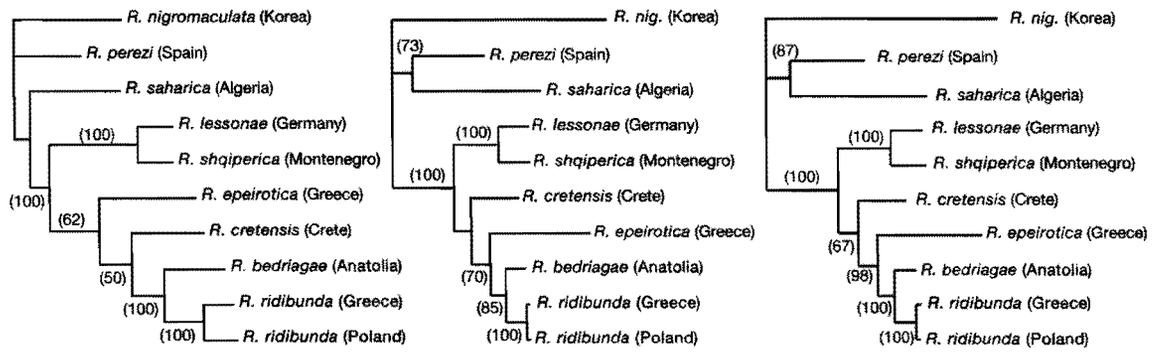


Fig. 24. Examples of results of bootstrap analyses using parsimony (left), likelihood (middle) and of posterior probabilities of the clades found in a Bayesian analyses (right). Numbers in parentheses are bootstrap probablilities (left, middle) or clade probabilities (right) in percentages.

Statistical evaluation of phylogenies using the bootstrap was introduced by Felsenstein (1985). There is still discussion about the interpretation of the clade bootstrap support. In principle one should interpret the bootstrap values as probabilities, but low support values have an upward bias and high support values have a downward bias (Hillis and Bull 1993, but see Felsenstein and Kishino 1993 for another opinion). Despite the problems of the exact interpretation of bootstrap values, they are useful for judging whether a clade has high or low support.

Recently, comparison of bootstrap support and Bayesian clade probabilities has shown that often the Bayesian clade probabilities have a tendency to overestimate the support, but there are counter-examples in cases where researchers were careful to analyse the convergence of the Bayesian inference (BI) runs (Cummings *et al.* 2003). A comparison of two bootstrap runs (parsimony and likelihood) with clade probabilities derived from a Bayesian analysis is shown in Figure 24. Differences among the bootstrap trees suggest that the parsimony method may yield less appropriate trees for this dataset.

3. Other Methods

Four main methods for finding the best phylogeny were discussed above. Other programmes use phylogenies to find sites under selection (for example PAML by Ziheng Yang, *http://abacus.gene.ucl.ac.uk/software/paml.html*), evaluate mutation models (MrBayes, Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2005), or estimate times of splits among lineages (multidivtime by Jeff Thorne and Hirohisa Kishino, *http://statgen.ncsu.edu/thorne/multidivtime.html*), among many others. Often researchers use phylogenetic trees to infer biogeographic relationships; this is very useful with divergences that happened long ago, for example splits of South America and Africa, but recent biogeographical inferences, such as effects of glaciations on species distributions, call for programmes that can take into account multiple gene lineages within a single species. Phylogenetic methods are often not very useful in this context and one would need to consider methods that bridge between phylogenetics (speciation events) and population genetics. Only a few programmes allow appropriate analyses on this interface, for example IM (Hey and Nielsen 2004). The field bridging between true phylogenetic inference and population genetic inference is in its infancy and it is easier to describe more problems than it is to find solutions.

B. Phylogenetics versus Population Genetics

Phylogenetics is the study of relationships among species whereas population genetics studies the relationship among individuals within a population within a species. Both fields are well developed, but the contact zone, when species hybridize, or the same species has regional genetic patterns that are incompatible, is not. An important quantity in population genetics is the effective size of a population (or species). It is an indicator of the available variability in a population. The larger the population the more different allele-lineages (haplotype-lineages) can be found in a population. All lineages in a population of a diploid species typically take about four times as many generations as the effective population size to coalesce when looking backwards in time. Therefore the species divergence is always more recent than the gene divergence. For some time it has been recognized that when speciation events are close together in time or close to the present, trees based on data from different individuals (within the same species) or from different genes do not necessarily agree. In fact, when the population size of the ancestral species is large compared to the divergence time then the risk of disagreement of the species-trees and the gene-tree is large (Edwards and Beerli 2000; Rosenberg 2002; Degnan and Salter 2005, among others). Figure 25 shows three scenarios that might occur within the same species phylogeny: A. The gene-tree matches the species-tree. B. The gene-topology matches the species-topology, but different individuals suggest different divergence times. C. The gene-tree does not match the species-tree. Good examples of all three patterns are given in the analyses by Takahata and Satta (1997) on the speciation sequence of humans, chimpanzees, and gorillas, for which many nuclear genes support the human-chimpanzee

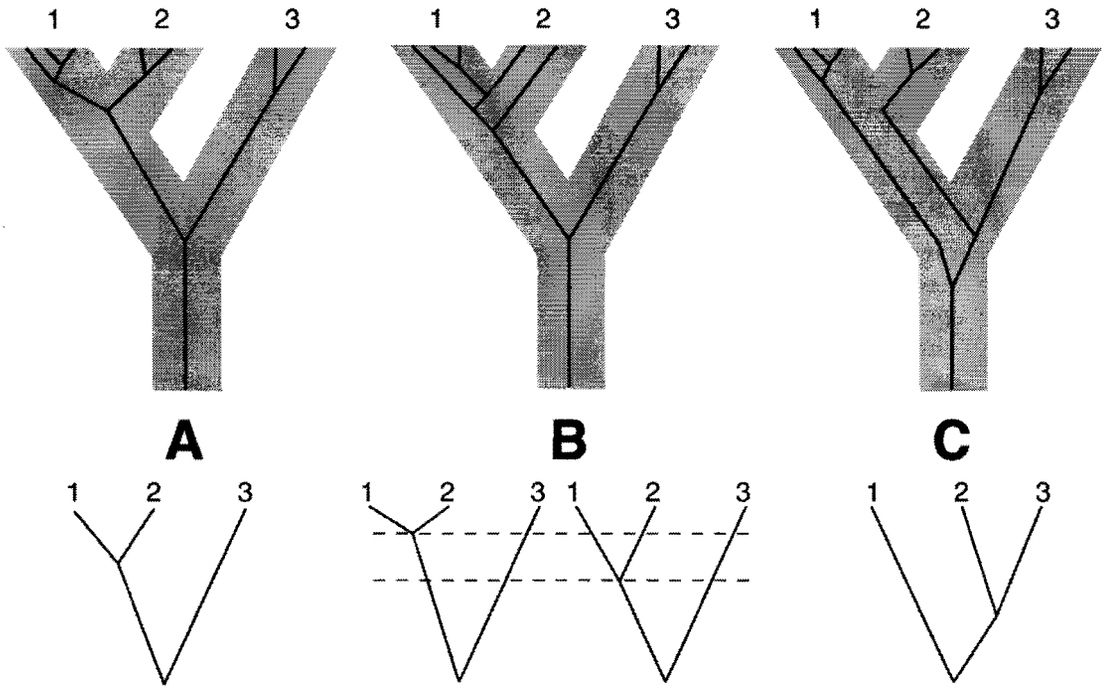


Fig. 25. Gene-trees and species-trees. (A) Species-tree and gene-tree are congruent and the inferred species-tree (bottom) is the same as the true tree (grey outline, top). (B) Ancestral population size of species 1 and 2 is large, and by chance branching time for different individuals results in different inferred species divergence times (bottom). (C) Very large ancestral population size of species 1, 2, and 3 makes it possible that the gene-tree and species-tree are incongruent. The inferred species tree is incorrect.

split as more recent, but a smaller number support the human-gorilla split. Using a single locus (for example complete mtDNA) does not necessarily reveal the species' phylogeny. The ancestral population size determines the chance of detecting the correct species phylogeny. Edwards and Beerli (2000) used a simple rule to establish how many loci are needed to establish that the confidence interval of the species divergence time does not include zero: $l\,g / Q > 0$, where l is the number of loci, g is the divergence time measured in expected mutations per generation and Q is four times the ancestral effective population size times the mutation rate per generation. Accurate estimation of the divergence time will guarantee good estimation of the topology. For example, if one believes that a species pair diverged at the end of the last glaciation period, 5 000 generations ago, and the ancestral population size was, say, 10 000, then one would certainly need more than one locus to make firm statements about divergence times. This measure does not really help ascertain whether there is resolution of the divergence times and topology of several species; it does not, for example, help determine to which groups *Rana cretensis*, and *R. epeirotica* belong. The mtDNA data at hand cannot resolve that question.

V. APPLICATIONS OF MOLECULAR METHODS IN AMPHIBIAN SYSTEMATICS

A. Determination and Identification of Species and Species Hybrids

Identifying taxa that constitute "species" requires a theoretical species concept. In the past more than 20 species concepts that became more or less popular have been developed (reviewed by Mayden 1997). For phylogenetic systematics *sensu* Hennig, the "Evolutionary Species Concept" (ESC), which was originally elaborated by Simpson (1961) and revised by Wiley (1978, 1981), seems most suitable (Ax 1984; Peters 1998). According to this concept *"An Evolutionary Species is a single lineage of ancestor-descendant populations that maintains its identity from other such lineages and that has its own evolutionary tendencies and historical fate".*

A lineage is considered as a group of populations with a common ancestry that is not shared with other populations. Isolating mechanisms, which have to be interpreted as side effects of infraspecific cohesion, the mate recognition systems, and the species-specific niche, are emergent properties of Evolutionary Species (Peters 1998).

The recognition of Evolutionary Species requires operational tools that provide indicators that may demonstrate the evolutionary independence of lineages. Such indicators are, in ideal circumstances, apomorphic characters that characterize ancestor-descendant populations and occur only within one lineage. Especially in recently evolved taxa, however, apomorphic characters are rare or remain unidentified. In groups of closely related species, morphological methods often fail because of a high morphological similarity of individuals, the lack of morphological features with diagnostic value, and/or the occurrence of species hybrids. In such cases molecular data may provide important information that underpins the coherence of ancestor-descendant lineages.

The importance of molecular methods for systematic research in morphologically uniform groups can be impressively demonstrated, for example by the western Palaearctic water-frog group. The discovery of the hybrid nature of the common European edible frog, *Rana esculenta*, and its taxonomic separation from one of its parental species, the pool frog *Rana lessonae*, by Berger (1967, 1968), inaugurated a period of intensive research on western Palaearctic water frogs (reviewed by Graf and Pols Pelaz 1989; Plötner 2005). Molecular methods, especially such as protein electrophoresis, immunology, and DNA sequencing, have had a great impact on water-frog systematics. As a result, our understanding of the biodiversity of the water-frog group has changed enormously; the number of recognized species has increased from two at the end of the 1960s to about 14–16 today (Dubois and Ohler 1994; Plötner and Ohst 2001), and relatively robust hypotheses on the phylogenetic relationships among the taxa are now available (e.g., Beerli 1994; Beerli *et al.* 1996; Plötner 1998; Plötner and Ohst 2001; Plötner 2005; but see section IV for contradictions and unsolved problems).

Genetic diversity detected in North Africa, the Mediterranean region, and central Asia indicates the existence of several additional undescribed species (Plötner and Ohst 2001; Plötner *et al.* 2001). For example, although it has been reported, on the basis of bioacoustic and morphological data, that *Rana bedriagae* occurs in Anatolia as well as in Israel and Syria (Joermann *et al.* 1988; Schneider *et al.* 1992; Schneider and Sinsch 1999; Sinsch and Schneider 1999) and that water frogs on Cyprus also belong to this species (Böhme and Wiedl 1994), marked differences occur between the mt genomes of (1) *Rana bedriagae* from Syria, Jordan, and Egypt, (2) water frogs distributed in Anatolia, and (3) individuals from Cyprus (Plötner and Ohst 2001; Plötner *et al.* 2001). These differences indicate that neither the Anatolian nor the Cypriote populations belong to *Rana bedriagae* but instead probably represent separate evolutionary species *sensu* Wiley (1978, 1981).

Morphologically very similar species that possess clear genetic differences have further been reported from other anuran (e.g., Hillis 1988; Donnellan *et al.* 1999; Glaw and Vences 2002) and salamander groups (e.g., Good 1989; Larson 1989). There is, however, no general distance threshold to differentiate between intraspecific and interspecific genetic variation, neither for proteins nor for nucleic acids (e.g., Avise and Aquadro 1982; Nishioka *et al.* 1987, 1992; Nishioka and Sumida 1992). Furthermore, there are cases where morphologically distinct taxa show little or no genetic divergence.

Although genetic distances obtained from different molecular datasets are often correlated (Fig. 26), discrepancies between various distance measurements can be observed for single population or species pairs; these may be caused by different evolutionary phenomena such as adaptive selection, genetic drift (especially bottleneck or founder effects in the case of small populations), introgressive hybridization, and/or incomplete lineage sorting. For the last two reasons, distance data resulting from mtDNA should be interpreted with caution (see Section on mitochondrial DNA). Nonetheless, mtDNA is often an appropriate tool for recognizing closely related species (e.g., Plötner 1998; Glaw and Vences 2002).

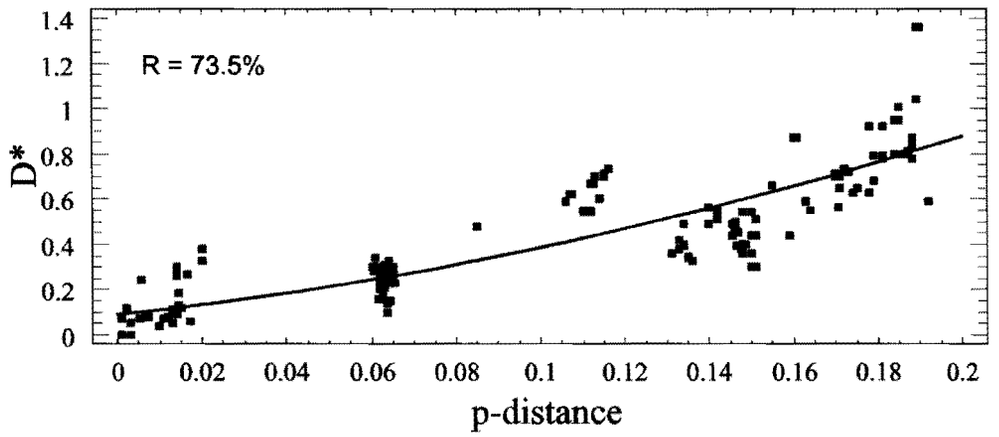


Fig. 26. The p distance (calculated on the basis of the mt ND2 and ND3 genes) versus modified Nei distance (D* values obtained from Beerli *et al.* [1996]), estimated for western Palaearctic water frog populations and species pairs.

Although fixed allelic differences are the best indicators for answering the question whether individuals, populations or forms represent distinct species, very different allele frequencies are operationally equivalent to fixed differences because they also indicate high genetic divergence. For allopatric populations and asexual populations, an important aim is to assess the extent of genetic divergence between the populations being tested in relation to geographic variation within species (Baverstock and Moritz 1996). Even if morphologically indistinguishable species (so called "cryptic species") are sympatrically distributed but completely isolated reproductively, their recognition is almost always relatively simple by analysis of codominant-inherited polymorphic markers. The number of specimens needed to look for the presence of "cryptic species" is relatively small because in a randomly breeding population, different alleles of a single locus should be in Hardy-Weinberg equilibrium, i.e., heterozygous individuals should exceed the frequency of at least one of the homozygotes (Richardson *et al.* 1986). For an hypothetical sample of ten conspecific individuals, Baverstock (1988) demonstrated that the probability of no individual being heterozygous is 0.1% for one locus with two alleles at frequencies of 0.4 and 0.6. The absence of heterozygotes, therefore, clearly indicates the presence of at least two species. It has to be emphasized, however, that small samples are only adequate when specimens of both species are included. Moreover, a lack of heterozygotes can also occur in asexual or haploid species, or if there is a high level of self-fertilization. Genetic effects such as imprinting or null alleles may also be responsible for an apparent lack of heterozygotes. Baverstock and Moritz (1996) therefore recommended a minimum of two loci showing patterns of fixed differences that are consistent between individuals and emphasized, that "in all cases it is more important to maximize the number of loci screened than to maximize the number of individuals examined".

If two evolutionary species are at the beginning of the divergence process (in prespeciation *sensu* Sperlich 1984), fixed allelic differences may not yet be developed. In such cases differences in allele frequencies of polymorphic loci or deficiencies of heterozygotes may be useful to test how far this process has progressed, especially in respect of prezygotic and postzygotic reproductive isolation.

Beside protein electrophoresis, which appears to remain the most generally applicable and efficient method for detecting "cryptic species", other molecular methods can be useful as well, e.g., the analysis of RFLPs, RAPDs, minisatellites, or microsatellites (Baverstock and Moritz 1996). First evidence for the existence of a "cryptic species" can even be obtained from genome-size measurements by DNA-flow cytometry. Because intraspecific variation in cellular DNA content among amphibians is considered to be typically low,

individuals with substantially different amounts of DNA probably are not conspecific. Based on 173 specimens, Borkin *et al.* (2001) identified two distinct groups of *Pelobates fuscus* with different genome sizes (8.7–9.0 pg per cell and 9.2–9.5 pg per cell, respectively) that are distributed in the western (the group with the smaller genome size) and the eastern part of Eastern Europe. These results speak in favour of two separate species. For a final systematic decision, however, more sophisticated data are needed.

If the distributional areas of closely related species overlap and if reproductive isolation is not complete, species-hybrids may occur. Natural hybrids have been detected in numerous amphibian groups, for example in salamanders (e.g., Karlin and Guttman 1981; Arntzen *et al.* 1998; Kozak and Montanucci 2001; Babik *et al.* 2003; Mikuliček *et al.* 2004), bufonids (e.g., Masta *et al.* 2002), hylids (e.g., Gartside 1980), and ranids (e.g., Berger 1967, 1968; Graf *et al.* 1977; Sage and Selander 1979). The proportion of hybrids and the dimensions of hybrid zones mainly depend on the degree of prezygotic and postzygotic reproductive isolation. In some cases, for example in ranid frogs and newts, hybridization is not limited to the formation of F_1 individuals (e.g., Sage and Selander 1979; Babik *et al.* 2003; Miculicek *et al.* 2004).

The detection and differentiation of species hybrids and hybrid zones requires information on the genetic variability of the parental species over their distributional ranges. During recent years many hybrid zones have been analysed, most of them by protein electrophoresis (e.g., Gartside 1972; Uzzell and Berger 1975; Sage and Selander 1979). In numerous hybrid zones, new allozyme variants have been detected that were not found in either parental species, for example in salamanders of the genera *Bolitoglossa* and *Ensatina* (Wake *et al.* 1980, 1986), European *Bombina* species (Gollmann *et al.* 1988), North American toads of the genus *Bufo* (Green 1984), and frogs of the genus *Rana* (Hotz 1983; Kocher and Sage 1986; Günther and Plötner 1994). The origin of these new allozymes remains speculative. Intracistronic recombination between different parental alleles during meiosis in hybrids has been discussed as one possible source of new alleles; such recombination may lead to an increased frequency of mutations resulting in new alleles.

Beside protein electrophoresis, hybrids can also be detected by means of nuclear DNA markers. For example, Miculiček *et al.* (2004) used RAPD to record natural hybridization and introgression between the newt species *Triturus cristatus* and *T. dobrogicus*. Furthermore, the DNA content, measured by DNA flow cytometry, can also provide first evidence for the occurrence of hybrids, especially in the case of polyploid forms (e.g., Borkin *et al.* 2002, 2004; Stöck *et al.* 2002) (Fig. 27). As emphasized by Streit *et al.* (1994), the combination of different kinds of markers usually provides superior resolution of genetic processes associated with polyploidy and interspecific hybridization. Combined with nuclear markers, the analysis of mtDNA can provide information on both the degree and biases in the direction of hybridization (Hillis *et al.* 1996).

Beside the detection and analysis of cryptic species and hybrid zones, molecular methods are particularly well suited for studying clonal diversity and the origin of unisexual species. Based on serological investigations, Uzzell and Goldblatt (1967) found that the triploid all-female forms of *Ambystoma tremblayi* and *A. platineum* arose by hybridization of *A. laterale* and *A. jeffersonianum*. Subsequent investigation revealed that the mtDNA of the hybrid forms did not derive from any of the parental species but originated from a distinct ancestral lineage (Kraus and Miyamoto 1990; Spolsky *et al.* 1992).

In general, conclusions concerning the systematic status of a population or form are almost all uncertain if they are drawn on the basis of limited data or a single method, but rather require a holistic approach; beside molecular markers, character sets such as bioacoustic parameters or morphological features should be analysed. Such analyses necessarily have to be conducted in a biogeographic context.

