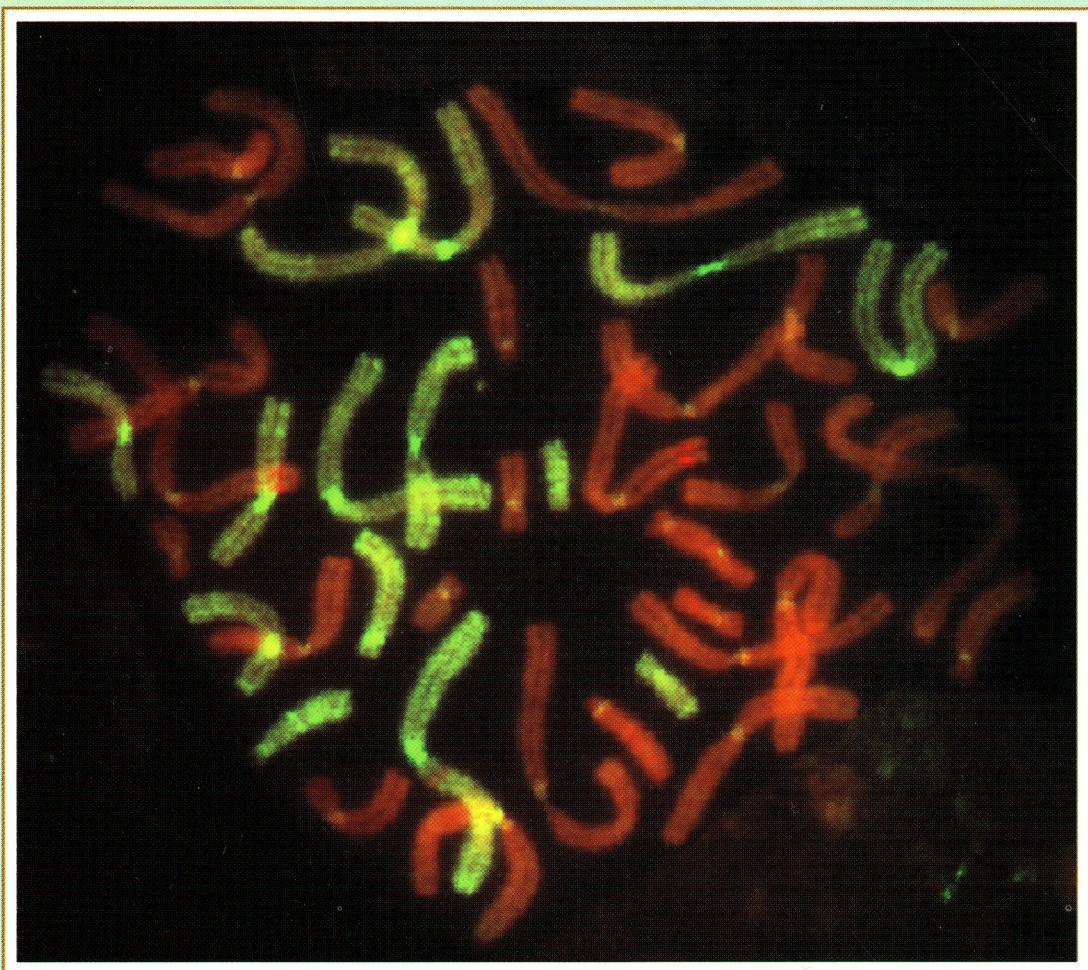


# AMPHIBIAN BIOLOGY

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Edited by

Harold Heatwole

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Michael Tyler

# Amphibian Biology

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Harold Heatwole

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Miguel Vences and David B. Wake

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## I. INTRODUCTION

### A. Species Concepts, Theories on Speciation and their Application in Amphibians

A RESULT of intense interest in species concepts during the past decade is the recognition that controversy has been focused more upon criteria for determining what species to recognize rather than upon what species, in general, are. The intellectual framework presented by de Queiroz (1998, 1999) suggested that despite the appearance of disagreement, there is fundamental agreement among the diverse definitions employed for species. De Queiroz argued that nearly all contemporary biologists accept the idea that species are segments of population-level evolutionary lineages. There remains the large question of what the defining properties of the taxonomic category “species” might be, but the primary issue is to discover when such lineages diverge and when lineages are finally split. The many species definitions — Mayden (1997) lists more than 20 — become

criteria for species recognition under this perspective. The issue of delimiting species remains, but there can be agreement on the goal — an understanding of when evolutionary lineages have irretrievably diverged. As de Queiroz (1998) noted, there are still unresolved issues relating to whether successive species can exist in unbranched lineages, and whether asexual organisms form species, but an intellectual advance has been achieved.