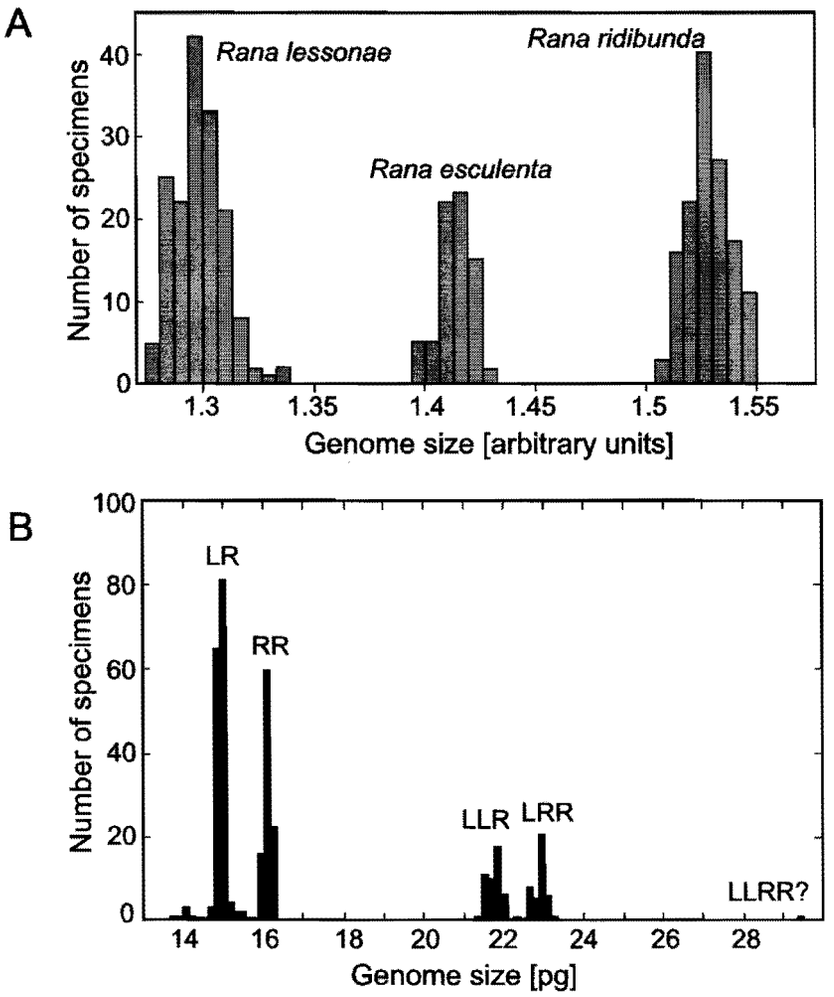


Fig. 27. A: Genome size in three forms of western Palaearctic water frogs expressed in arbitrary units in relation to the genome size of *Rana temporaria* (Rt-index) (modified from Borkin *et al.* 2002). B: Genome sizes of diploid (LR, RR) and triploid (LLR, LRR) water frogs. L.: *Rana lessonae* genome, R: *Rana ridibunda* genome, LR: *Rana esculenta*, RR: *Rana ridibunda*. Adapted from Borkin *et al.* (2004).

B. Molecular Phylogeny of Living Amphibians

1. The Phylogenetic Relationships between Gymnophiona, Urodela, and Anura

Based on morphological and molecular genetic evidence, the monophyly of Amphibia is commonly accepted, as is that of its three constituting orders Gymnophiona (caecilians), Urodela (salamanders), and Anura (frogs) (e.g., Duellman and Trueb 1986; Hedges and Maxson 1993; San Mauro *et al.* 2004; Zhang *et al.* 2005; Frost *et al.* 2006). The phylogenetic relationships among these three orders, however, have continuously been among the most intensively debated aspects of vertebrate phylogeny (reviewed by Duellman and Trueb 1986; Meyer and Zardoya 2003). Because each of the three major lineages acquired its distinctive body plan early in its evolutionary history, few synapomorphic characters have been identified, which renders a phylogenetic reconstruction on the basis of morphological data difficult. Even the application of molecular data faces serious difficulties in resolving the relationships among caecilians, salamanders, and frogs.

The first molecular-phylogenetic studies of interordinal relationships of Amphibia were based on ribosomal sequences of the mitochondrial (mt) genome, although nuclear (n)

DNA sequences were also soon used (Hedges *et al.* 1990; Larson 1991; Hedges and Maxson 1993; Feller and Hedges 1998). Most of these studies suggested that salamanders and caecilians are sister groups (Procera hypothesis, Fig. 28, right), although analyses of a combined dataset of 12S and 16S mtDNA sequences postulated instead a sister-group relation between frogs and salamanders (Batrachia hypothesis, Fig. 28, left). The latter was not well supported by bootstrap analyses (Hay *et al.* 1995). At that time, most molecular studies favoured the Procera hypothesis. Nevertheless this disagrees with most (e.g., Gardiner 1983; Milner 1983, 1988, 1993; Duellman and Trueb 1986; Trueb and Cloutier 1991), but not all (e.g., Carroll and Holmes 1980; Bolt 1991; Carroll *et al.* 1999) morphology-based studies.

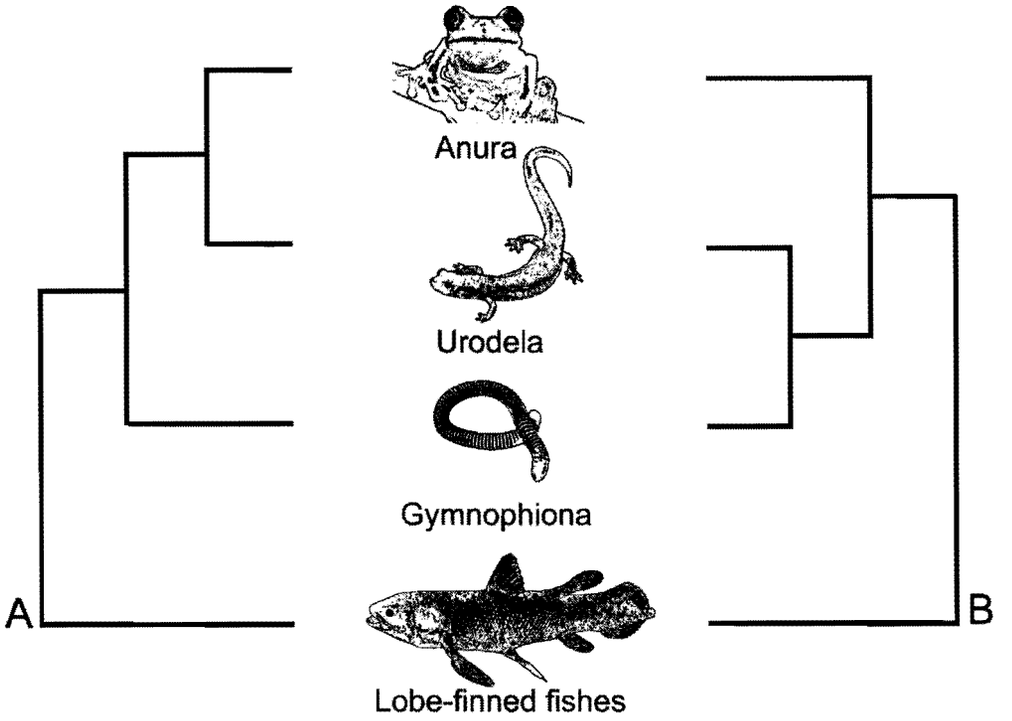


Fig. 28. Batrachia (A) or Procera (B) — the two alternative hypotheses on the phylogenetic relationships of extant amphibians.

Very recently, molecular phylogenies have been presented that are based on whole mt genomes or on protein-encoding nuclear genes, such as RAG1 and RAG2 (recombinase activating genes 1 and 2), or on a combination of both (Zardoya and Meyer 2001; San Mauro *et al.* 2004, 2005; Zhang *et al.* 2005). In contrast to the pre-2001 studies, these have provided evidence in favour of the Batrachia hypothesis.

The incongruence between molecular phylogenies of living amphibians can mainly be attributed to (1) the use of different genetic characters, (2) the different lengths of sequences analysed, and (3) the varying taxon sampling. Long-branch-attraction has been discussed as another source of incongruence (e.g., Zhang *et al.* 2005). This may explain why two different analyses of entire mt genomes provided conflicting results — once in favour of the Batrachia hypothesis (Meyer and Zardoya 2001) and once against it (Zhang *et al.* 2003a,b). In both cases, only one mt genome per major amphibian group was analysed, which may have caused long-branch-attraction in one or the other tree. Two more recent studies attempted to overcome this problem by including complete mt genomes from more than one taxon per clade. Both studies favoured the Batrachia hypothesis (San Mauro *et al.* 2005; Zhang *et al.* 2005). The trees shown in Figures 29 and 30 reveal Anura as a monophyletic sister group of the Urodela, with Gymnophiona as the most basal lineage of extant amphibians.

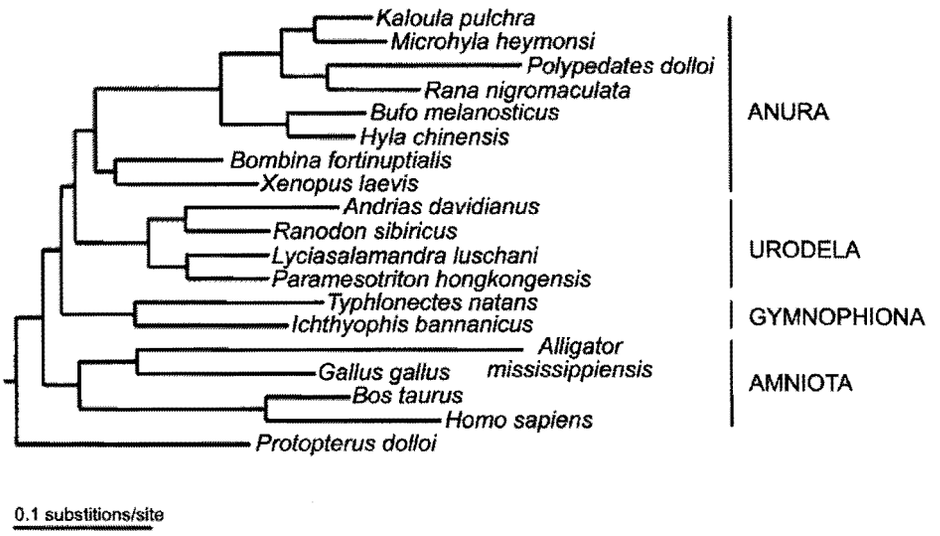


Fig. 29. Phylogenetic tree of living amphibians as revealed by Bayesian Inference of complete mt genomes. Adapted from Zhang *et al.* (2005).

In summary, the dataset supporting a sister-group relationship of frogs and salamanders is more extensive both in regard to lengths of sequences analysed and comprehensiveness of taxon sampling than any dataset supporting the Procera hypothesis. The Batrachia hypothesis is supported not only by evidence from molecular analyses but also by a majority of morphological and palaeontological studies. By present knowledge, therefore, the Batrachia hypothesis seems likely to reflect the real phylogenetic relationships among living amphibians.

2. The Early History and Diversification of Amphibians

A diversity of hypotheses has been proposed for the divergence times of extant amphibian lineages based on both morphological and molecular genetic data (e.g., Bolt 1969, 1977, 1991; Feller and Hedges 1998; Kumar and Hedges 1998; Reisz and Müller 2004; Blair and Hedges 2005; San Mauro *et al.* 2005; Zhang *et al.* 2005). According to Blair and Hedges (2005) the evolutionary history of extant amphibians dates back to Palaeocene (late Devonian to early Carboniferous, i.e., 386–398 My BP). Molecular studies by San Mauro *et al.* (2005) and Zhang *et al.* (2005) suggested that the split between Gymnophiona and Batrachia occurred about 337 to 367 My BP; Table 5). Estimates of the age of the extant amphibians based on analyses of molecular data thus exceed the age of the earliest known fossil amphibian by about 100 My (San Mauro *et al.* 2005).

Molecular studies also indicate that Gymnophiona, Urodela, and Anura originated soon after the stem group of Amphibia arose, and rapidly radiated about 360 My ago (San Mauro *et al.* 2005; for dating of this event see also Benton 1990; Milner 1993; Carroll *et al.* 2004). This conclusion is mainly drawn from the comparatively short branches that connect the basal nodes in the phylogenetic tree of amphibians. Given there was such a rapid radiation in the ancestral lines of amphibians, this would explain the lack of fossils that represent plausible ancestors or morphologically intermediate sister taxa of all three extant amphibian orders and would also account for the difficulties in unambiguously resolving basal branching among caecilians, salamanders, and frogs (San Mauro *et al.* 2005). Furthermore, the studies based on protein-encoding nuclear genes or entire mt genomes suggest a much more ancient origin of the extant amphibians than was proposed by Feller and Hedges (1998) based on ribosomal sequences (but without application of a molecular clock). The latter authors hypothesized an early Mesozoic origin of living amphibians and assumed that the separation of the three extant orders was directly linked to the break-up of

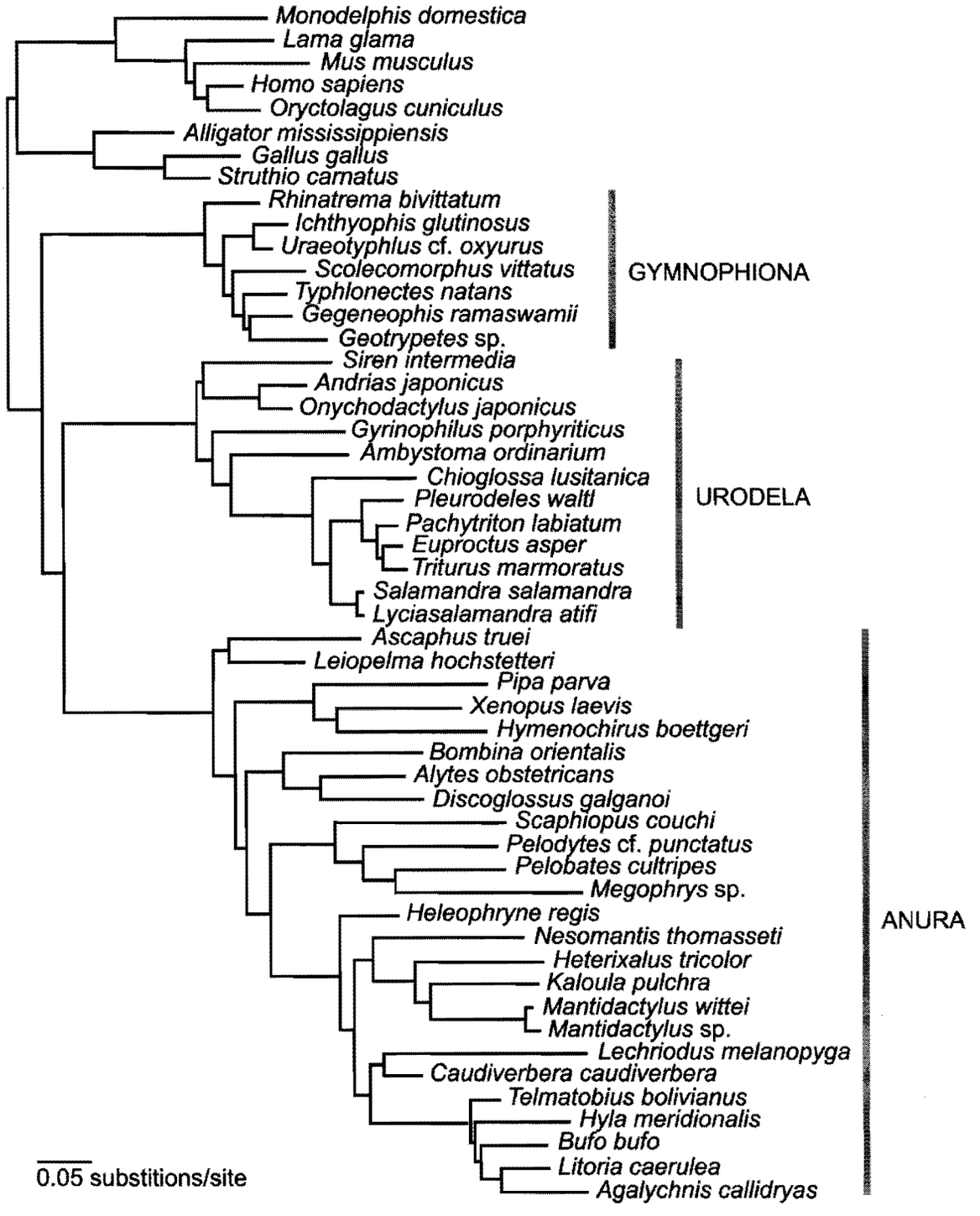


Fig. 30. Unconstrained, non-ultrametric maximum-likelihood phylogram of living amphibians as presented by San Mauro *et al.* (2005).

Pangaea, with salamanders being of Laurasian origin and caecilians essentially of Gondwanan origin. Current age estimates as presented by San Mauro *et al.* (2005) and Zhang *et al.* (2005) disprove these assumptions and rather suggest that the origin of extant amphibian orders predates the fragmentation of Pangaea (Fig. 31). If a Palaeozoic origin of amphibians is assumed and the assumption that the Pangaean break-up led to the formation of the three major amphibian lineages is rejected, the presence of the putative

Table 5. Age estimates of the origin of extant amphibians and of its basal nodes, with 95% confidence intervals, obtained by a) Bayesian analysis of amino acid sequences of 191 proteins (Blair and Hedges 2005), b) relaxed Bayesian clock analysis for 1 368 positions of the nuclear RAG1 gene (San Mauro *et al.* 2005) and c) relaxed Bayesian clock analysis for entire mt genomes (Zhang *et al.* 2005).

Event	Years Before Present [My]			Reference
	Lower limit	Mean	Upper limit	
Split between amniotes and amphibians	398	392	386	a
Split Gymnophiona-Batrachia	328	367	417	b
	321	337	353	c
Split Urodela-Anura	317	357	405	b
	289	308	328	c
Initial split within the Gymnophiona	177	214	256	b
	224	250	274	c
Initial split within the Urodela	238	273	312	b
	176	197	219	c
Initial split within the Anura	223	262	305	b

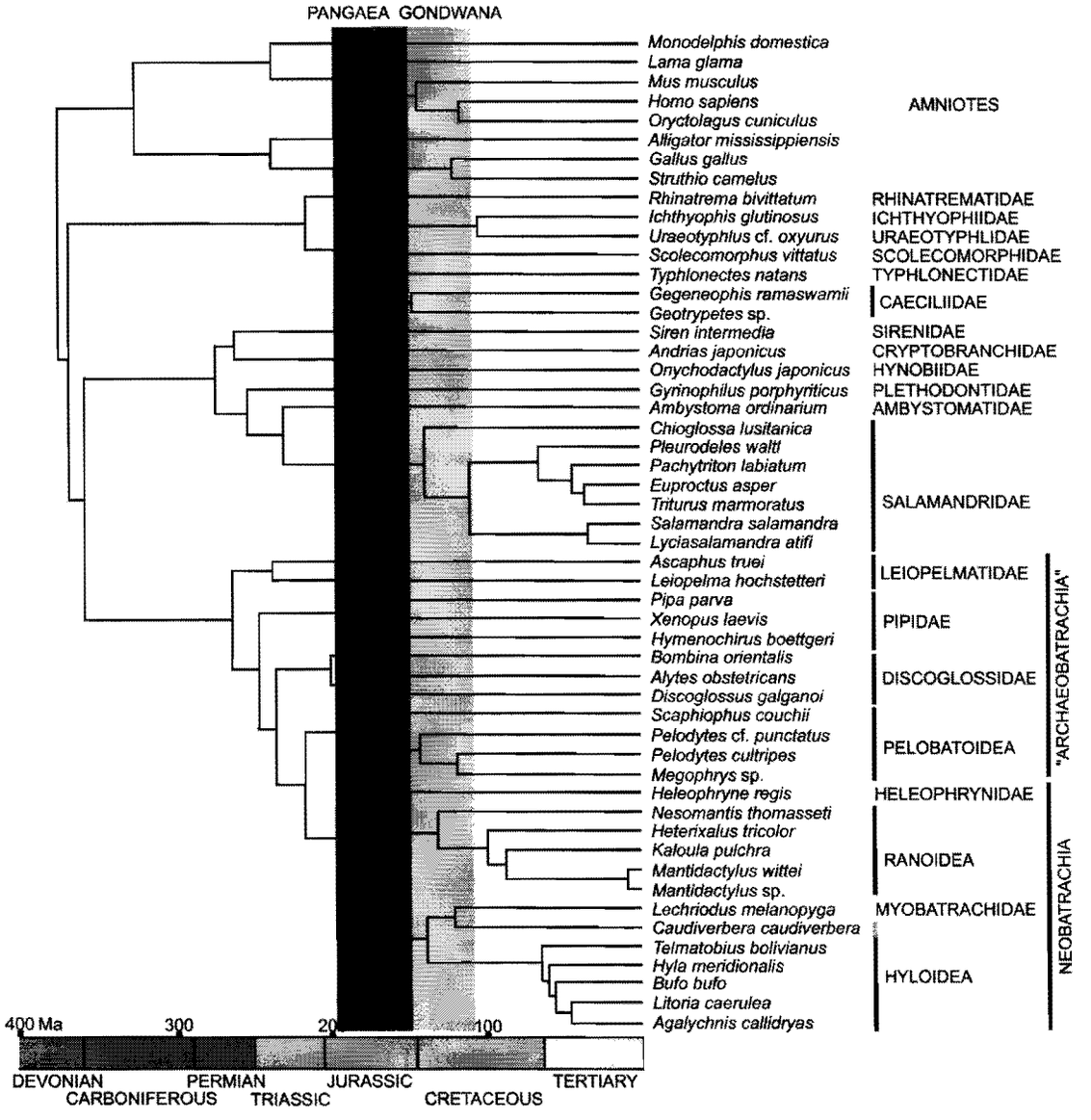


Fig. 31. Average age estimates of the main amphibian lineages: Maximum-likelihood phylogeny based on 1 368 nucleotide bases of the RAG1 gene. Multiple calibration points were used to employ a Bayesian relaxed molecular clock model (simplified from San Mauro *et al.* [2005]; confidence intervals are not shown).

stem-group caecilian *Eocaecilia* in the early Jurassic of Northern America (Jenkins and Walsh 1993) is no longer bewildering. In contrast, to reconcile this fossil record with the scenario suggested by Feller and Hedges (1998) is not easy.

Choosing between an early Mesozoic and a late Palaeozoic origin to explain the evolutionary history of amphibians, including the relevance of tectonic events during the break-up of Pangaea, essentially depends on the trustworthiness of the molecular clock estimates suggested by a variety of investigations. In general, the use of molecular clocks has been controversial because of the frequent discrepancies between molecularly estimated dates and those based on the fossil record. It has been found that molecular clocks often exaggerate the age of lineages, whereas paleontological dates necessarily give only a minimum age and thus lead to underestimations of a lineage's age (e.g., Benton and Ayala 2003). There are many publications available on the pros and cons of molecular clocks, and the entire discussion cannot be recapitulated here. Methods and theories of molecular-clock estimation have themselves evolved (Arbogast *et al.* 2002; Kumar 2005). Authors who employ molecular clocks claim that considerable progress has been made in recent years, especially because Bayesian analytical techniques allow relaxation of molecular-clock assumptions by incorporating multiple independent calibration points within a single analysis (Renner 2005; San Mauro *et al.* 2005; Yang and Rannala 2006). Nevertheless, accuracy, number, and preferably even distribution of the calibration points used to date the internal nodes of a given phylogeny remain essential for reliable relaxed-clock estimates. All more recent attempts to calibrate the tree of vertebrate life (e.g., San Mauro *et al.* 2005) fit it into the frame of various calibration points, including such earlier events as the coelacanth-tetrapod split (429 My BP; Zhu *et al.* 2001) or the divergence of diapsids and synapsids (338–228 My BP; Graur and Martin 2004), and more recent events such as the last contact between Australia and South America (86 My BP; Pitman *et al.* 1993). Thus, it is no longer necessary to assume constant evolutionary rates across all clades of a given phylogeny, which helps avoid unrealistic estimates of ages. In Figure 32 a schematic comparison of two hypotheses of the phylogenetic history of extant amphibians is shown based on a figure published by San Mauro *et al.* (2005).

Resolving the phylogenetics of amphibians is a pre-requisite for understanding their evolution. In the mid-1980s molecular studies were concerned mainly with smaller datasets, but in the past decade the accumulation of molecular data has been accelerating. To date,

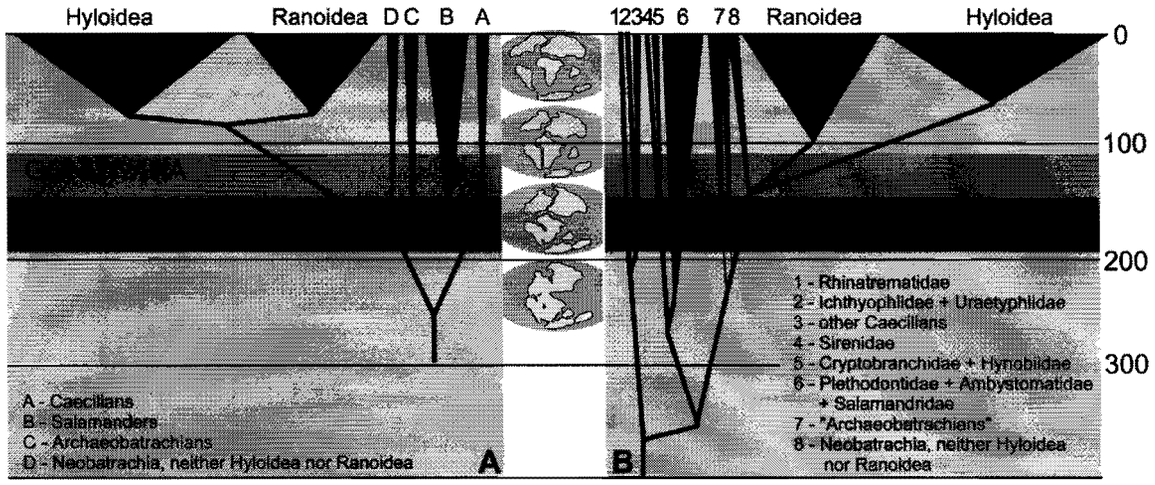


Fig. 32. Schematic comparison of two hypotheses about the evolutionary history of amphibians summarizing the views of (A) Feller and Hedges (1998), assuming that the diversification of amphibian lineages is connected to the break-up of Pangaea, and (B) of San Mauro *et al.* (2005), who suggested that it pre-dates this palaeotectonic event. The cross sections of the cones indicate relative species diversity within each group. Shaded bands show the approximate stages of the break-up of Pangaea and Gondwana, respectively. Drawing adapted from San Mauro *et al.* (2005).

the most complete molecular study in regard to amphibians was presented by Frost *et al.* (2006). It covers 522 species representing all amphibian families with the exception of only one. A simplified version of their comprehensive tree is depicted in Figure 33.

There follows a cursory review of the impact of molecular studies on amphbian systematics and on current understanding of the phylogenetic relationships of the major amphibian lineages.

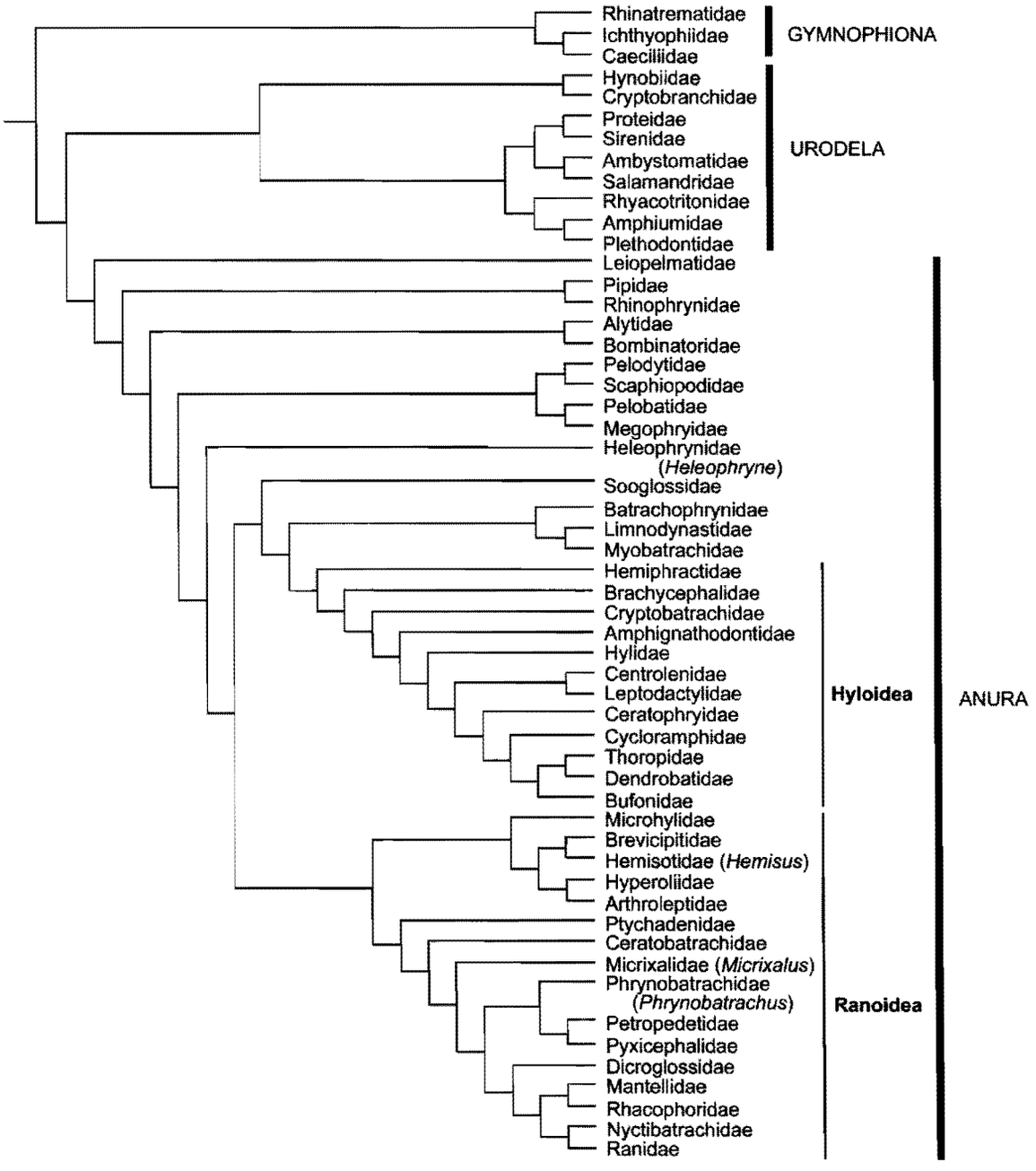


Fig. 33. Simplified phylogenetic tree of the living amphibians resolved only to the family-level as presented by Frost *et al.* (2006). Consensus of four equally parsimonious cladograms based on analysis of a concatenated data set of the mitochondrial transcription unit H1 (12S and 16S ribosomal RNA and $tRNA^{Val}$ genes, about 2 400 bp) and the nuclear genes histone H3, rhodopsin, tyrosinase, and seven in absentia, and the large ribosomal subunit 28S (about 2 300 bp).

3. Molecular Phylogeny of the Gymnophiona

Currently about 170 species of caecilians are recognized (Frost 2004; Frost *et al.* 2006), most of them dwelling in the soils of the wet tropics. Based on morphological characters, usually six caecilian families (Table 6) are distinguished (Duellman and Trueb 1986; Nussbaum and Wilkinson 1989; Wilkinson and Nussbaum 1999). Because of their secretive life style, Gymnophiona certainly is one of the least-known groups of vertebrates in regard to their reproductive biology, behaviour, and ecology (Carroll 1988; Nussbaum and Wilkinson 1989). The group is characterized by a very distinct morphology, including among other features an elongate, annulated, and limbless body and sensory tentacles. In fact, the monophyly of this group is based on so many morphological characters (e.g., Nussbaum and Wilkinson 1989) that it never has been seriously questioned. In concordance with the morphology, molecular phylogenetic studies generally corroborate the monophyly of Gymnophiona. Nevertheless, many areas of caecilian systematics and evolution remain insufficiently understood. Phylogenetic analyses of morphological data are dogged by the scarcity of obvious external characters. In addition, these may be particularly prone to homoplasy because of their adaptive nature in the context of a burrowing life style. Furthermore, the limited availability of material hampers an investigation of infraspecific morphological and genetic variation. In recent years, however, substantial progress in reconstructing gymnophionan relationships has been achieved by the application of molecular methods.

Table 6. Classification of the recent Gymnophiona (adapted from Pough *et al.* 2003; Frost 2004).

Family	Distribution	Numbers of Genera	Numbers of Species
Rhinatrematidae	South America	2	9
Typhlonectidae	South America	5	14
Uraeotyphlidae	India	1	5
Scolecomorphidae	Africa	2	6
Ichthyophiidae	South and Southeast Asia	2	39
Caeciliidae	Cosmopolitan	22	93

The earliest molecular genetic study used what nowadays seems a ludicrously short fragment of the mt 12S ribosomal RNA gene (Hedges and Maxson 1990). It did not result in a satisfactory hypothesis of the phylogenetic relationships of the gymnophionan families because of incomplete taxon sampling and of the presumably high degree of homoplasy in the dataset. Subsequent work was based on longer sequences of 12S and 16S mtDNA, but still suffered to a varying degree from incomplete taxon sampling in respect to higher-ranked groups; Hedges *et al.* (1993) analysed representatives from only four of six families and seven of 21 genera of Caeciliidae, and Wilkinson *et al.* (2002) added sequences of three further species, now covering at least five families (Fig. 34).

Because they used the same molecular markers, it is not surprising that the studies of Hedges *et al.* (1993) and Wilkinson *et al.* (2002) delivered similar results. The basal status of Rhinatrematidae was corroborated and Ichthyophiidae and Uraeotyphlidae were shown as a sister group to the next, more derived clade, which corresponds to the results of morphological analyses by Wilkinson (1997) and Wilkinson and Nussbaum (1997). The trees based on ribosomal sequences of the mt genome, however, did not unambiguously support the morphologically based assumptions of the relationships of Scolecomorphidae, Caeciliidae, and Typhlonectidae. The Caeciliidae, for which the largest number of representatives was analysed, are revealed as a paraphyletic group with respect to *Typhlonectes natans*, the only included species of Typhlonectidae. To complicate matters, subsequent studies that incorporated mt ribosomal sequences of additional taxa from South Asia (i.e., Ichthyophiidae; Gower *et al.* 2002) and from Africa (i.e., Scolecomorphidae; Wilkinson *et al.* 2003), presented phylogenies that did not unambiguously support the monophyly of those caecilian families that were represented by a larger number of species.

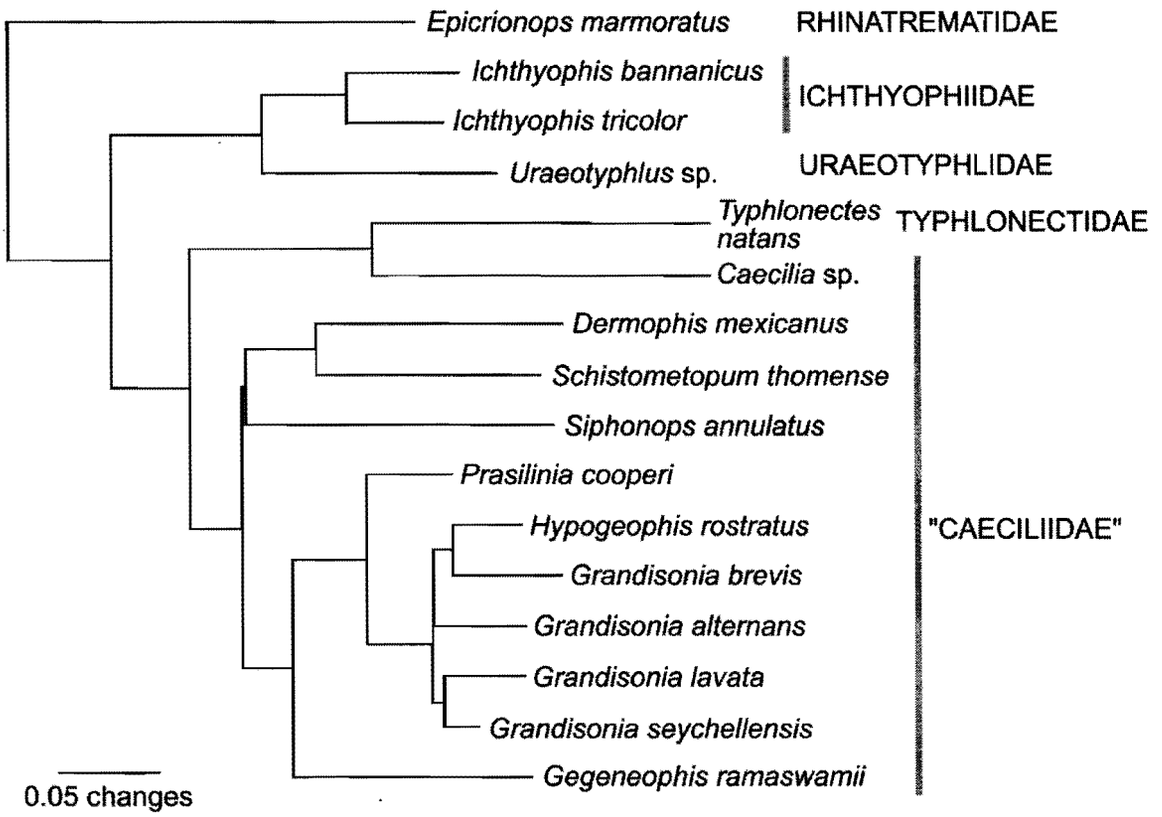


Fig. 34. Phylogenetic hypothesis about relationships among main lineages of Gymnophiona based on analyses of sequences of the 12S and 16S mtDNA. From Wilkinson *et al.* (2002).

According to Gower *et al.* (2002) and Frost *et al.* (2006), Ichthyophiidae are not monophyletic with respect to Uraeotyphlidae. Wilkinson *et al.* (2003) were not able to unequivocally resolve the relationships of scolecomorphids, which are shown to cluster with the caecilians (Frost *et al.* 2006).

A recent phylogenetic study used complete mt genomes and sequences of the nuclear protein-encoding gene RAG1 of a single species from each of the six recognized caecilian families (San Mauro *et al.* 2004). The topology of the tree (Fig. 35) obtained for a concatenated dataset of RAG1 nucleotide sequences as well as amino-acid sequences of all mt protein-coding genes corresponds with a topology calculated only with amino-acid sequences of mt genes and is also concordant with the phylogenetic reconstructions based on morphological data (Wilkinson and Nussbaum 1997; Wilkinson 1997). This makes the tree a plausible hypothesis of the phylogenetic relationships among the main caecilian lineages. In order to retain only monophyletic clades, Frost *et al.* (2006) suggested that (1) Uraeotyphlidae be subsumed under Ichthyophiidae and that (2) Thyplonectidae and Scolecomorphidae be subsumed under Caeciliidae, thereby reducing the number of recognized caecilian families to three.

In summary, during the past two decades molecular data have promoted remarkable progress in an understanding of the systematics of the Gymnophiona by recognizing cryptic species but most importantly by providing robust and testable hypotheses not only on the relationships among the principal lineages but also in regard to their phylogenetic age, origin, and processes of evolutionary diversification. The goal of generating a trustworthy phylogenetic system for the Gymnophiona has, however, not yet been achieved. The phylogenetic status of the Caeciliidae, the most diverse but also most poorly known group of caecilians, for example, remains to be clarified. In molecular phylogenies, this group is frequently shown as paraphyletic or even polyphyletic. Subsuming Typhlonectidae and

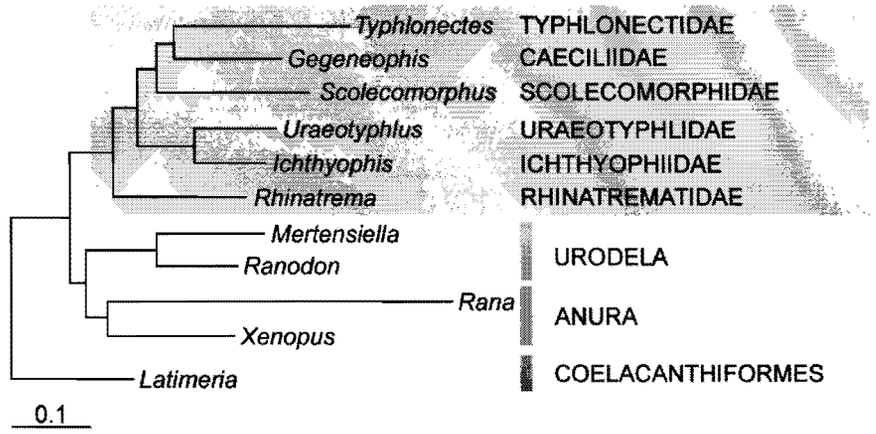


Fig. 35. Phylogenetic relationships among the six currently recognized families within the Gymnophiona inferred from a combined dataset of deduced amino acid sequences of all mt protein-coding genes and nucleotide sequences of the nuclear RAG1 gene (San Mauro *et al.* 2004). Among all molecular trees presented in the past few years, this tree is based on the most comprehensive molecular dataset regarding the lengths of the analysed sequences.

Scolecomorphidae under Caeciliidae (Frost *et al.* 2006) avoids the polyphyly of the Caeciliidae as traditionally encompassed. This proposal, however, neglects the morphologically well-corroborated and derived status of the two synomymized taxa. The molecular phylogenetic studies indicate that the current systematic treatment, as inferred from morphology, may not properly reflect the true relationships within the Caeciliidae. This probably means that some of the morphological features used to define the taxon, such as the presence of vestigial eyes, reflect concerted homoplasy caused by adaptation to a burrowing life style.

Furthermore, currently available phylogenies suffer from incomplete taxon sampling (e.g., San Mauro *et al.* 2004) or homoplasy problems (Hedges and Maxson 1993; Hedges *et al.* 1993; Gower *et al.* 2002; Wilkinson *et al.* 2002, 2003). Future studies aiming to resolve relationships among caecilians should therefore focus on slowly evolving nuclear genes and try to broaden the basis of sampled taxa, particularly in regard to the under-represented caeciliids.

Like the relationships among recent caecilians, their evolutionary origin and the patterns of their early diversification have been unclear because the fossil record is scant (Evans and Sigogneau-Russell 2001). The almost exclusive occurrence of caecilians in the Southern Hemisphere has commonly been interpreted as an indication that the diversification of the recent lineages has essentially been caused by the break-up of Gondwanaland (e.g., Gower *et al.* 2002). While this may hold true for some more derived clades, such as Southeast Asian Ichthyophiidae, which were shown to originate from India (Gower *et al.* 2002), the assumption that all recent caecilians are essentially of Gondwanan origin is in conflict with the newest age estimates based on molecular data, which place the origin of caecilians back to late Devonian or early Carboniferous times, thus predating both the break-up of Pangaea (San Mauro *et al.* 2005; Zhang *et al.* 2005) and the estimated time of origin based on the fossil record (Evans and Sigogneau-Russell 2001). At present, the most likely scenario is that the basal lineages, i.e., rhinotrematids, ichthyophiids, and uraeotyphlids, are relicts of groups that had been widespread in those parts of Pangaea that gave rise to Gondwana, whereas more derived clades, such as scolecomorphids and typhlonectids, may have evolved subsequent to large tectonic events and thus never achieved a wider distribution (Duellman and Trueb 1994; San Mauro *et al.* 2004). Because caecilians already inhabited areas in Pangaea before this landmass was fragmented, a simple vicariance scenario invoking the separation, drift, and later amalgamation of terranes in

the course of Gondwanan fragmentation may fail to correctly explain the causes and processes leading to the current distributional patterns of gymnophionans. Estimates of divergence times of the recent gymnophionan lineages are given in Table 5.