Delimiting species has been identified as a Renaissance issue in systematic biology by Sites and Marshall (2003), because of a number of new methods that result largely from the new kinds of data that have been introduced in recent years. This started about 30 years ago when use of allozyme data became common. Sites and Marshall (2004) dealt with twelve methods for delimiting species; there are more and no doubt new methods will arise as data generation and analysis become more sophisticated. It can be agreed, however, that there is something to be determined, establishment of permanently diverged lineages, and then use whatever evidence is available to make decisions in individual cases. A major issue is determining whether different researchers studying the same larger lineage, in the present case the clade Lissamphibia, have recognized similar entities as species.

The useful reviews by Sites and Marshall (2003, 2004) contrasted population-based (their “non-tree”) versus tree-based methods of determining species’ status. This designation parallels what has emerged as a dichotomy concerning patterns or modes of species formation, that for the purposes of their present review are identified as *vicariant species formation* and *adaptive species formation*.

Vicariant species formation refers to the physical, usually geographic, isolation of fragments of a lineage, by whatever means, and their subsequent history of divergence. Included are the categories termed dichopatric (Bush 1994) and peripatric (Mayr 1954). Both are forms of allopatric species formation, the former referring to the splitting of a lineage into two fragments by some historical disruption of a formerly continuous geographic range, and the latter to what is sometimes called founder species formation, when a new geographic range is invaded. These two kinds of separation can lead to divergence in isolation, and tree-based methods typically are employed to determine whether such fragments should be recognized as species.

Adaptive species formation refers to the situation in which spatially adjacent or overlapping populations have diverged while maintaining the possibility of genetic interaction. Included are parapatric and sympatric modes, and also hybrid species formation. Typically, population-based methods are used to determine whether the diverging units are separate populations or species; criteria include estimation of the degree of genetic exchange taking place.

There is reason to think that vicariant and adaptive species formation might both be functioning in a given lineage experiencing continuing episodes of isolation and recontact. This kind of mixed perspective appears to cause the greatest controversy among systematists. In the end, the most direct evidence evinced in support of species-status is when populations of what are determined as lineages occur in sympatry. When there is hybridization in such circumstances controversy can and does arise. Hybridization is well known and much studied in amphibians, and often leads to differences in how species are determined. Littlejohn and Watson (1993) reported that hybrid zones are known in one-third of southeastern Australia’s frog genera. In contrast to mammals (but not to other animal groups, e.g., birds), hybridizable species of amphibians can be relatively old, averaging an estimated 21 million years (Wilson *et al.* 1974; Prager and Wilson 1975). Accordingly, the observation of a natural hybridization zone between two species is not necessarily indicative of a recent speciation event.

An explicit analysis of the frequency of modes of speciation in vertebrates was made by Lynch (1989). He presented a method for determining which of three modes of species formation had occurred: vicariant, peripheral isolate (these two he recognized as ends of a continuum and both fall into the present vicariant category) and sympatric (largely corresponding to the present adaptive category). Detailed information on geographic distribution and robust phylogenetic hypotheses are necessary elements of his method. Lynch

identified his assumptions and presented case examples that are considered later in this review. His pioneering effort anticipated the explosion of phylogeographic information resulting from techniques that readily generate large amounts of data on DNA sequences.

### B. Characters Used to Define Amphibian Species

In the past, morphology was often the sole criterion used to determine species' status, and it remains the most general criterion. When two distinctive morphs, identifiable by at least two unrelated characters, occur in a single population with age and sex taken into account, they are considered species. Bioacoustics long has been employed as a criterion for frogs, and ever more sophisticated means of discrimination are employed (Schneider and Sinsch Chapter 8 this volume). The idea is that if two distinctive call types are found in a single population and when size of caller and such environmental variables as temperature can be excluded as explanations for the differences, two species are assumed to be involved, even if no morphological differences are detected. In most cases of cryptic species detected by bioacoustics in the tropics, the differences in call structure are distinct, easily recognizable by a trained observer, and leave little doubt that two reproductively isolated species are present.

Starting about 1970, allozymes were employed to determine whether a population included two or more genetic subpopulations. Allozymes have been used to determine the status of species, e.g., *Plethodon dorsalis* was found to be sympatric with a greatly diverged form that resembled it closely in morphology, and that subsequently was named *Plethodon websteri* (Highton 1979). Allozymes have also been used to show the invalidity of taxa previously recognized as separate species on morphological grounds, e.g., *Plethodon gordoni* was shown not to differ allozymically from sympatric *Plethodon dunni*, from which it differed in coloration (Feder *et al.* 1978).