4. Molecular Phylogeny of the Urodela

Urodela (more informally, salamanders) is a group of typically four-limbed amphibians with a long tail. Its monophyly is evidenced by a number of morphological autapomorphies (e.g., Duellman and Trueb 1986, 1994; Pough *et al.* 2003) and molecular characters. The current distribution patterns indicate that recent urodelan lineages arose mainly from the Laurasian part of Pangaea (Duellman and Trueb 1994; San Mauro *et al.* 2004). An exclusively Laurasian origin, however, seems questionable for various reasons. First, Mesozoic fossils from South America (*Noterpeton*) and Africa (*Kababisha*) provide evidence that salamanders did not exclusively inhabit Laurasian areas (Evans *et al.* 1996). Second, latest-age estimates based on sequence data suggest that the initial splitting within modern salamanders occurred during the late Palaeozoic, i.e., between 312 and 238 My ago (Table 5). This considerable period of time should have provided opportunities for earlier range expansions into the Gondwanan parts of Pangaea long before this landmass was fragmented (San Mauro *et al.* 2005). The Palaeozoic ancestry of salamanders is also evidenced by fossil data. Schoch and Carroll (2003) showed that fossil larvae of putative ancestors of salamanders from Permo-Carboniferous sediments of Germany exhibit ontogenetic features that are still found in primitive living salamanders. These features thus provide evidence in favour of a Palaeozoic origin of the Urodela and considerably reduce the persisting gap between the first appearance of salamanders in the fossil record (Jurassic) and the age estimates based on molecular genetic data (Devonian; Table 5).

Current classification schemes usually distinguish ten major lineages of extant salamanders that usually are ranked as families (Table 7).

The systematics of Urodela has emerged as very difficult. Attempts to infer the phylogenetic relationships among the major lineages by analysing morphological and life-history data (e.g., Regal 1966; Wake 1966; Edwards 1976; Duellman and Trueb 1986, 1994; Sever 1991a,b, 1992, 1994), also in combination with relatively restricted molecular-sequence data of mt ribosomal genes (Larson 1991; Larson and Dimmick 1993), resulted in a plethora of possible trees that rendered most aspects of urodelan phylogeny unresolved. There are only two monophyletic groups that are concordantly revealed by morphologically based analyses: (1) Cryptobranchidae and Hynobiidae and (2) a group consisting of Ambystomatidae, Salamandridae, and Plethodontidae. Faced with the high number of conflicting hypotheses, it has been concluded that paedomorphosis produces a huge

Table 7. Classification of the recent Urodela (according to Pough *et al.* 2003; Frost 2004; Min *et al.* 2005).

Taxon	Distribution	Number of Genera	Number of Species
Sirenidae	Southern North America	2	4
Cryptobranchidae	Eastern Asia, North America	2	3
Hynobiidae	Central to Eastern and Southern Asia	7	45
Amphiumidae	South-eastern North America	1	3
Plethodontidae, Desmognathinae	Eastern North America	2	21
Plethodontidae, Plethodontinae	Western North America to Brazil, Southern Europe, Korean peninsula	26	297
Rhyacotritonidae	North-western North America	1	4
Proteidae	Southern Europe, eastern North America	2	6
Salamandridae	Cosmopolitan	15	62
Ambystomatidae	North America	1	30
Dicamptodontidae	Western North America	1	4

number of convergent traits in respect to both morphology and life history (Wake 1991; Duellman and Trueb 1994; Wiens *et al.* 2005). The pervasiveness of this phenomenon in salamanders has made it almost impossible to achieve a consensus on their phylogeny, based only on morphological characters (Pough *et al.* 2003). To give an example: Sirenidae show a varying number of character reversals and character convergences depending on which tree is considered to reflect its relationships (Duellman and Trueb 1986). This holds true also for other salamander groups. A consensus hypothesis, based on the analysis of morphological and life-history data, is presented in Figure 38 (Wiens *et al.* 2005).

The inability to generate robust phylogenies by use of exclusively morphological characters underscores the potential and importance of molecular studies to gain insights into urodelan phylogeny and evolution. Because of the considerable age of the whole group, however, earlier molecular studies failed to overcome the problem of homoplasy, which has also been one of the major obstacles in morphologically based analyses. From a current perspective, the relatively short fragments of mt genes that were analysed in initial studies were not adequate to address the deep splits between the major salamander lineages and thus increased the number of suggested trees but did not necessarily improve the understanding of phylogenetic relationships (e.g., Larson 1991). In an attempt to reconcile morphology with molecular genetics, Larson and Dimmick (1993) performed phylogenetic analyses of a combined dataset of 177 phylogenetically informative characters of ribosomal sequences and 32 anatomical characters. Phylogenetic analyses (Maximum Parsimony) of the entire dataset resulted in 40 equally parsimonious trees. The strict consensus tree provided evidence for the monophyly of each family (one to four species per family were investigated) and revealed the Sirenidae to be the most basal group, being the sister group of all other extant salamanders. In addition, two major groups were recognized, referred to as Cryptobranchoidea (Cryptobranchidae + Hynobiidae) and Salamandroidea (all residual families). A 50%-majority-rule consensus tree generated after elimination of three morphological characters was considered to represent the most plausible phylogenetic hypothesis for Urodela at that time (Fig. 36).

Recent studies used either entire mt genomes (e.g., Chippindale *et al.* 2004; Mueller *et al.* 2004) or more conserved nuclear protein-encoding genes (San Mauro *et al.* 2005; Wiens *et al.* 2005; Frost *et al.* 2006) to address the phylogenetic relationships within the Urodela. These analyses revealed fascinating new insights into the systematics and evolution of salamanders. For example, Wiens *et al.* (2005) provided evidence for morphological convergences related to the retention of larval (paedomorphic) characters in some lineages. Whereas in trees based purely on morphological data, taxa possessing paedomorphic

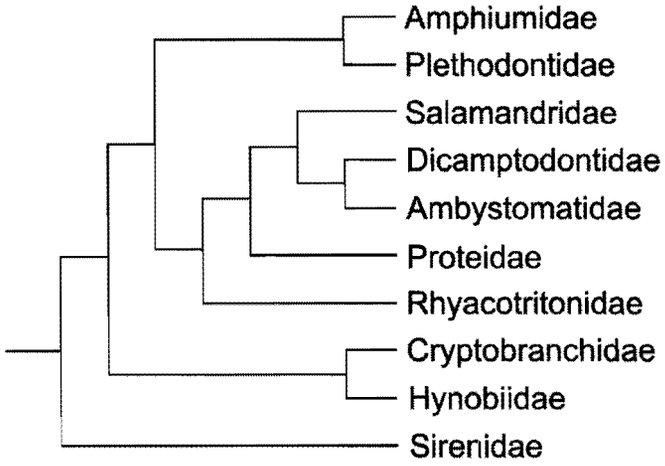


Fig. 36. Hypothesis on the phylogenetic relationships among salamanders based on the combined analyses of mt ribosomal DNA sequences and morphological characters (Larson and Dimmick 1993; Pough *et al.* 2003).

characters always clustered together (Fig. 37), these taxa were more evenly distributed across the tree topology when only molecular data or combined molecular and morphological data (without paedomorphic characters) were analysed (Fig. 38). Wiens *et al.* (2005) provided robust evidence in favour of the monophyly of all recognized salamander families, including the Proteidae, the monophyly of which has been disputed. It was impressively shown, however, that analyses based on deviant datasets produce strikingly different topologies. Among the various reconstructions presented, the trees based only on molecular data and the Bayesian tree based on a combined set of molecular and morphological data with paedomorphic characters coded as unknown for adult morphology showed the widest congruence. Accordingly, Rhyacotritonidae + (Amphiumidae + Plethodontidae) are suggested to form the most derived clade. Proteidae and Salamandridae + (Dicamptodontidae + Ambystomatidae) form the next more basal clades to which in turn (Hynobiidae + Cryptobranchidae) and Sirenidae represent the most basal clades (Fig. 38). These hypotheses deviated considerably from those presented by San Mauro *et al.* (2005), who suggested that Sirenidae do not

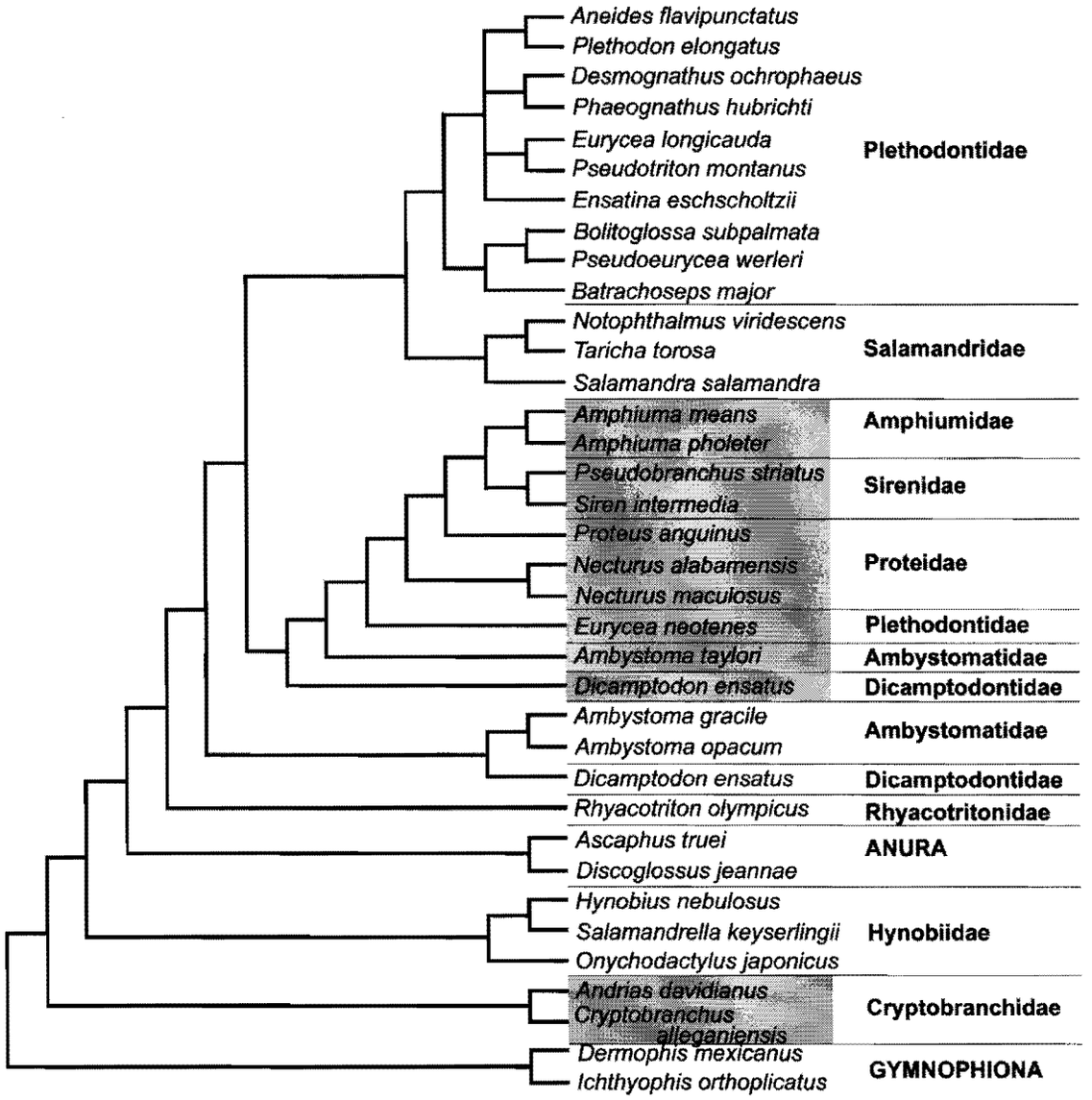


Fig. 37. Strict consensus tree of four equally parsimonious trees representing the phylogenetic relationships among extant Urodela based on parsimony analysis of adult morphological characters. Taxa with paedomorphic features are shaded. After Wiens *et al.* (2005).

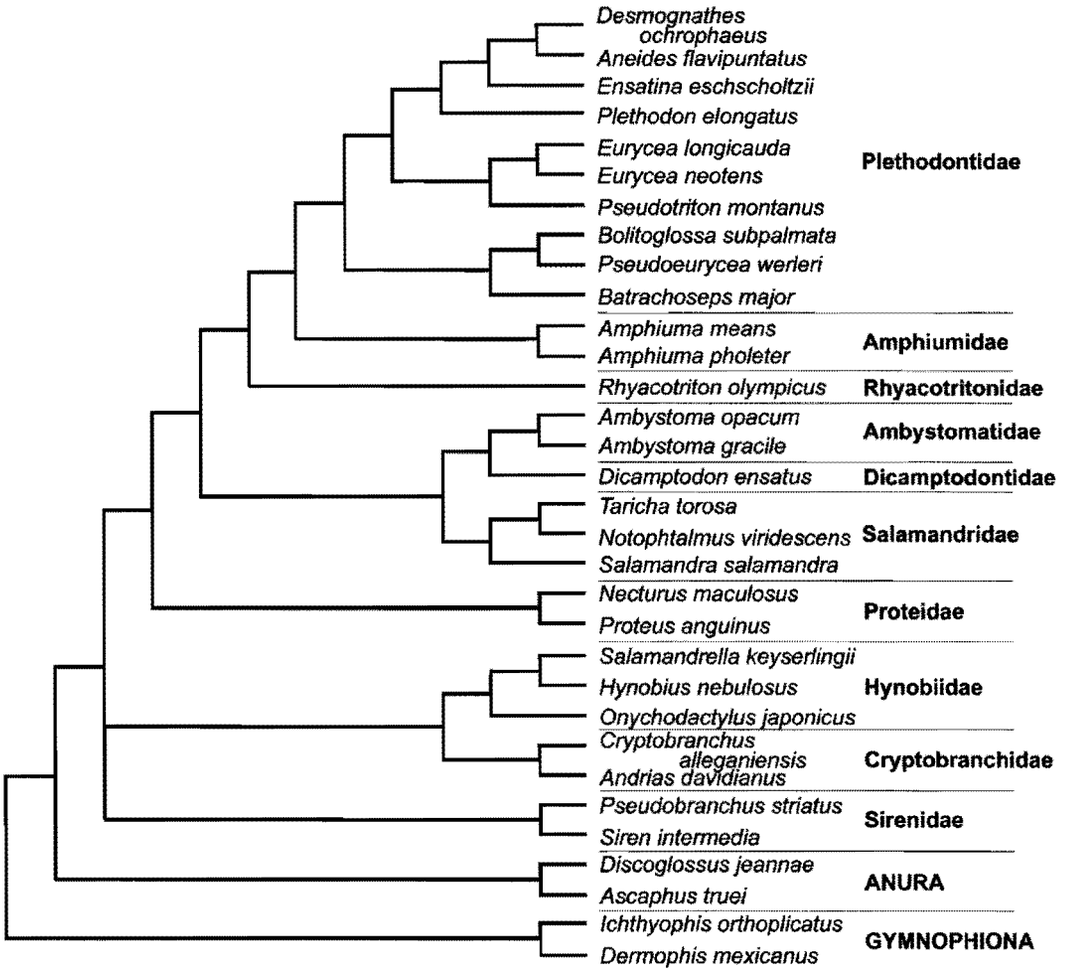


Fig. 38. Phylogenetic hypothesis based on a Bayesian analysis of a combined dataset of 1 530 nucleotide bases of the nuclear protein-encoding gene RAG1, 212 characters of ribosomal sequences, and 326 morphological characters. Paedomorphic characters were coded as unknown for adult morphology. Adapted from Wiens *et al.* (2005).

represent the most basal group of living salamanders. Instead, a basal bifurcation into two principal clades was shown, one comprising Sirenidae + (Cryptobranchidae + Hynobiidae), the other Plethodontidae + (Ambystomatidae + Salamandridae) (Fig. 30). A similar tree was suggested by Frost *et al.* (2006) except for the position of the Sirenidae, which are shown as the sister-group of the Proteidae (Fig. 33).

The current state of knowledge of the molecular systematics of the recognized families of salamanders is briefly presented below; the families are listed in alphabetical order.

Ambystomatidae: Phylogenetic relationships among ambystomatid species were inferred by means of morphological characters (Kraus 1988) as well as 26 allozyme loci (Shaffer *et al.* 1991). When analysed separately, the morphological and molecular data support different phylogenies. Shaffer *et al.* (1991) presented a useful but rather tentative working hypothesis based on a combined analysis of morphological and allozyme data. As shown in Figure 39 the relationships of the Mexican species related to *Ambystoma tigrinum* could not be resolved on the basis of morphological data, allozymes, or mtDNA sequences (Shaffer and McKnight 1996) and therefore are drawn as an unresolved star topology in the tree. Two species, *A. platineum* and *A. tremblayi*, are chromosomal triploids originating from ancient hybridization events between *A. jeffersonianum* and *A. laterale* (Hedges *et al.* 1992; Spolsky *et al.* 1992); consequently, they cannot be displayed in a bifurcating phylogenetic tree. Recently, Samuels *et al.* (2005) showed that the structures of five complete ambystomatid mt genomes are

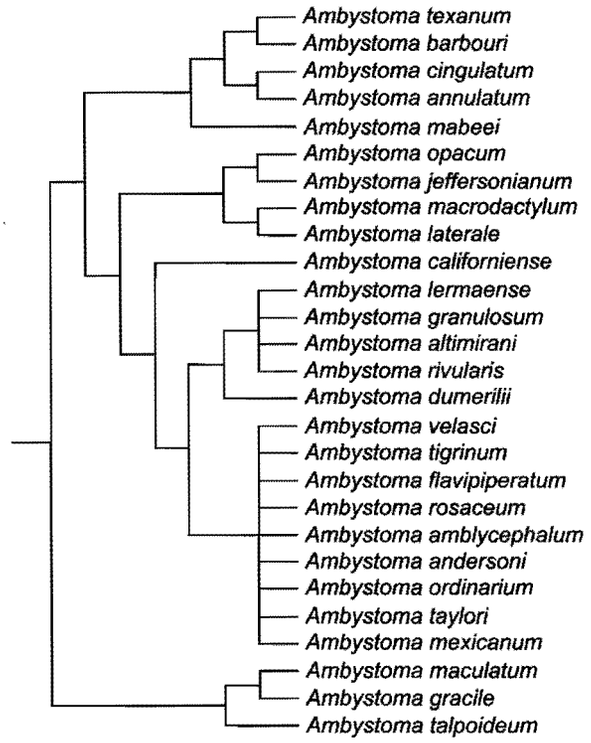


Fig. 39. Hypothesis of phylogenetic relationships among extant ambystomatid salamanders combining the results of Shaffer *et al.* (1991) and Shaffer and McKnight (1996). Adapted from Larson (1996).

very similar and suggested that Ambystomatidae are more closely related to Cryptobranchidae and Hynobiidae than they are to Salamandridae, which stands in conflict with the results of other phylogenetic studies. Because Samuels *et al.* (2005) analysed only a limited number of non-ambystomatid mt genomes, their hypothesis on the phylogenetic position of Ambystomatidae may be distorted by long-branch attraction.

Amphiumidae: Amphiumidae encompass only three known species and clearly represents a monophyletic group. With a high probability it is the sister taxon of Plethodontidae (Larson 1991; Larson and Dimmick 1993; Wiens *et al.* 2005; Frost *et al.* 2006). Using allozymes, Karlin and Means (1994) showed that *Amphiuma means* and *A. tridactylum* are sister taxa; their clade is the sister taxon of *A. pholeter*.

Cryptobranchidae: Only three species are known, *Andrias japonicus* (Japanese giant salamander), *A. davidianus* (Chinese giant salamander), and the North American *Cryptobranchus alleganiensis* (Hellbender). Cryptobranchidae is regarded as monophyletic; the relationships among species are consistent with biogeographic findings. Cryptobranchidae are closely related to the Asian family Hynobiidae as concordantly revealed by various phylogenetic analyses (e.g., Larson and Dimmick 1993; Mueller *et al.* 2004; San Mauro *et al.* 2005; Wiens *et al.* 2005; Frost *et al.* 2006). Wiens *et al.* (2005) and Frost *et al.* (2006) showed that these two taxa are sister groups, and that their clade is the adelphotaxon to all other extant salamanders.

Dicamptodontidae: Relationships among the four extant dicamptodontid species were inferred by Good (1989) using allozymes. The species occurring in Idaho (*Dicamptodon aterrimus*) is more distantly related to a group including the three residual species that are distributed in the Pacific coastal region of North America (*D. copei*, *D. ensatus* and *D. tenebrosus*). The exact placement of the three coastal species relative to each other remained tentative. After analysing mt sequences, Steele *et al.* (2005) suggested that *D. aterrimus* indeed represents the most basal group within the phylogenetic tree of the genus followed by *D. copei* + (*D. ensatus* + *D. tenebrosus*). They concluded that speciation within *Dicamptodon* is attributable to ancient geologic events, while more recent Pleistocene glaciation has shaped the genetic variation and the distributional areas of the extant species. In their

molecular phylogeny of living amphibians, Frost *et al.* (2006) hypothesized *Dicamptodon* and *Ambystoma* to be sister taxa and proposed that *Dicamptodon* be subsumed under Ambystomatidae.

Hynobiidae: To date, Hynobiidae have not been subject to a comprehensive molecular phylogenetic study. Members of the family were, however, included in various studies addressing the general phylogenetic relationships within the Urodela. These studies consistently provided evidence for the monophyly of this group and a sister-group relationship with Cryptobranchidae (e.g., Larson and Dimmick 2003; Mueller *et al.* 2004; San Mauro *et al.* 2005; Wiens *et al.* 2005; Frost *et al.* 2006).

Plethodontidae: In recent years attention has been paid particularly to the Plethodontidae, the most diverse group of living salamanders. Based on morphology, two subfamilies are recognized, the Desmognathinae and Plethodontinae, the latter comprising Hemidactyliini, Bolitoglossini, and Plethodontini. After extreme homoplasy was detected not only in morphological characters (Parra-Olea and Wake 2001; Mueller *et al.* 2004) but also in regard to reversals in life-history traits (Chippindale *et al.* 2004), doubts were raised whether the morphologically based classification accurately reflects the natural relationships among plethodontid salamanders. On a smaller systematic scale (i.e., on the generic level), molecular studies have widely supported previous classification schemes based on morphology (i.e., Jackman *et al.* 1997) and led to the recognition of additional, previously unidentified lineages (e.g., Mahoney [2001] in *Plethodon* and Parra-Olea *et al.* [2004] in *Bolitoglossa*). On a larger systematic scale, however (i.e., on the familial level), molecular phylogenies stand in conflict with those based on morphological characters. In fact, the analysis of entire mt genomes of 29 plethodontid species by Chippindale *et al.* (2004) provided evidence in favour of the monophyly of the Plethodontidae as a whole but also suggested that major groups recognized by their morphology are not monophyletic. Accordingly, the monophyly of the Plethodontinae, Hemidactyliini, and Plethodontini as previously delineated was rejected. The mitochondrial phylogeny suggested instead the existence of four major clades with (1) Desmognathinae as previously encompassed, (2) its sister group composed of a number of taxa previously affiliated with the Hemidactyliini but without *Hemidactylium* itself, (3) a clade composed of *Aneides* and *Plethodon*, which might be referred to as Plethodontini *sensu stricto* and (4) a clade comprising *Ensatina*, *Hemidactylium* and the Bolitoglossini. A widely congruent phylogeny has been presented by Mueller *et al.* (2004) in which taxa affiliated with the above mentioned clades 1+2 and 3+4, respectively are shown as sister groups of each other (Fig. 40). Frost *et al.* (2006) treated these four lineages as subfamilies within the Plethodontidae. They suggested restricting the names Hemidactylinae and Bolitoglossinae to those lineages that contain their respective type genera, to subsume Desmognathinae under Plethodontinae, and to name the above-mentioned clade 2 Spelerpinae.

Proteidae: Proteidae encompass two genera with six paedomorphic (neotenic) species. Proteidae exhibit a disjunct distribution in eastern North America (*Necturus*) and Southern Europe (*Proteus*). Monophyly of the family was originally suggested on the basis of morphology, but their shared features have also been suggested to result from convergence. Monophyly of Proteidae was suggested by analyses of allozymes (Guttman *et al.* 1990), but mt sequences failed to clarify this aspect (Weisrock *et al.* 2005). Very recently, a combination of nuclear and mt ribosomal sequences with morphological data provided new evidence in favour of the monophyly of this group (Wiens *et al.* 2005).

Salamandridae: Analyses of the phylogenetic relationships within the Salamandridae based on different kinds of data, such as behavioural, morphological, or mt sequence characters, resulted in controversial phylogenetic hypotheses. The most comprehensive molecular genetic study was presented by Titus and Larson (1995), who analysed a combined data set of 12S and 16S mtDNA sequences from 18 species (Fig. 41). More basal relationships within the family, however, have not yet been adequately resolved. Moreover, the monophyly of two genera recognized by traditional classification, i.e., *Mertensiella* and *Triturus*, was not

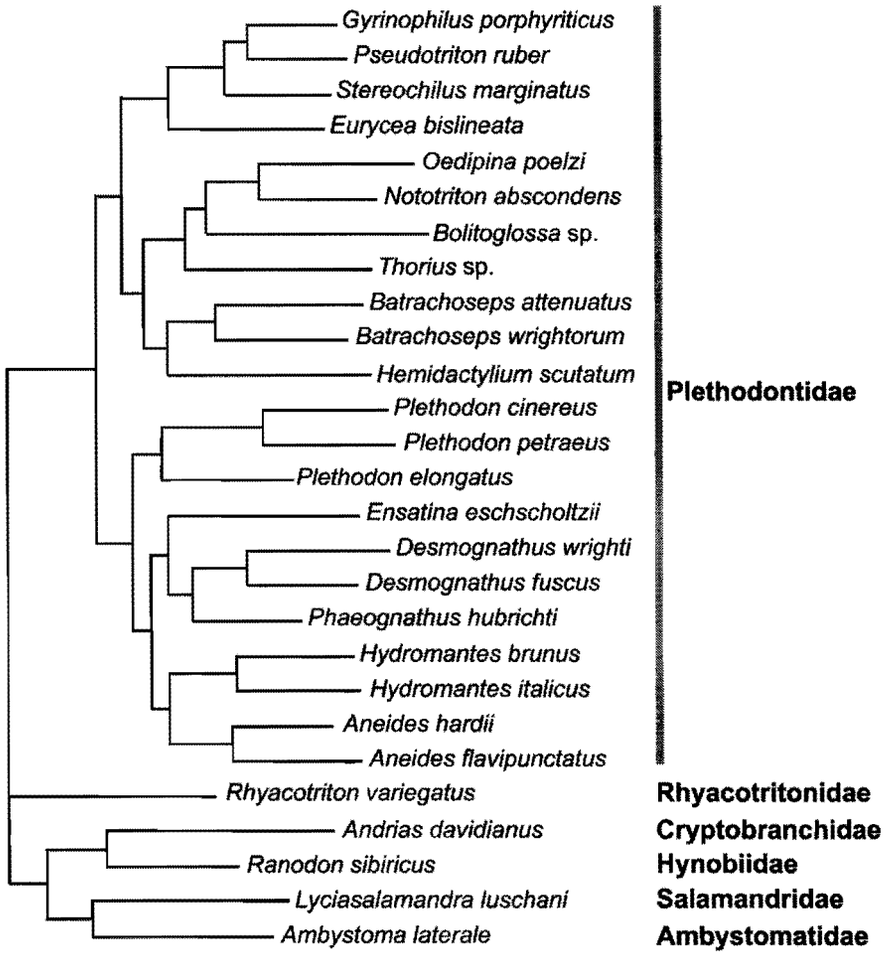


Fig. 40. Phylogenetic relationships among plethodontid salamanders as revealed by the analysis of entire mt genomes. From Mueller *et al.* (2004).

supported (e.g., Titus and Larson 1995; Veith *et al.* 1998; Steinfartz *et al.* 2002). Accordingly, García-París *et al.* (2004) suggested partitioning *Triturus* as traditionally encompassed into three different genera (*Triturus sensu stricto*, *Lissotriton*, and *Mesotriton*). There are indications among the Salamandridae that further genera also may not be monophyletic (García-París *et al.* 2004). At present, Salamandridae is considered the least known urodelan group in terms of the phylogenetic relationships among its members.

Sirenidae: The monophyly of Sirenidae is corroborated by the molecular phylogenies presented by Larson and Dimmick (1993) as well as by Wiens *et al.* (2005). The phylogenetic relationships of the family, however, remain controversial (Wiens *et al.* 2005 presented varying phylogenetic positions of this family in trees based on varying datasets).

Future systematic studies in Urodela should aim at completing the taxon sampling, especially within the more diverse lineages. Furthermore, additional sequence data are necessary to continue with the analysis of the phylogenetic relationships of salamanders. Beside mtDNA, which is still an appropriate tool to infer the relationships within more derived clades, the analysis of nuclear genes may offer new insights into the phylogeny of this diverse amphibian group.

5. Molecular Phylogeny of the Anura

Like caecilians and salamanders, frogs are also a comparatively old group (Table 8). The earliest known fossils date back to early Triassic from Madagascar and Poland, respectively (Rage and Roček 1989; Evans and Borsuk-Bialynicka 1998). This finding

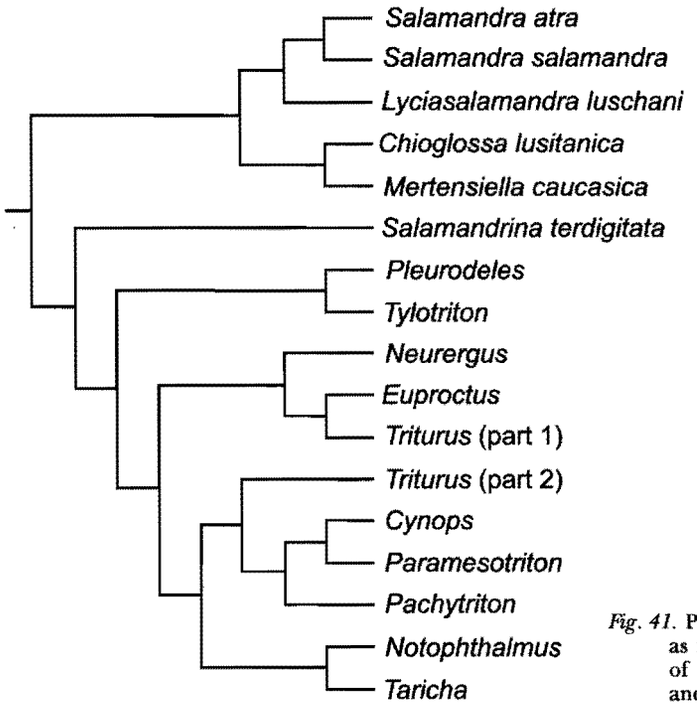


Fig. 41. Phylogenetic relationships among Salamandridae as revealed by the analysis of a combined dataset of the 12S and 16S mtDNA. According to Titus and Larson (1995).

implies that their common stem group must have occurred throughout Pangaea. Solely based on molecular age estimates San Mauro *et al.* (2005) proposed that the basal splits within the group even predate the break-up of Pangaea and that frogs occurred in all parts of the landmass before it became fragmented. It is hypothesized that urodeles and anurans separated in late Devonian to early Carboniferous (San Mauro *et al.* 2005) or at least in late Carboniferous (Zhang *et al.* 2005) (Table 8). Thus, the molecular genetic estimates on the origin of frogs exceed the minimum age suggested by the fossil record by up to 100 My.

According to San Mauro *et al.* (2005), the major lineages of living frogs diversified during the Permian (Table 8; Figs 31–32, 42). Nonetheless, it is accepted that the splitting of Pangaea (and later of Gondwana) were important for the formation of specific Mesozoic frog faunas on the different isolated landmasses (Roelants and Bossuyt 2005). Despite recent compelling evidence for the capacity of frogs for transmarine dispersal (Evans *et al.* 2003; Vences *et al.* 2003b), there is little doubt that continental drift has had a major influence on the current distribution and phylogeny of anurans. Age estimates based on differently

Table 8. Age estimates for different anuran groups, with 95% confidence intervals, suggested by various molecular studies under application of Bayesian clock methodology.

	Million years [My] before present			
Event	upper limit	mean	lower limit	Reference
Initial splits within the Anura	223	262	305	San Mauro *et al.* (2005)
Origin of Amphicoela	198	225	257	Roelants and Bossuyt (2005)
Split between Ascaphidae and Leiopelmatidae	154	183	215	
Origin of Discoglossoidea	184	211	243	
Origin of Pipoidea	177	204	237	
Split between Rhinophrynidae and Pipidae	154	183	215	
Origin of Pelobatoidea	159	185	217	
Split between Hyloidea and Ranoidea	152	173	195	Zhang *et al.* (2005)
	108	152	202	Biju and Bossuyt (2003)
Origin of Heleophrynidae and Myobatrachidae	109	150	198	
Origin of the Sooglossidae/Nasikabatrachidae lineage	131	178	233	
Split between Sooglossidae and Nasikabatrachidae	93	131	177	
Origin of Ranoidea	93	134	177	Van der Meijden *et al.* (2005)

composed sequence data of either nuclear or mt genes consistently suggested that the more basal lineages within the most speciose clade of frogs — the Neobatrachia — originated during the end of the Jurassic to the early Cretaceous (Biju and Bossuyt 2003; San Mauro *et al.* 2005; Zhang *et al.* 2005) at a time when Gondwanan fragmentation was still in its initial phase. It has been shown, for instance, that India played a significant role in the trans-Tethys passage of advanced frogs to southeastern Asia (Duellman and Trueb 1986; Bossuyt and Milinkovitch 2001; Biju and Bossuyt 2003).

As for caecilians and salamanders, the monophyly of Anura is well established by both morphological and molecular characters. Based on morphology, earlier phylogenetic hypotheses proposed two principal anuran lineages, referred to as Archaeobatrachia and Neobatrachia (e.g., Duellman 1975). In order to retain monophyletic clades, the Pipoidea and the Pelobatoidea subsequently were allocated to a third group, called Mesobatrachia (e.g., Duellman and Trueb 1986). Other systematists, however, have not followed this treatment, consistently. Frost *et al.* (2006) complained that subdivision into "primitive", "transitional" and "advanced" groups is rather subjective and reflects traditional preoccupation with certain groupings, thereby interfering with the aim of a phylogenetic system.

The earliest molecular studies used mt ribosomal (r) DNA sequences to infer anuran phylogeny and at first seemed to corroborate a bifurcation of the anuran tree into two major clades, the Archaeobatrachia and the Neobatrachia (Hedges and Maxson 1993; Hay *et al.* 1995). It was demonstrated by Hertwig *et al.* (2004), however, that because of homoplasy the phylogenetic signal of mt rRNA genes is weak in respect to old (Mesozoic) splits within the Anura. Consequently, analyses of such sequences do not result in robust phylogenetic hypotheses. Subsequent studies employing nuclear genes delivered contrasting results by revealing the so-called Archaeobatrachia as a paraphyletic assemblage (e.g., Hillis *et al.* 1993; Kjer 1995; Hoegg *et al.* 2004; Roelants and Bossuyt 2005; San Mauro *et al.* 2005; Fig. 43). Because it is supported by analyses of a variety of different nuclear markers, a paraphyletic arrangement is considered to be a more reliable hypothesis of the relationships among the more primitive frog taxa.

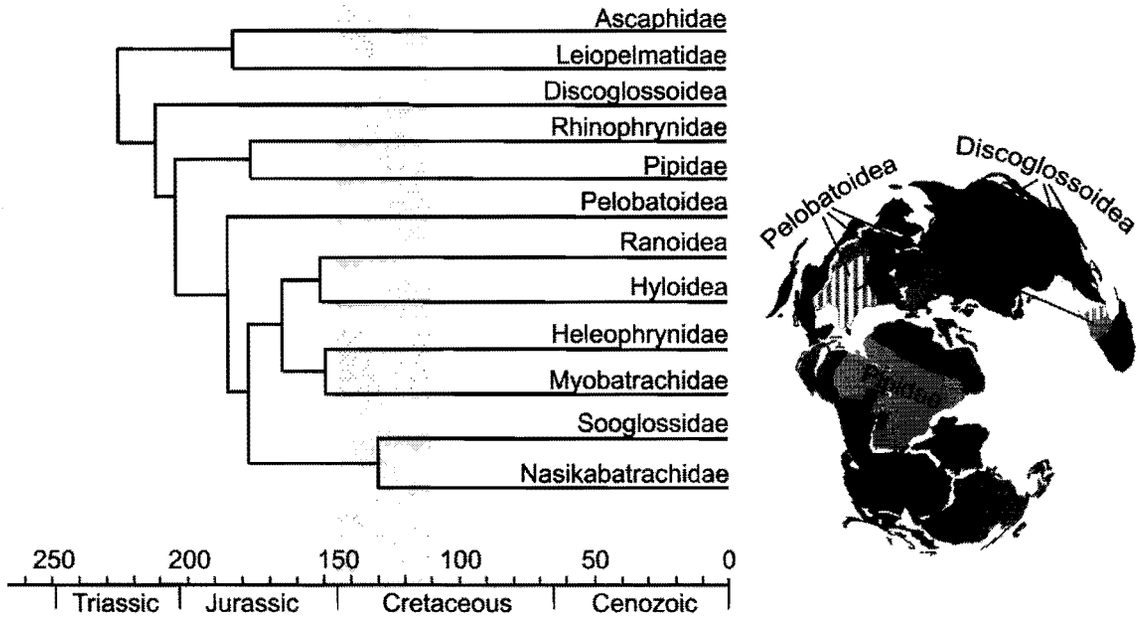


Fig. 42. Early evolution of frogs. Evolutionary tree showing estimates of divergence times for some major lineages of "archaeobatrachian" and neobatrachian frogs combined from two different studies (Biju and Bossuyt 2003; Roelants and Bossuyt 2005). The shaded area marks the period of Gondwanan separation. The lines indicate only tentative phylogenetic relationships and various relationships indicated here are disputable. The figure on the righthand side shows a tectonic reconstruction of Gondwana at about 150 My with the approximate recent distributional areas of some main lineages shaded. Adapted from Biju and Bossuyt 2003; Roelants and Bossuyt 2005).

Phylogenies based on nuclear genes consistently reveal the same lineages among the more primitive frogs (Figs 44, 46): Amphicoela, Discoglossoidea, Pipoidea, and Pelobatoidea. There is broad consensus that Amphicoela, a group consisting of Ascaphidae and Leiopelmatidae, represents the most primitive lineage of living frogs. The phylogenetic relationships among the remaining more basal clades are not unambiguously resolved. Hoegg *et al.* (2004), using sequences of the nuclear protein-encoding genes RAG1 and RAG2, suggested that Discoglossoidea, Pelobatoidea including Scaphiopodidae, and Pipoidea are successively the next more derived clades (Fig. 44), but their study included no amphicoelans. Roelants and Bossyut (2005) presented a tree in which Pelobatoidea with Scaphiopodidae are shown as the most derived archaeobatrachian group, being the sister taxon to the Neobatrachia, while the Pipoidea form a more basal group (Fig. 44). This relationship has also been shown by Frost *et al.* (2006). The trees in Figures 44 and 46 show that very short branches separate these major groups and branch-support values are also low. Consequently, it remains uncertain whether Pipidae or Pelobatoidea represent the sister group of the Neobatrachia.

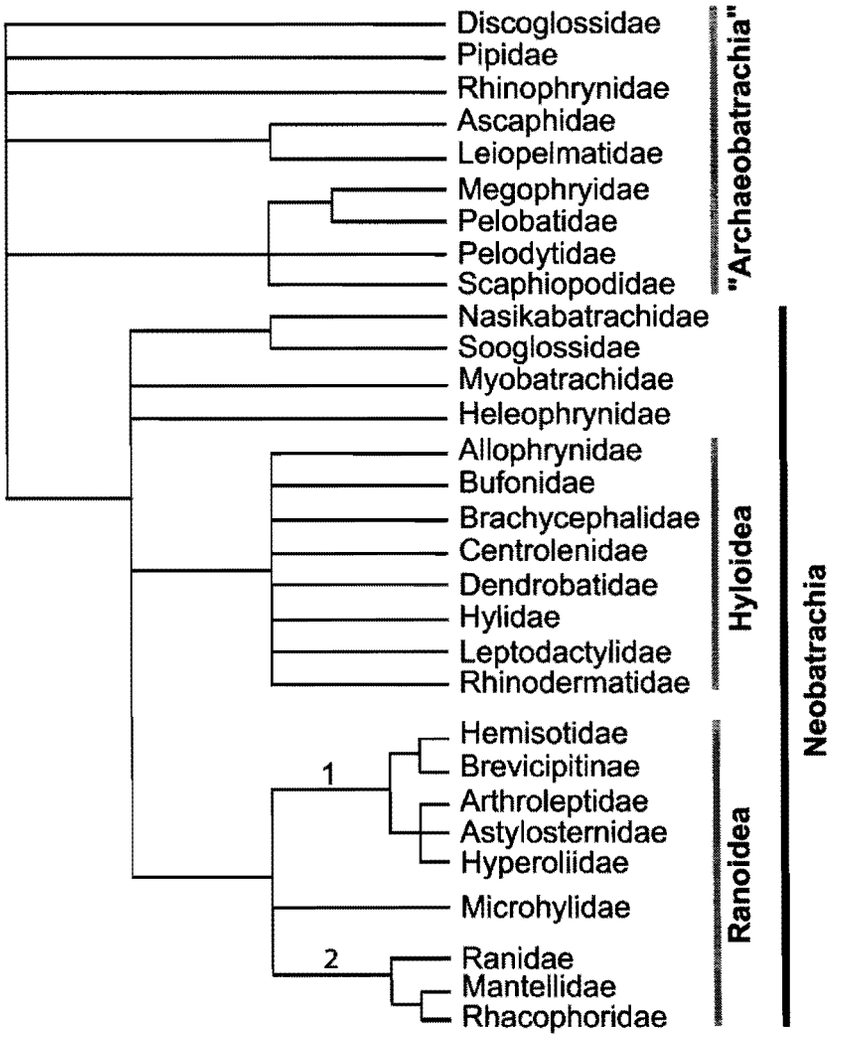


Fig. 43. Schematic classification of frogs. Simplified reconstruction adapted from Van der Meijden *et al.* (2004) with consideration of the results of Vences and Glaw (2001), Biju and Bossuyt (2003), Hoegg *et al.* (2004), Roelants *et al.* (2004), and Van der Meijden *et al.* (2004). Phylogenetic relationships of most families are still controversial (Bombinatoridae are included in Discoglossidae; Limnodynastidae and Rheobatrachidae are included in Myobatrachidae). 1: "Arthroleptidei" or "Arthroleptoidae". 2: "Ranidei" or "Ranoidae".

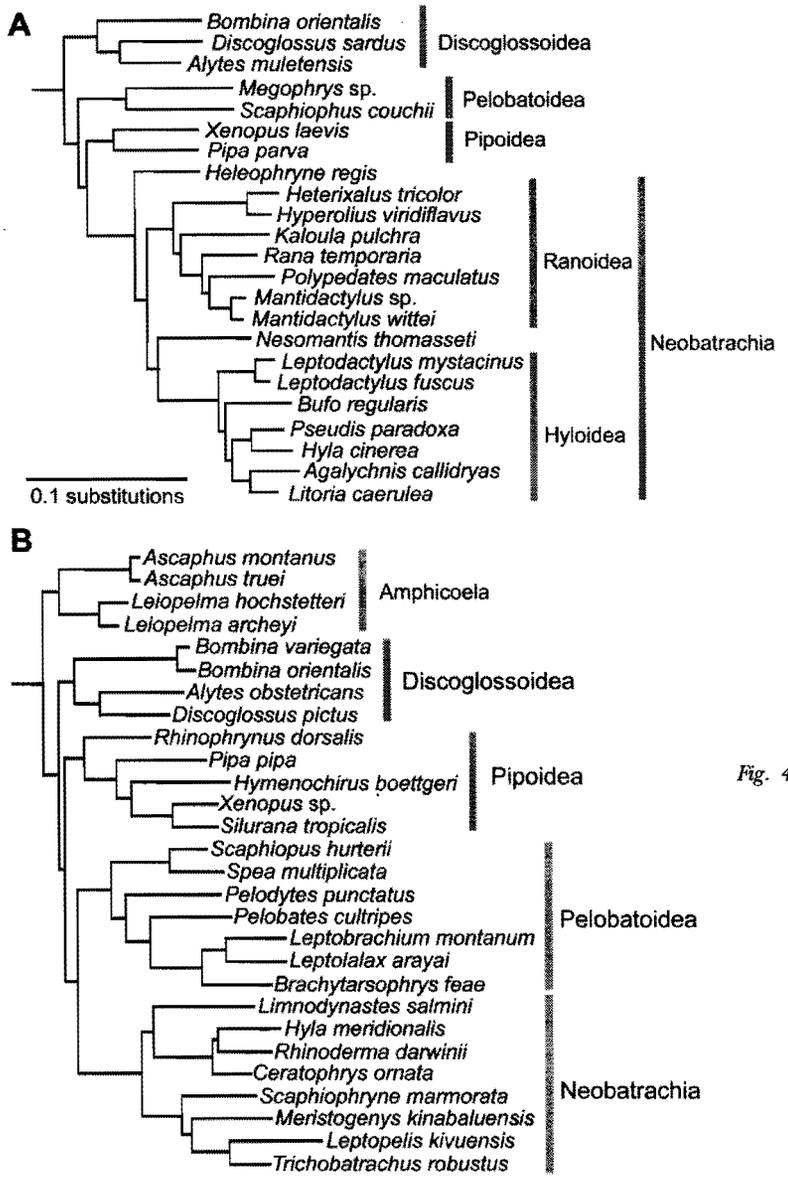


Fig. 44. Phylogenetic relationships of the major anuran lineages. Outgroup taxa are not shown. A: Maximum-likelihood phylogram obtained from analysis of a combined dataset of the nuclear genes RAG1, RAG2, and rhodopsin. Adapted from Hoegg *et al.* (2004). B: Maximum-likelihood phylogram obtained from analysis of a combined dataset of nuclear sequences (RAG1, exon 2 of the chemokine receptor 4, exon 2 of the sodium-calcium exchanger 1 gene) and 16S mtDNA sequences under a GTR+G+I model of sequence evolution. Adapted from Roelants and Bossyut (2005).

In contrast to so-called archaeobatrachians, which represent only 4% of living species of frogs, the monophyly of neobatrachian frogs has generally not been questioned. Neobatrachia usually is subdivided into two major clades, Hyloidea (previously named Bufonoidea) and Ranoidea with current distributions centres in the Neotropics and the Old World, respectively (Duellman and Trueb 1986). Besides these two huge groups, there are some lineages that contain comparatively few species. Presented below are the main results of molecular studies on recent frogs and conclusions for their systematics, starting with the more primitive groups.

Ascaphidae and **Leiopelmatidae**: Based on allozymes, Green *et al.* (1989) concluded that *Leiopelma* was the sister-group to all other frogs including *Ascaphus*. The alternative hypothesis, that *Ascaphus* is the sister taxon to all remaining frog taxa, could not, however, be excluded (Cannatella and Frost 1995). Based on 12S and 16S mtDNA sequences, Hay *et al.* (1995) found that *Ascaphus* and *Leiopelma* form a sister pair that is the sister taxon to all other frog taxa. This finding was corroborated by Roelants and Bossuyt (2005), who employed a comprehensive set of nuclear sequence data. Accordingly, there is convincing

evidence that *Leiopelma* and *Ascaphus*, subsumed by some authors under the term Amphicoela, represent the sister group of all living frogs (Fig. 44). Frost *et al.* (2006) suggested that the lineage containing the two sister taxa *Ascaphus* and *Leiopelma* should be treated as a single family, Leiopelmatidae.

Discoglossidae: Molecular studies provide strong evidence for the monophyly of this group, which next to the Leiopelmatidae belongs to the more basal group of living frogs. (e.g., Biju and Bossuyt 2003; Hoegg *et al.* 2004; Roelants and Bossyut 2005; Frost *et al.* 2006) (Fig. 44). There is dissent, however, whether *Alytes* and *Discoglossus* on the one hand and *Bombina* and *Barbourula* on the other hand should be placed within different families Discoglossidae *sensu stricto* (some authors prefer the name Alytidae) and Bombinatoridae, respectively. The molecular trees consistently show these two groups as being separated by a relatively deep bifurcation and it is rather a matter of taste whether one considers the two sister groups as separate families or not.

Pipoidea: There is considerable confusion as to the definition of the Pipidae, mainly because of the unresolved relationships of the fossil taxa. Nevertheless, molecular data, both mt ribosomal sequences (Hertwig *et al.* 2004), nDNA sequences (Hoegg *et al.* 2004; Roelants and Bossuyt 2005) and combined mt and nDNA sequences (Frost *et al.* 2006) strongly support the monophyly of the recent pipids. According to Roelants and Bossuyt (2005) and Frost *et al.* (2006), Pipidae and Rhinophrynidae form a monophyletic group, Pipoidea, which is the sister group of (Pelobatoidea + Neobatrachia). In contrast, Hoegg *et al.* (2004) showed Pelobatoidea as the sister to a clade formed by Pipoidea + Neobatrachia. The clustering of Pipidae and Rhynophrynidae into a monophyletic group was first suggested by Hay *et al.* (1995) and has recently been corroborated by Roelants and Bossuyt (2005) and Frost *et al.* (2006) (Fig. 44).

Pelobatoidea: As discussed under Pipoidea, phylogenetic analyses have delivered conflicting results as to whether Pipoidea or Pelobatoidea form the sister taxon of the Neobatrachia. Irrespective of this discrepancy, the monophyly of Pelobatoidea, including Scaphiopidae, Pelobatidae, and Megophryidae, has been strongly supported by a variety of molecular studies (e.g., Garcia-Paris *et al.* 2003; Hertwig *et al.* 2004; Hoegg *et al.* 2004; Roelants and Bossuyt 2005; Frost *et al.* 2006). The most comprehensive datasets have been provided by Garcia-Paris *et al.* (2003) in respect to taxon sampling and by Roelants and Bossyut (2005) in respect to the lengths of sequences analysed. Both works consistently suggest that Scaphiopodidae represent the most basal clade within the Pelobatoidea, successively followed by the branches of Pelodytidae + (Pelobatidae + Megophryidae), the latter two forming the most derived clade (Fig. 45). In contrast, Frost *et al.* (2006) suggested a sister-group relationship between Pelodytidae and Scaphiopodidae.

Neobatrachia: About 96% of all living frog species belong to the Neobatrachia, a clade that traditionally is separated into two large groups, the Hyloidea and the Ranoidea — a system that has been accepted by most systematists since the mid-1800s (Lynch 1973). Morphological studies have suggested that Hyloidea are paraphyletic with respect to Ranoidea (Kluge and Farris 1969; Lynch 1971, 1973). However, considering the more restricted definition of Hyloidea as suggested by Darst and Cannatella (2004) who excluded Heleophrynidae, Limnodynastidae, Myobatrachidae, Nasikabatrachidae, Sooglossidae and Rheobatrachidae, this taxon is consistently recognized as monophyletic by molecular studies (e.g., Ford and Cannatella 1993; Hay *et al.* 1995; Ruvinsky and Maxson 1996; Vences *et al.* 2000a; Darst and Cannatella 2004; Hoegg *et al.* 2004; San Mauro *et al.* 2005). For a deviant opinion on the Hyloidea, see Frost *et al.* (2006). The placement of the more basal neobatrachian clades, such as Heleophrynidae, Myobatrachidae, Sooglossidae, and Nasikabatrachidae has largely remained uncertain (e.g., Austin *et al.* 2002; Biju and Bossuyt 2003; Darst and Cannatella 2004; Wiens *et al.* 2005; Frost *et al.* 2006). San Mauro *et al.* (2005) stated that all these comparatively species-poor clades originated in much earlier periods than did the radiations of the Hyloidea and Ranoidea. Accordingly, it has been suggested that *Heleophryne*, *Nasikabatrachus*, and *Caudiverbera* were separated from other

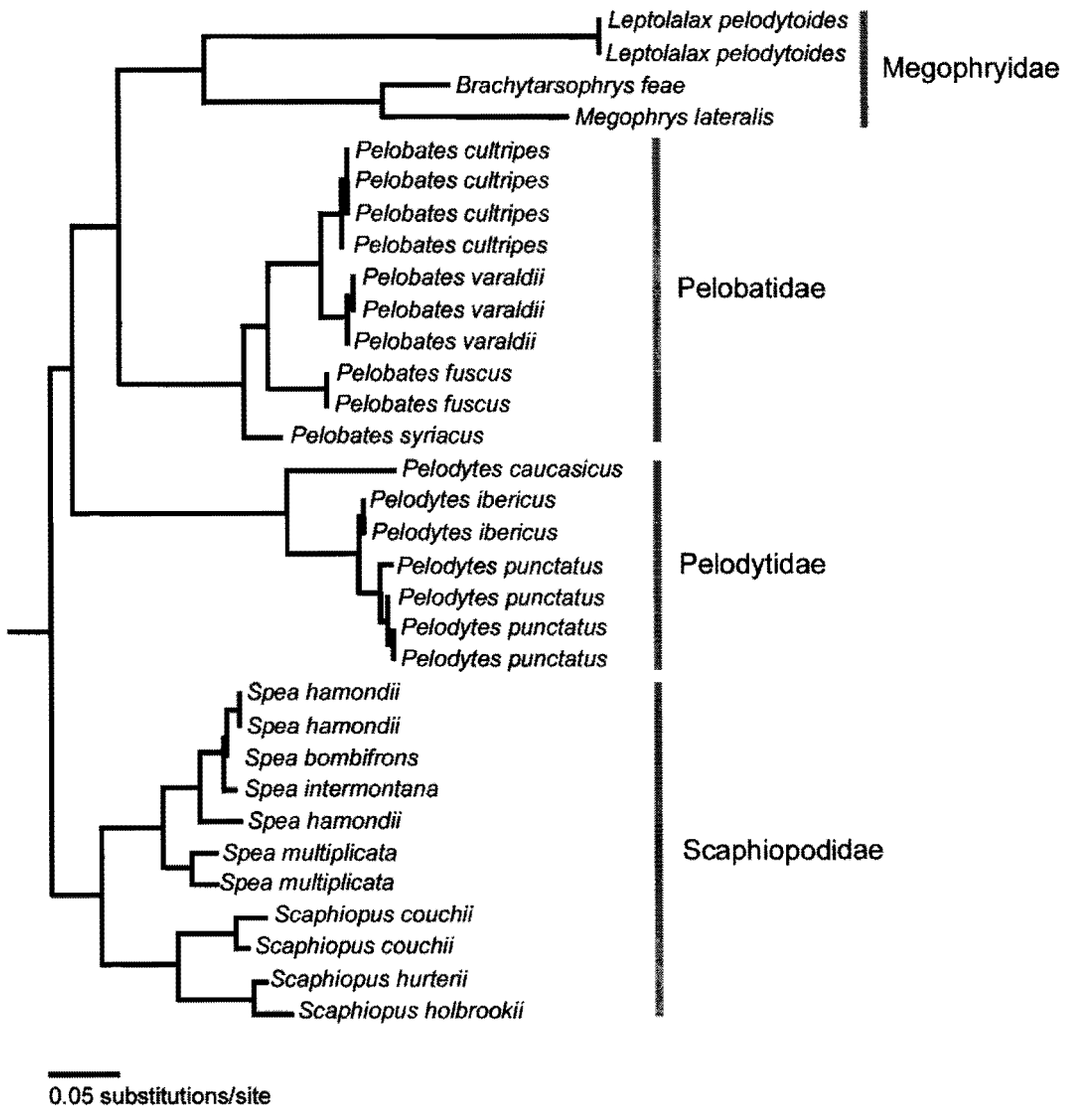


Fig. 45. Maximum-likelihood phylogeny of the Pelobatoidea based on the analysis of a combined dataset of mt sequences of the cytochrome *b* and 16S mtDNA. Adapted from Garcia-Paris *et al.* (2003).

neobatrachians between 162 (199 to 128) My BP and 120 (154 to 91) My BP (San Mauro *et al.* 2005), which means that their initial diversification occurred before the break-up of Gondwana. This scenario points to their wide, though localized, recent distribution. It has further been anticipated that their current restriction to geographic refuges indicates that these early neobatrachians may once have been more widespread but were outperformed by the more advanced hyloid and ranoid radiations in large parts of their original distributional area (San Mauro *et al.* 2005).

It has been argued that the derived *Bauplan* of advanced frogs in concert with a rather limited morphological plasticity leads to a high degree of homoplasy which hinders the establishment of a commonly accepted systematization based on morphology (Wallace *et al.* 1973; Maxson and Wilson 1974; Emerson 1988; Bossuyt and Milinkovitch 2000; Vences *et al.* 2000a). On the other hand, earlier molecular genetic investigations suffered from incomplete taxon sampling (Emerson *et al.* 2000). In addition, the predominant use of mt sequences that bear a relatively high degree of homoplasy if more ancient splits are inferred, has produced equivocal results (Hertwig *et al.* 2004). The debate on the phylogenetic position of the hyloid *Allophryne* may be taken as just one impressive example for the

problems with which systematists are faced. Different morphologically based studies suggested a placement of the taxon in five different families (Bufonidae, Leptodactylidae, Centrolenidae, Hylidae, and Allophrynidae) (reviewed by Austin *et al.* 2002). Based on a mitochondrial phylogeny, Austin *et al.* (2002) proposed that *Allophryne* represents a primitive member of Centrolenidae but at the same time it was stated that the phylogeny as presented suffered from poor taxon sampling. Later, the basal position of *Allophryne* within (or next to) Centrolenidae was corroborated by Wiens *et al.* (2005). In contrast to the former authors, however, Wiens *et al.* (2005) treated *Allophryne* as an independent and monotypic family.

Below, the systematics of the neobatrachian groups will briefly be reviewed from a molecular genetic perspective. A comprehensive phylogeny of the Neobatrachia was published by Biju and Bossuyt (2003) covering all larger and important groups and paying particular attention to the phylogenetic relationships of the relatively species-poor lineages that are members neither of the Hyloidea or Ranoidea (Fig. 46). This phylogeny shows both the hyloid and the ranoid radiations to be isolated by relatively long basal branches. Myobatrachidae, Heleophrynidae, Sooglossidae and Nasikabatrachidae accordingly form old lineages that are distinct from, and basal to, both of these two large groups (Fig. 42; Table 8). By contrast, in the phylogenetic tree presented by Frost *et al.* (2006) Heleophrynidae are shown as the most basal neobatrachian group. Furthermore, Sooglossidae and a clade containing Batrachophrynidae, Limnodynastidae and Myobatrachidae represent the successively more basal branches of a large monophyletic group consistent with the Hyloidea. This group is known as the sister group of the Ranoidea (Fig. 33).

Sooglossidae and **Nasikabatrachidae**: Sooglossidae comprises the two Seychellian genera *Sooglossus* and *Nesomantis*. The newly discovered *Nasikabatrachus* from the Western Ghats, South India, is considered to be the closest relative to the Sooglossidae in the rank of an independent family (Biju and Bossuyt 2003). The phylogenetic position of sooglossids within the advanced frogs has been subject to debate for decades. While there is little doubt that they belong in the Neobatrachia, molecular data suggests either (1) an unresolved trifurcation between sooglossids, ranoids, and all other neobatrachians (Hay *et al.* 1995), (2) a basal placement within the Hyloidea (Ruvinsky and Maxson 1996; Frost *et al.* 2006), (3) a position basal to Hyloidea and Ranoidea (Vences *et al.* 2003b; Hoegg *et al.* 2004), or (4) a sister-group relationship with Nasikabatrachidae, both comprising the most basal position within the Neobatrachia (Biju and Bossuyt 2003). It has been suggested that both groups, Sooglossidae and Nasikabatrachidae, have faced a long and joint history as an isolated lineage that has emerged from a rapid and basal radiation within the neobatrachian clade. Their ancestors likely were already present in Indo-Madagascar during its trans-Tethys drift that followed Gondwanan fragmentation, and subsequently became extinct in those areas that are not inhabited by their recent representatives, such as Madagascar (Biju and Bossuyt 2003). This scenario is corroborated by molecular-clock analyses indicating that Nasikabatrachidae and Sooglossidae diverged well before the break-up of India and the Seychelles at the Cretaceous/Tertiary boundary (Table 8, Fig. 42).

Heleophrynidae: Previous molecular studies placed this taxon within the Hyloidea (Hay *et al.* 1995; Ruvinsky and Maxson 1996), whereas Hoegg *et al.* (2004) suggested a basal position of *Heleophryne* within the Neobatrachia but not as a member of the Hyloidea. In the phylogenetic trees presented by San Mauro (2005) and Frost *et al.* (2006), *Heleophryne* is shown to comprise a position within the Neobatrachia basal to Ranoidea and Hyloidea. The correct placement of Heleophrynidae thus remains ambiguous and deserves further attention.

Myobatrachidae: This group comprises about 100 species from Australia, Tasmania, and New Guinea that are placed within two groups, Limnodynastinae and Myobatrachinae. Similar to the Sooglossidae, Nasikabatrachidae, and Heleophrynidae, Myobatrachidae hold a very basal position within the Neobatrachia. Currently, a robust, commonly accepted hypothesis on their phylogenetic relationships is lacking. In a tree based on 12S and 16S mtDNA sequences, *Limnodynastes* clusters with *Heleophryne*, both forming the sister group

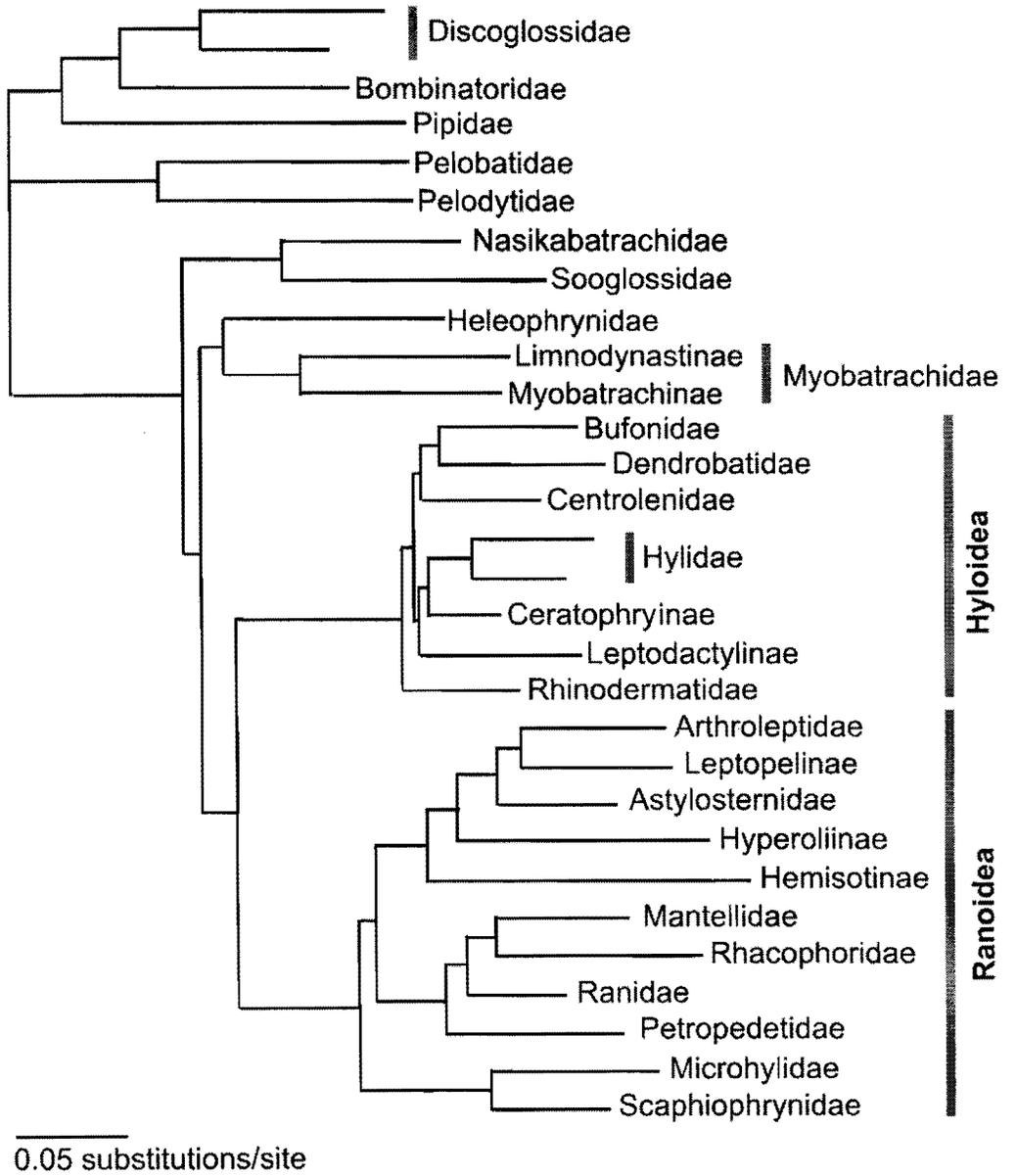


Fig. 46. Phylogenetic relationships within the neobatrachian clade as revealed by Bayesian analysis of 2 335 bp of a combined dataset of mtDNA (partial 12S and 16S mtDNA plus $tRNA^{Val}$) and nuclear DNA (exon 1 of rhodopsin, single exon of RAG1, and exon 2 of Cxcr4). Adapted from Biju and Bossyut (2003).

to all other neobatrachians (Darst and Cannatella 2004). Correspondingly, Biju and Bossuyt (2003) suggested a sister-group relationship of Myobatrachidae and Heleophrynidae on the basis of nuclear and mt genes. The analysis of only nuclear genes, however, revealed *Limnodynastes* as being the most basal taxon within the Hyloidea (Roelants and Bossuyt 2005). According to Frost *et al.* (2006), Limnodynastidae and Myobatrachidae should be recognized as independent families clustering together with Batrachophrynidae at a position basal to the Hyloidea as restricted by Darst and Cannatella (2004), but derived in respect to the Sooglossidae (Fig. 33).

Hyloidea: Hyloidea is a huge group comprising some 2 000 species in about ten families (e.g., Duellman and Trueb 1986). Various systematization schemes have been suggested, and more recent studies tend to treat Hyloidea in a more restrictive way than proposed earlier (e.g., Biju and Bossuyt 2003; Darst and Cannatella 2004). On the other hand, analyses of sequence data provided evidence that Dendrobatidae lies within the Hyloidea

and not within the Ranoidea as was formerly assumed (Darst and Cannatella 2004; Faivovich *et al.* 2005; Wiens *et al.* 2005; Frost *et al.* 2006). The monophyly of some groups (Centrolenidae, Bufonidae, Dendrobatidae) is strongly supported by molecular data while the systematics of the two most speciose groups recognized by traditional methods, Hylidae and Leptodactylidae, has remained highly enigmatic.

Hylidae: Hylid frogs are the second largest family of amphibians (exceeded only by leptodactylid frogs) with at least 861 species in 42 genera currently recognized (Wiens *et al.* 2005). The patterns of species diversity within the Hylidae are intriguing. While the majority of hylid species are found in the New World tropics, the representatives of the subfamily Hylinae that comprises genera such as *Hyla* and *Pseudacris*, show highest species richness in midtemperate regions of northern America, Europe, and eastern Asia where they have undergone extensive and parallel radiations (Wiens *et al.* 2005). This distributional pattern has been discussed as reflecting specialized tolerance for temperate climates by this group (Smith *et al.* 2005).

Hylid phylogeny still poses a challenging problem. Until recently there have been no detailed phylogenetic analyses of the whole family. Molecular studies were mostly based on relatively rapidly evolving mtDNA markers and suffered from a limited sampling of species (e.g., Chek *et al.* 2001; Darst and Cannatella 2004; Faivovich *et al.* 2004; Moriarty and Cannatella 2004). Although there have been several noteworthy points of congruence between the results of molecular and morphologically based studies, such as the monophyly of phyllomedusines and pelodryadines, molecular data have not supported the monophyly of Hylidae as a whole. In order to resolve the problematic phylogeny of this group, Wiens *et al.* (2005) examined differently composed datasets of pure or combined morphological and molecular sequence data from 54 species of hylids and 27 representatives of other anuran families, and a second dataset comprising 12S rDNA sequences of 193 hyloid species. Their study indicated that the current taxonomic treatment in fact reflects phylogeny very poorly; Hylidae have been demonstrated to be polyphyletic and Hylinae have been retained as a monophyletic group only by the inclusion of the Pseudinae. Further monophyletic taxa accordingly are: (1) Pelodryadinae, (2) Phyllomedusinae, (3) a group comprising clades 1 and 2, (4) Centrolenidae with *Allophryne* as its sister taxon, (5) Hemiphractidae, and (6) Bufonidae. Leptodactylidae as currently defined are polyphyletic (Fig. 47). Suggestions as to the taxonomic treatment of the non-monophyletic groupings have been presented by Frost *et al.* (2006).

Faivovich *et al.* (2005) paid special attention to the phylogenetic relationships within the Hylinae by analysing 5 100 bp of concatenated sequences of four mt genes (12S and 16S mtDNA, $tRNA^{Val}$, cytochrome *b*) and five nuclear genes (rhodopsin, tyrosinase, RAG1, seventh in absentia, 28S). In this study 279 taxa were considered that represent 40 of 41 recognized genera of Hylidae and 39 of 41 recognized species-groups within *Hyla*. As an important result, Hemiphractidae has been excluded from the Hylidae, which in turn includes only the three monophyletic lineages Hylinae, Pelodryadinae, and Phyllomedusinae. The latter two groups form a sister taxon to the Hylinae. The study also showed that in respect to Hylinae previous systematization schemes were widely incorrect. Various genera recognized by their morphology have been shown not to be monophyletic. In summary, it was suggested that Hylinae consists of four major clades: (1) Andean stream-breeding Hylinae, *Aplastodiscus*, Gladiator frogs, and a Tepuian lineage (South American clade I), (2) 30-chromosome "*Hyla*", *Lysapsus*, *Pseudis*, *Scarthyla*, *Scinax*, *Sphaenorhynchus*, *Xenohyla* (South American clade II), (3) *Nyctimantis*, *Phrynohyas*, *Phyllodytes*, South American and West Indian casque-headed frogs (South American-West Indian clade) and (4) most Middle American and Holarctic species-groups of "*Hyla*", *Acris*, *Anotheca*, *Duellmanohyla*, *Plectrohyla*, *Pseudacris*, *Ptychohyla*, *Pternohyla*, *Smilisca*, and *Triprion* (Middle American-Holarctic clade) (Fig. 48). Of the species previously subsumed under "*Hyla*," many were transferred to other genera. *Hyla* was restricted to *H. femoralis* and the *H. arborea*, *H. cinerea*, *H. eximia*, and *H. versicolor* species groups. Further systematic and taxonomic implications are provided in the original work.

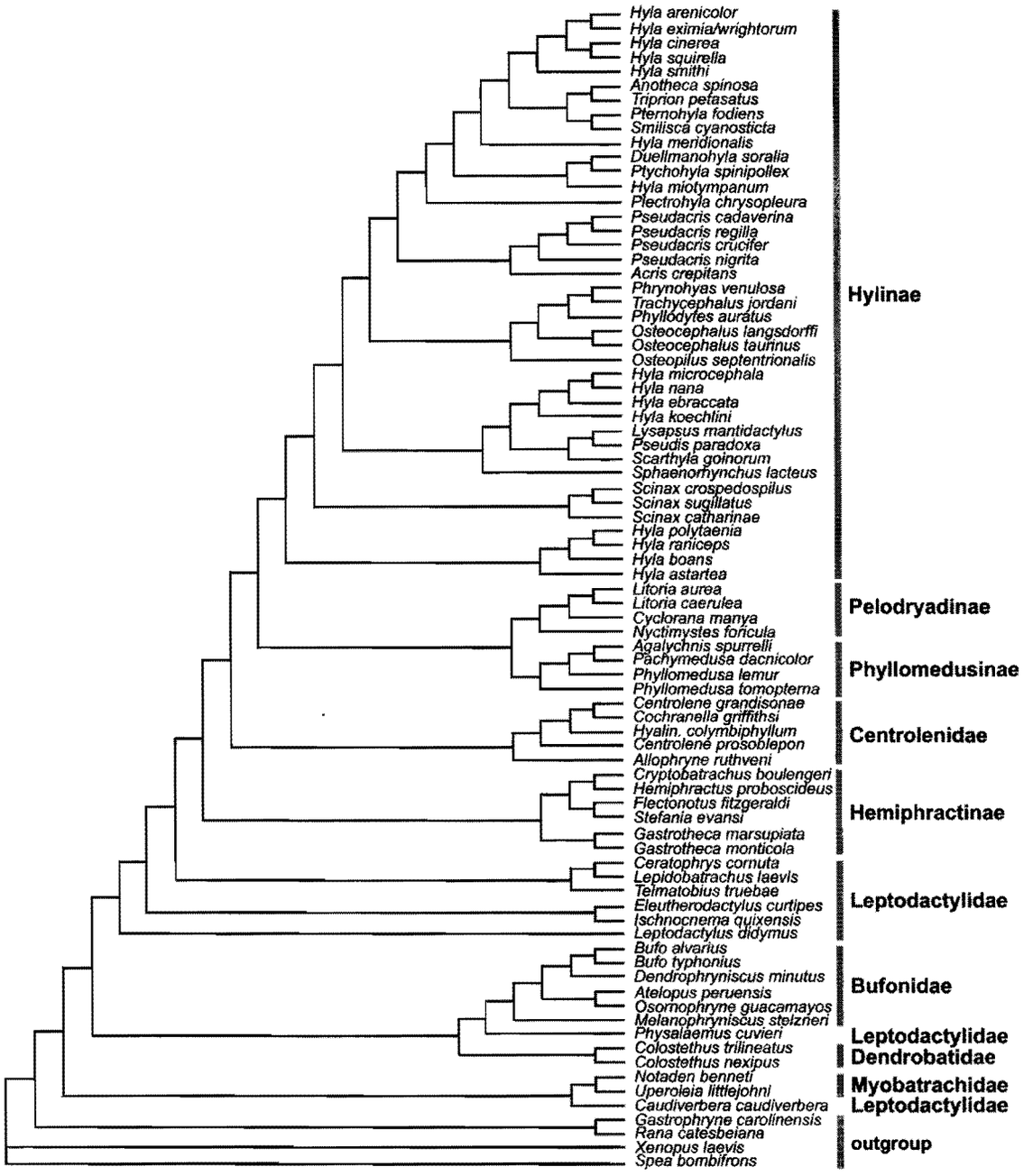


Fig. 47. Hypothesis on the phylogeny of hylid frogs based on Bayesian analysis of a combined set of molecular and morphological data, including 81 taxa. Adapted from Wiens *et al.* (2005).

Centrolenidae: Austin *et al.* (2002) proposed that Centrolenidae are monophyletic and that *Allophryne* represents the sister taxon of all centrolenid species. This finding has been corroborated by the studies of Wiens *et al.* (2005), Faivovich *et al.* (2005) and Frost *et al.* (2006) and is thus founded on an extensive molecular dataset. Both Wiens *et al.* (2005) and Faivovich *et al.* (2005), however, treat *Allophryne* as a separate family, Allophrynidae, whereas Frost *et al.* (2006) affiliated it with the Centrolenidae with subfamilial rank. The phylogenetic relationships of Centrolenidae (including *Allophryne*) remain unclear. Depending on the kind of data analysed, the family has been regarded either as the sister taxon of the Hylidae (by combined morphological and molecular data; Wiens *et al.* [2005]), as closely related to the Bufonidae (by molecular genetic data alone; Wiens *et al.* [2005]), or as the sister taxon of the "Leptodactylinae" or Leptydactylidae as restricted by Frost *et*

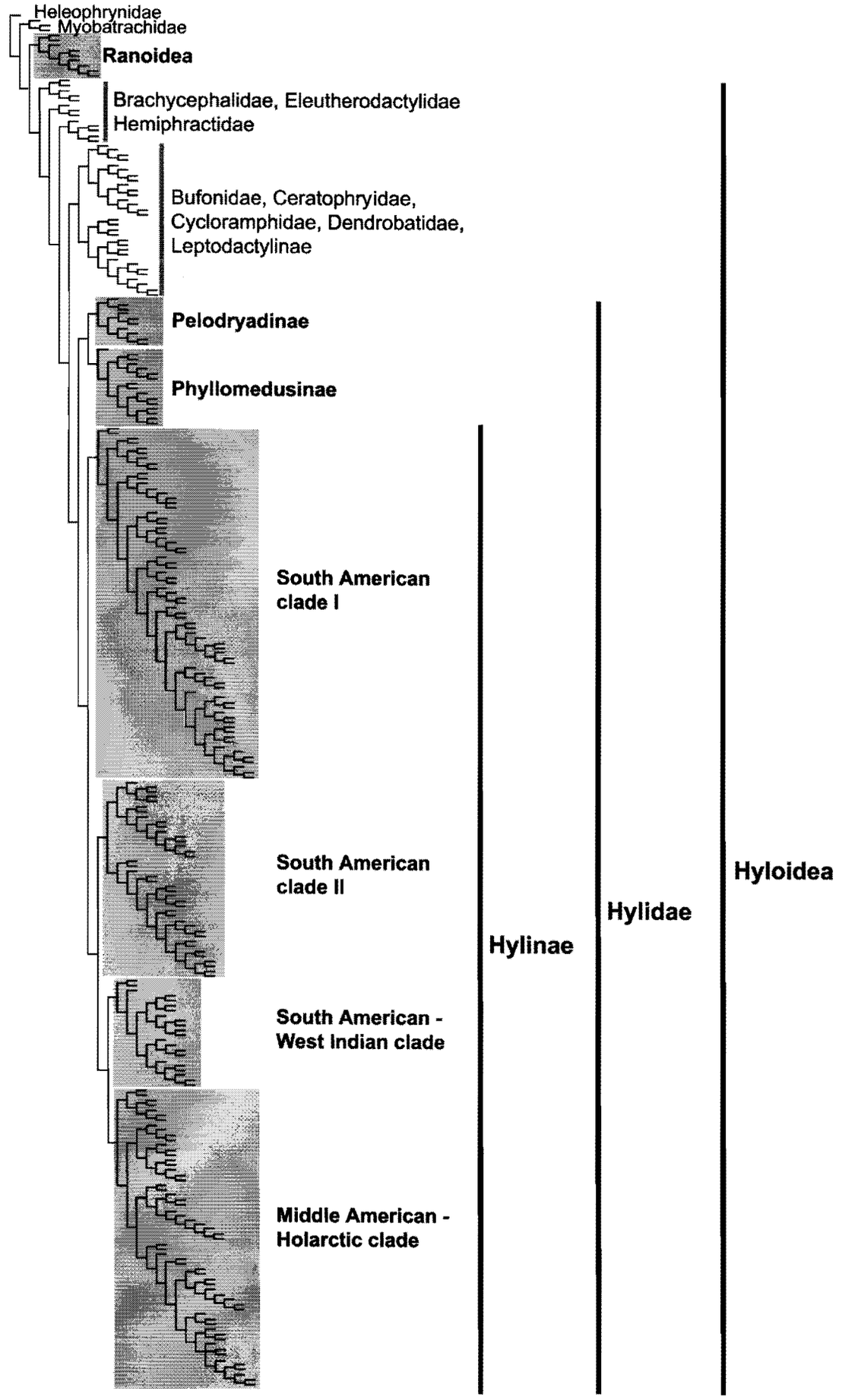


Fig. 48. Phylogenetic relationships among the Hylinae, as presented by Faivovich *et al.* (2005). Strict consensus maximum parsimony tree based on the analysis of concatenated sequences of four mitochondrial and five nuclear genes.

al. (2006) on the basis of additional molecular and morphological data (Faivovich *et al.* 2005; Frost *et al.* 2006). Both taxa form part of a large South American clade that consists of some species traditionally assigned to Leptodactylidae, Centrolenidae, and Bufonidae. Suggestions as to the taxonomic treatment of the non-monophyletic groupings have been presented by Frost *et al.* (2006).

Hemiphractidae: Hemiphractidae are shown as a monophyletic clade basal to a group consisting of Centrolenidae and Hylidae (with Hylinae, Phyllomedusinae, Pelodryadinae) in a phylogeny based on combined morphological and molecular genetic data (Wiens *et al.* 2005). If only sequence data are analysed, however, Hemiphractidae are found at a surprising position in the phylogenetic tree, forming the sister group of some leptodactylid species (Eleutherodactylinae; subsumed under Brachycephalidae by Frost *et al.* [2006]). Analyses based only on mt 12S and 16S sequences even show the Eleutherodactylinae as nested within the hemiphractine subclade (Wiens *et al.* 2005). The phylogeny of Faivovich *et al.* (2005) shows Hemiphractidae as a polyphyletic group with the taxa subsumed under this name being distributed across three different clades. Accordingly, (1) *Hemiphractus helioi* clusters together with two *Eleutherodactylus* species, *Phrynopus* sp. (both Eleutherodactylinae), and *Brachycephalus epihippium* (Brachycephalidae), (2) *Cryptobatrachus* and *Stefania* form a sister group to all Hyloidea except for Eleutherodactylinae and Brachycephalidae, whereas the third hemiphractine clade composed of (3) *Flectonotus* and *Gastrotheca* is revealed as the sister group of the remaining Hyloidea. The confusing systematics has been revised by Frost *et al.* (2006) in order to define monophyletic taxa within this polyphyletic assemblage (Fig. 33). Further studies of the Hemiphractidae are badly needed.

Leptodactylidae: Originally delineated by morphology, this family encompasses a large and varied group of frogs with most of their diversity in South and Central America and the West Indies. There are about 50 genera with 700 species; the genus *Eleutherodactylus* alone comprises about 400 species (Cannatella 1995). Molecular studies, however, provide consistent evidence that Leptodactylidae is polyphyletic (Darst and Cannatella 2004; Faivovich *et al.* 2005; Wiens *et al.* 2005) and not even the formerly recognized subfamilies, such as Leptodactylinae, Eleutherodactylinae, and Telmatobiinae, are monophyletic (Faivovich *et al.* 2005). Frost *et al.* (2006) have suggested discriminating between Leptodactylidae *sensu stricto* and Ceratophryidae, Cyclorhamphidae, and Thoropidae in order to retain only monophyletic taxa (Fig. 33).

Bufonidae: The monophyly of this group is consistently revealed by various studies (e.g., Darst and Cannatella 2004; Wiens *et al.* 2005; Faivovich *et al.* 2005; Frost *et al.* 2006). Bufonidae is composed of 33 genera with a nearly cosmopolitan distribution, except for Australia where the only species is the introduced cane toad (*Bufo marinus*). More than half of all bufonids are members of the genus *Bufo* (Frost 2002). Although phylogenetic relationships within this speciose family are not yet entirely clear, Bufonidae, which were subject to some pioneer studies (e.g., Maxson 1984; Graybeal 1993), certainly belongs to the better-documented anuran groups. A considerable number of papers address mainly the phylogenetic relationships of different bufonid groups, such as Nearctic toads (Pauly *et al.* 2004), bufonids in South and Central Asia (Maxson 1984), the *Bufo peltocephalus*-group from the West Indies (Pramuk *et al.* 2001; Pramuk 2002), eastern Asian species (Liu *et al.* 2000), and African species (Cunningham and Cherry 2004). Ambiguity exists about the monophyly of some genera and species-groups, especially those known to have undergone more recent radiations (e.g., Estoup *et al.* 2004; Fu *et al.* 2005; Igawa *et al.* 2005). Pauly *et al.* (2004) supposed, on the basis of approximately 2.5 kb of mtDNA sequence data for the 12S, 16S, and intervening $tRNA^{Val}$ gene from 56 species, that Nearctic species of *Bufo* are monophyletic and nested within a large clade of New World *Bufo* to the exclusion of Eurasian and African taxa. This suggest that Nearctic *Bufo* result from a single colonization from the Neotropics.

Dendrobatidae: Systematists emphasizing morphology were not sure whether these South American frogs should be placed within the Ranoidea or the Hyloidea (e.g., Duellman and Trueb 1986), but molecular studies clearly showed that dendrobatids are correctly

placed within the Hyloidea (Vences *et al.* 2000b; Darst and Cannatella 2004). While the monophyly of the group is generally undisputed, its phylogenetic position within the Hyloidea is not yet clear. Vences *et al.* (2000b) analysed partial sequences of the mt 16S rRNA gene (582 bp) of 20 poison-frog species and suggested a closer relationship with bufonid and leptodactylid frogs. According to Faivovich *et al.* (2005), the family is part of a larger South and Central American clade comprising also Centrolenidae and certain leptodactylids. In the phylogenetic trees presented by Wiens *et al.* (2005), Dendrobatidae is shown either as one of the most basal splits within the Hyloidea (based only on sequence data) or as the sister taxon of Bufonidae (based on combined molecular and morphological data). In complete disagreement with these results, Dendrobatidae were revealed as the most derived group within the Hyloidea by Cannatella and Darst (2004), who analysed sequences of the 12S and 16S mtDNA.

Within the Dendrobatidae, the monophyly of *Phyllobates* has been supported but all other genera have been indicated as being either paraphyletic or polyphyletic (Vences *et al.* 2000b). This contradicts assumptions based on morphological and behavioural characteristics and suggests that certain features have evolved convergently.

Ranoidea: The neobatrachian superfamily Ranoidea as delineated by Ford and Cannatella (1993) comprises a large group of predominantly Old World species. Originally placed in the only poorly defined family Ranidae, many groups such as Mantellidae, Rhacophoridae, or Hyperoliidae were subsequently split off (e.g., Duellman and Trueb 1986). Still, the phylogenetic relationships among the ranoid groups are inadequately understood (e.g., Emerson 2000; Vences *et al.* 2003b; Hoegg *et al.* 2004); this is reflected in their very confusing taxonomy. In conflict with morphological studies, molecular investigations revealed that Dendrobatidae do not belong to the Ranoidea (Ruvinsky and Maxson 1996), quite in contrast to the Microhylidae, which were formerly considered as independent and basal to this group (Emerson *et al.* 2000). More comprehensive molecular studies frequently have recognized three major lineages among the Ranoidea: (1) Microhylidae and Scaphiophrynidae, (2) a lineage comprising Brevicipitidae, Hemisotidae, Arthroleptidae, Astylosternidae, Hyperoliidae, Heterixalidae, for instance, and (3) a lineage to which groups such as Petropedetidae, Ranidae, Mantellidae, and Rhacophoridae belong (Vences *et al.* 2003a,b; Biju and Bossuyt 2003; Scott 2005; Frost *et al.* (2006), Figs 49–51). For a taxonomic revision see Frost *et al.* (2006).

In a comprehensive study employing 2,325 bp of concatenated sequences of the two mt rRNA genes as well as three nuclear genes, Biju and Bossuyt (2003) suggested that Ranoidea are constituted of (1) Microhylidae and Scaphiophrynidae, being basal and the sister taxon of the two following groups, (2) Petropedetidae + (Ranidae + (Mantellidae + Rhacophoridae)) and (3) Hemisotidae + (Hyperoliidae + (Astylosternidae + (Leptopelidae + Arthroleptidae))). Only a single species was incorporated, from each of these groups, however, which renders it impossible to address their monophyly. Analyses of RAG1 sequences alone provided some conflicting evidence and placed clade (3) basal and as the sister group of clade (1) + (2) (Van der Meijden *et al.* 2004; Scott 2005; Figs 50, 51).

(1) Microhylidae and Scaphiophrynidae: Formerly assumed to be members of the taxon Hyloidea by morphologically oriented systematists, molecular studies revealed that Microhylidae are members of the Ranoidea representing, together with Scaphiophrynidae, a distinct monophyletic group (Biju and Bossyut 2003; Van der Meijden 2004) at a basal position in the phylogenetic tree of Ranoidea (Fig. 46). While Scaphiophrynidae is a relatively depauperate taxon containing only eleven species in two genera endemic to Madagascar, Microhylidae are more speciose comprising over 300 species in 67 genera with a pantropical distribution. Given their taxonomic and ecological diversity, microhylids are a rather neglected group; their phylogenetic relationships remain unsatisfactorily resolved. While microhylids have often been employed as outgroup-representatives in phylogenetic studies focusing on other groups, their relationships have not so far been emphasized in the same comprehensive way.

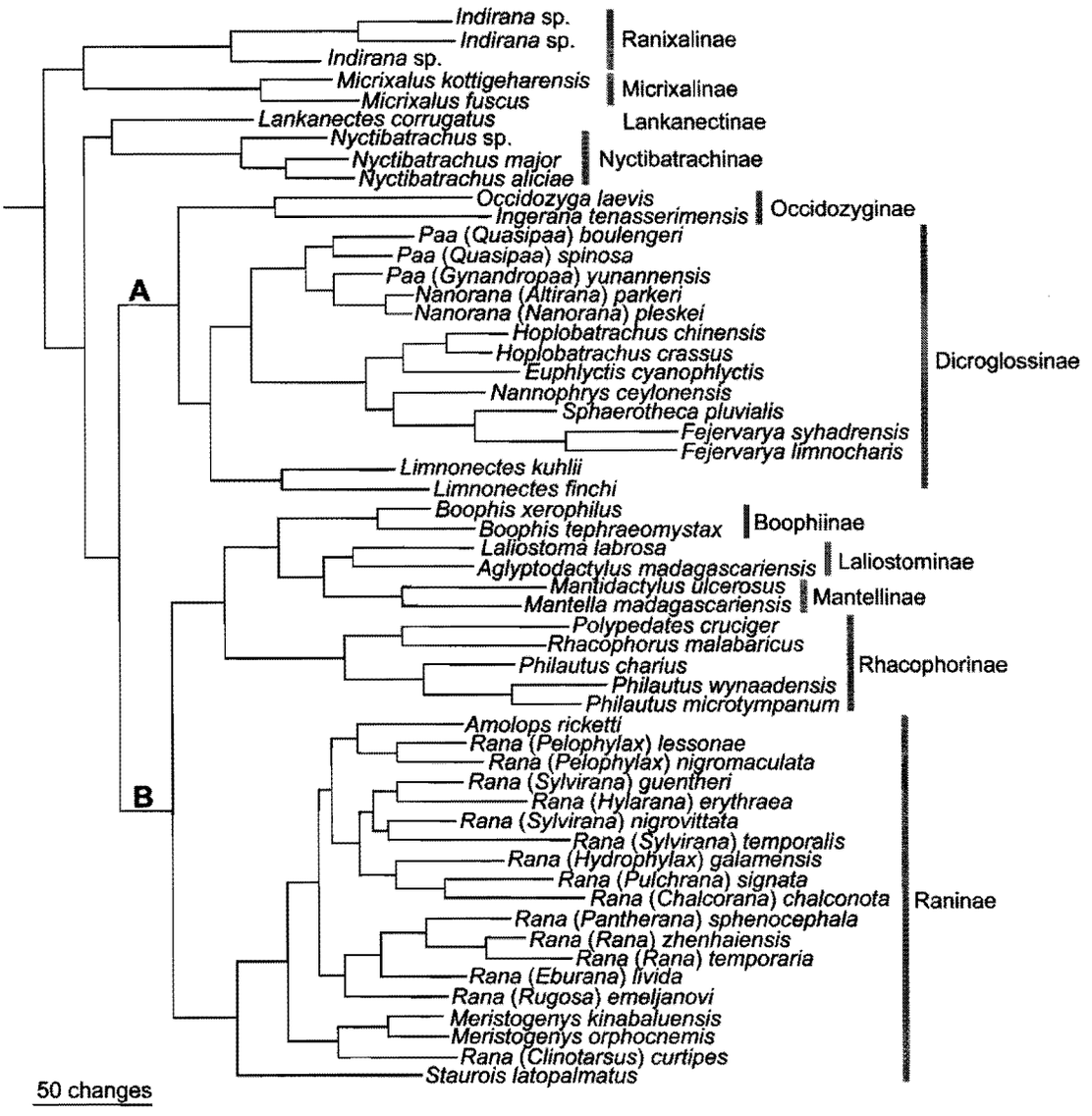


Fig. 49. One of 24 best trees of Ranidae calculated under maximum parsimony as presented by Roelants *et al.* (2004). All maximum parsimony trees revealed two large clades containing ranid subfamilies (A and B). Subfamilies endemic to the Indian subcontinent have basal positions (outside A and B). The original paper provides further details.

(2) "Ranidae": Ranidae *sensu lato*, or True Frogs, comprise more than 700 species, distributed throughout the world. Recent systematic studies consistently reveal that Ranidae as traditionally encompassed are polyphyletic. In order to establish monophyletic groups, major systematic rearrangements were instituted during the past two decades. Most of these attempts, however, were not based on a phylogenetic background, which is why a coherent system is not yet available (Scott 2005). While most systematists working with morphological characters subsume a wide range of taxa within the Ranidae, molecularly oriented researchers tend to recognize various sub-clades to which taxonomic names and ranks have been attributed in a confusing, inconsistent, and subjective way. To give just one example, true ranids, mantellids, rhacophorids, and dicroglossids were often subsumed under a larger taxon, referred to as "Ranidei" (e.g., Vences *et al.* 2003b), or as a so-called "epifamily Ranoidae" (e.g., Dubois 1992; Van der Meijden *et al.* 2004). Some authors still prefer to use the term Ranidae in a wider sense with its daughter taxa being ranked as subfamilies

Fig. 50. Maximum-likelihood phylogram of the Ranoidea based on a combined dataset of nuclear and mitochondrial sequences and rooted with hierarchical outgroups *Latimeria, Homo, Gallus, Lyciasalamandra, Alytes, Agalychnis,* and *Litoria* (not shown), as presented by Van der Meijden *et al.* (2005).

(e.g., Roelants *et al.* 2004). For a recent proposal on the taxonomic treatment of this group, see Frost *et al.* (2006).

Most phylogenetic studies agree in the recognition of a larger monophyletic group that comprises the closely related Ranidae *sensu stricto* (or Raninae), Mantellidae (or Mantellinae), Rhacophoridae (or Rhacophorinae), Dicroglossidae (or Dicroglossinae) as well as further lineages (Biju and Bossuyt 2003; Roelants *et al.* 2004; Scott 2005; Van der Meijden *et al.* 2005). Furthermore, Roelants *et al.* (2004) suggested that Ranidae *sensu stricto* are the sister taxon of a group comprising Rhacophoridae + (Boophiinae + (Laliostominae + Mantellinae)), together forming the sister group of Dicroglossinae + Occidozyginae (Fig. 49). Ranixalinae, Micrixalinae, Lankanectinae, and Nyctibatrachinae are shown to occupy phylogenetic positions basal to this group. Similarly, Van der Meijden *et al.* (2005) found three large ranid clades that are widely congruent with the Ranidae *sensu stricto*, Mantellidae + Racophoridae, and Dicroglossidae, respectively, as recognized by traditional classifications. Additionally, the existence of a further ranid group, composed of predominantly South African species that are thus far affiliated with different ranid subfamilies by the current, morphologically based systematization (e.g., Cacosterninae, Strongylopinae, Phrynobatrachinae, Tomopterinae and others), was hypothesized. The relationships among these large clades (Ranidae, Mantellidae, Rhacophoridae, Dicroglossidae, and the South African clade), however, remain unresolved (Fig. 50). Because of the basal position of this "African clade", Van der Meijden *et al.* (2005) assumed that Ranidae may have an African origin, a hypothesis that remains to be validated because other phylogenetic studies (Biju and Bossuyt 2003; Roelants *et al.* 2004; Van der Meijden *et al.* 2004; Scott 2005) do not support the existence of such an African clade. In the phylogeny obtained from combined

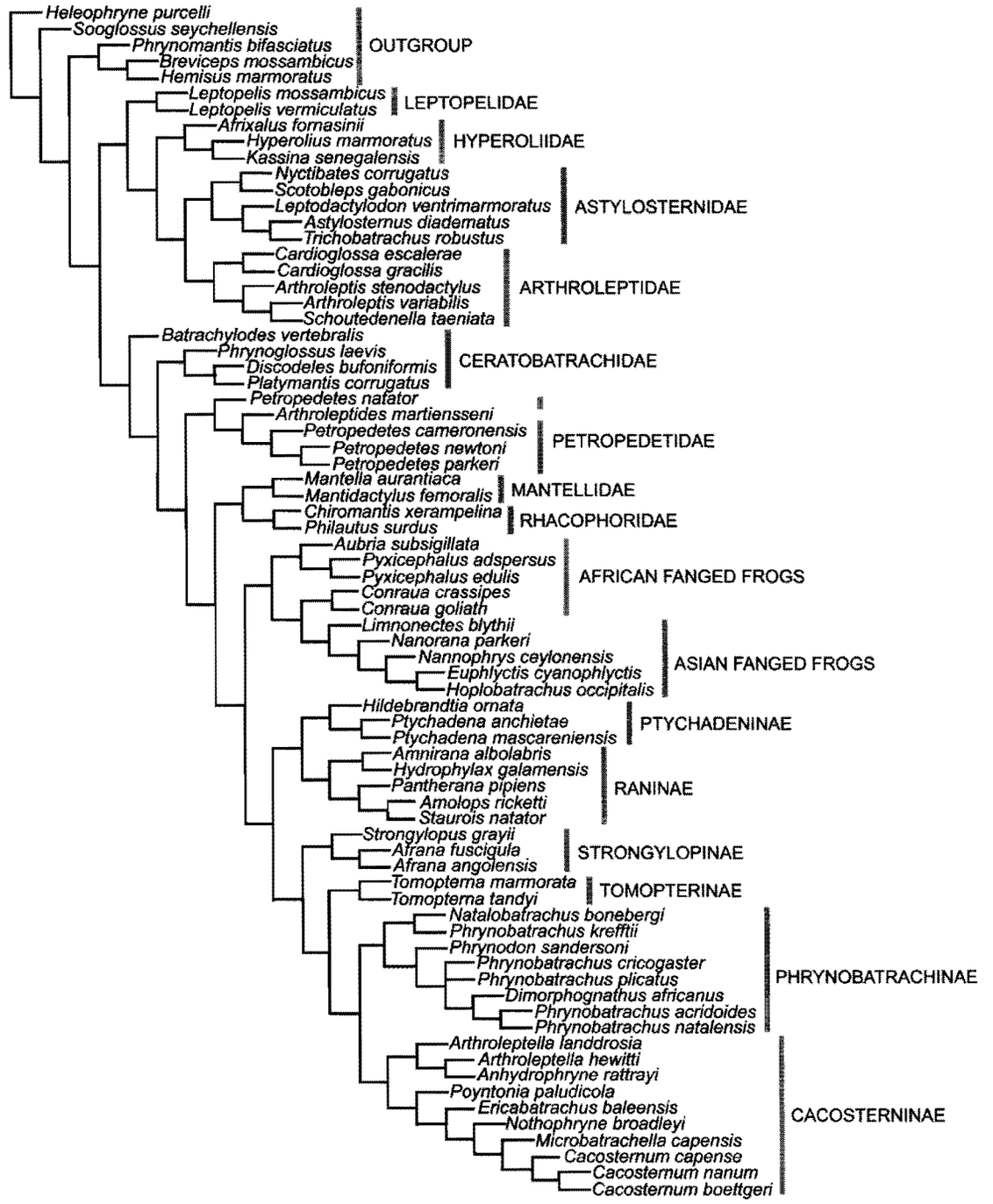


Fig. 51. Phylogenetic relationships within the Ranidae. Strict consensus tree of two equally parsimonious trees retrieved by simultaneous analysis of unweighted morphological (178 organismal characters) and molecular data (about 1 000 bp of 12S and 16S mtDNA). Adapted from Scott (2005).

morphological and molecular data (Scott 2005), taxa that form the South African clade as proposed by Van der Meijden (2005) are almost evenly dispersed across the different ranid lineages (Fig. 51).

It should be emphasized, however, that all aforementioned clades are separated by comparatively short basal branches and that the corresponding branch-support values are comparatively low. This holds true for all molecular reconstructions presented so far (Roelants *et al.* 2004; Van der Meijden *et al.* 2004, 2005). The general lack of resolution among the basal ranid splits, in contrast to the comparatively well-resolved relationships

at the levels above (i.e., among the major neobatrachian groups) and below (i.e., among most recognized ranid genera), was discussed as indicating a "hard polytomy" caused by a rapid radiation of the Ranidae *sensu lato* (Roelants *et al.* 2004; Van der Meijden *et al.* 2005). This low resolution certainly hampers the establishment of robust hypotheses on the phylogeny and evolution of ranid frogs.

In contrast to the results of previous, purely molecular studies, Scott (2005) presented a phylogenetic hypothesis based on a simultaneous analysis of molecular and morphological data. In contrast to all former studies, a derived position for a number of taxa was suggested, e.g., for Cacosterninae, Phrynobatrachinae, and Tomopterinae. According to this phylogenetic hypothesis, a monophylum was recognized, composed of Ceratobatrachidae + (Petropedetidae + ((Mantellidae + Rhacophoridae) + ((African + Asian fanged frogs) + ((Ptychadenidae + Ranidae) + (Strongylopinae + (Tomopterinae + (Phrynobatrachinae + Cacosterninae))))))) (Fig. 51).

(3) Arthroleptidae and related taxa: Most molecular phylogenetic studies agree in the recognition of a large clade that is distinct from the above-mentioned groups. This clade, by some authors referred to as Arthroleptidei and by others as Arthroleptoidae, is frequently shown to comprise Arthroleptidae, Astylosternidae, Hyperoliidae, and Leptopelidae (e.g., Biju and Bossuyt 2001; Vences *et al.* 2003b; Van der Meijden *et al.* 2004; Scott 2005). Except for one study (Van der Meijden *et al.* 2004), where Microhylidae are shown as sister group of the Ranidae *sensu lato* there is also wide agreement that "Arthroleptidei" form the sister group of "Ranidae *sensu lato*". The hypotheses on the phylogenetic relationships within this group, however, are not consistent. Biju and Bossuyt (2003) suggested that Hemisotidae are basal to Hyperoliidae + (Astylosternidae + (Leptopelidae + Arthroleptidae)) (Fig. 46), whereas Scott (2005) proposed that Hemisotidae as well as Brevicipitidae are distinct and that Leptopelidae are basal to a group comprising Hyperoliidae + (Astylosternidae + Arthroleptidae) (Fig. 51).

In summary, the application of molecular data has remarkably influenced the perception of ranid phylogenetics and evolution (e.g., Bossuyt and Milinkovitch 2000, 2001; Vences *et al.* 2003a,b; Roelants *et al.* 2004; Scott 2005; Van der Meijden *et al.* 2004, 2005), but at the same time both morphological and molecular studies thus far have failed to generate a consistent and generally accepted systematization of ranoid frogs. Despite the fact that not all recognized ranid diversity has been studied, the potential of ranids to decipher general patterns of biogeography and evolution was demonstrated by a number of valiant contributions. For example, Bossuyt and Milinkovitch (2001) and Roelants *et al.* (2004) identified India as a reservoir of ancient ranid lineages and proposed these animals as a model for "Out of India" vicariance of vertebrates. Recently, Africa was highlighted as another potential area for the origin of ranid frogs (Van der Meijden *et al.* 2005). This does not necessarily stand in conflict with the Indian origin of other ranids if a Gondwanan history of the entire group is accepted. Future comprehensive studies are desperately needed in order to obtain a better-resolved and more reliable phylogenetic system of ranid frogs.

VI. EVALUATION OF MOLECULAR SYSTEMATICS AND FUTURE PROSPECTS

Molecular data have undoubtedly had a significant impact on the understanding of biodiversity and phylogeny. Today, because of their advantages over classical approaches in which morphological, physiological, or ethological characters are used (e.g., Hillis 1987; Moritz and Hillis 1996; Nei and Kumar 2000), they are an inherent part of systematic research in any group of living organisms. The principle advantages of molecules over non-molecular features are that (1) the structure of DNA is relatively simple and thus allows for studying any group of organisms, (2) the evolutionary change of DNA and proteins follows a rather regular pattern, so that it is possible to compare homologous DNAs or proteins even from distantly related species, (3) the genomes of all organisms consist of long nucleotide sequences and thus contain a much larger amount of phylogenetic information than do morphological characters, i.e., the total dataset is limited only by the genome size, and (4) molecular data are confounded less by environmental influences than

are morphological or physiological data. With an increasing accumulation of molecular data during the past decades, the complexity of molecular evolution became obvious as was expressed for example, in long discussions about the constancy of rates of molecular evolution or the selective neutrality of molecular markers. Such phenomena, however, do not diminish the value of molecular data for systematic research.

When conflicts between molecular and morphological data occur, they may result from homoplasy as well as from non-independence of characters (Shaffer *et al.* 1991). Ideally, phylogenetic studies should be performed at the phenotypic as well as the genotypic level. Such combined approaches usually provide much better descriptions and interpretations of biological diversity than those that focus on just one approach (von Haeseler *et al.* 1993; Moritz and Hillis 1996). Because almost nothing is known about the genetic basis of morphology it is still impossible to analyse genes that are expressed in morphological structures. As shown by Wilson *et al.* (1974, 1977) morphological change and molecular divergence are quite independent, responding to different evolutionary pressures and following different rules. The evolutionary change of morphological characters is extremely complicated and complex, even for short evolutionary time, and it is not clear whether various assumptions required for morphological phylogenetics are really satisfied or not. Nevertheless, morphological characters continue to play an important role in systematic research. The same is true for bioacoustic characters and, to a minor extent, for physiological parameters. As suggested by Moritz and Hillis (1996), the "conflicts" between non-molecular and molecular evidence should not be overemphasized; incongruence between results obtained from different datasets or methods is at least a common phenomenon in almost all fields of biological science.

The central assumption of molecular systematics is that a phylogeny estimated from a set of gene sequences (the gene phylogeny or gene tree) represents the phylogeny of the organisms (the species tree) from which the genes originated (Cotton and Page 2002). A phylogenetic inference based on DNA-sequence variation can, however, be erroneous even though the gene tree has been correctly resolved, i.e., the gene tree may not be congruent with the species tree (Moore 1995). In such cases difficulties arise from the sharing of molecular polymorphisms across species, the sorting of ancestral polymorphisms among species, and interspecific introgression — all of which can lead to discordance between gene trees and species trees (Harrison 1991). As shown by Funk and Omland (2003), a significant proportion of mt gene trees do not deliver a reliable estimate of phylogenetic relationships in the groups examined. Because mt genes are inherited as a single linkage group they do not provide independent estimates of the species tree. In contrast, non-linked nuclear genes from different chromosomes provide independent estimates of the species tree (Moore 1995). Nuclear genes are therefore thought to be better suited to estimating the phylogenetic relationships among taxa and populations than are mt genes, especially when older groups are investigated (Albertson *et al.* 1999). On the other hand, the mt gene tree has a higher probability of representing the species tree than a single nuclear gene tree because the effective population size of mt genes is smaller than that of nuclear-autosomal genes. Especially in the case of short internodes, an mt tree is substantially more likely to be identical with the species tree than a tree based on a single nuclear gene. When a phylogenetic inference based on mtDNA is in doubt, a prohibitively large number of independent nuclear gene trees would be needed to resolve the species tree (Moore 1995). If different loci lead to different phylogenetic hypotheses or if differences between a gene tree and a commonly accepted species tree appear, the reasons may be found in either the method by which gene phylogenies have been constructed or in sampling errors (Cotton and Page 2002).

Progress in technological development, in particular the simplification of molecular techniques and the reduction in expense, will almost certainly lead to a further increase of molecular systematic studies of amphibians. Beside complete mt genomes, nuclear genes will be increasingly analysed in the future. A general problem that will always remain is to find appropriate genes that allow a phylogenetic analysis of the taxa under investigation.

As a first step, the evolutionary patterns of the selected genes have to be investigated to estimate their suitability for phylogenetic reconstructions. Furthermore, future studies should aim to analyse interactions between genes and phenotypic characters to evaluate phylogenies based on morphological data.

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