Populational (non-tree based) methods (Sites and Marshall 2003, 2004) depend largely on genetic distances inferred from allozymic or microsatellite differentiation among populations. For many taxa and regions where populational data are unavailable because of low sample sizes, however, pairwise distances between individuals based on DNA sequences of mitochondrial genes (mtDNA, apparently strictly maternally inherited in amphibians) increasingly are used for species discrimination (section IV-B). Such data are primarily used, however, to construct phylogenetic hypotheses of the relationships of species and segments of species and to assess species' boundaries. In "total evidence" approaches (Wiens and Penkrot 2002) tree-based methods include other kinds of data as well. MtDNA haplotype clades recovered using established algorithms show relatively rapid coalescence and thus provide much useful data that can be interpreted historically (Avise 2004). Haplotype trees are often interpreted as if they are decisive in finding clades that when phylogeographically distinct are recognized as species. Single populations, however, can share two or more haplotypes, and presumptive species can be composites of paraphyletic or even polyphyletic haplotype lineages (Funk and Omland 2003). Sites and Marshall (2004) concluded that all methods will sometimes fail to delimit species' boundaries properly, and virtually all methods will require researchers to make qualitative judgements. In a well-studied example of closely related species of fruit flies (*Drosophila*), Machado *et al.* (2002) and Machado and Hey (2003) concluded that a limited amount of gene flow can continue even after completion of speciation, and that simple bifurcating trees may in some cases be unable to reflect the complex history of species formation. Due to the many combinations of deterministic and stochastic processes associated with speciation, such fuzzy boundaries are unavoidable (Sites and Marshall 2004). This circumstance warns against oversimplified approaches to delimitation of species.

### C. The Concept of Phylogeography

Since its formalization as a distinct field (Avise *et al.* 1987), phylogeography has been closely related to systematics. The key element in phylogeography is the development of

intraspecific phylogenies, based mainly on mitochondrial DNA. Taken together with conceptual advances in population genetics (Avise 2000, 2004), modern phylogeography has greatly enhanced the abilities of systematists to function by facilitating the identification of historical population-level units within what have been recognized as species. Phylogeographic patterns are important elements of systematic analyses and increasingly there is a focus on the search for concordant patterns of distribution of allozymes, morphology and mitochondrial DNA haplotypes. Very large databases now exist that permit comparisons of different kinds of data with respect to patterns of geographic variation. When morphology, allozymes and mtDNA haplotypes show congruent patterns (e.g., Jockusch *et al.* 2001), determination of species seems straight-forward. Such patterns are central to understanding vicariant elements of species formation. When, however, incongruence is observed, as is becoming increasingly common (Funk and Omland 2003), the old problems of making determinations of species' status for geographically isolated populations return. While mtDNA is often treated as if it were a neutral marker, Ballard and Whitlock (2004) argued that adaptation often influences the course of its evolution, and they caution against many of the assumptions made by systematists.

## II. VICARIANT SPECIES FORMATION

### A. Dichopatric versus Peripatric

A subtle shift in emphasis with respect to vicariant species formation has taken place in recent years. Earlier, range subdivision, now frequently termed dichopatric species formation, was emphasized, but founder effect, or peripatric, species formation has gained much attention. The former is based on the idea that a once continuous range is disrupted and some barrier to dispersal forms. Latent in the framework of this conception is the idea that species are to some degree maintained as entities by gene flow. When gene flow is disrupted, now isolated populational segments are free to diverge under the influence of natural selection as well as by neutral factors that require either small populational sizes, much time, or both. Eventually, when the two groups are reunited, a sympatry test occurs, and either the units are now fully independent or they demonstrate some degree of reproductive compatibility, ranging from production of sterile hybrids grading to situations in which essentially free interbreeding leads to merger of the once distinct units. Of course, in many instances the units have diverged to some degree and no sympathy test has happened. In such situations different criteria of independence of lineages are used, and controversy is inevitable. Wiens (2004a) argued that geographically isolated segments of species become species when populations are unable to penetrate marginal, peripheral environments. He also argued that the failure of populations to adapt to ecological conditions at the edge of the species' range is the fundamental issue in geographic isolation and thus sets up lineage-splitting, which is tantamount to species formation when accompanied by some degree of differentiation (how much and what kind remains controversial). In other words, dichopatric species formation places an emphasis on isolation where parapatric species formation focuses on adaptive divergence.

Peripatric species formation contrasts with dichopatric in that previously unoccupied regions are invaded by small numbers of migrants and small populations are established that start with reduced variability and then, because they are small, experience ongoing genetic drift. Without going into detail concerning this somewhat more controversial mode of vicariant species formation, one can simply ask which pattern is most frequently encountered in amphibians. Using molecular markers, Avise (2004) has given criteria for determining which mode of speciation has occurred. Available data strongly suggests that most species formation in amphibians is vicariant, principally dichopatric. The most common evidence is the parapatric or allopatric distribution patterns of extant sister species (e.g., Roberts and Maxson 1985; Watson and Littlejohn 1985; Lynch 1989).

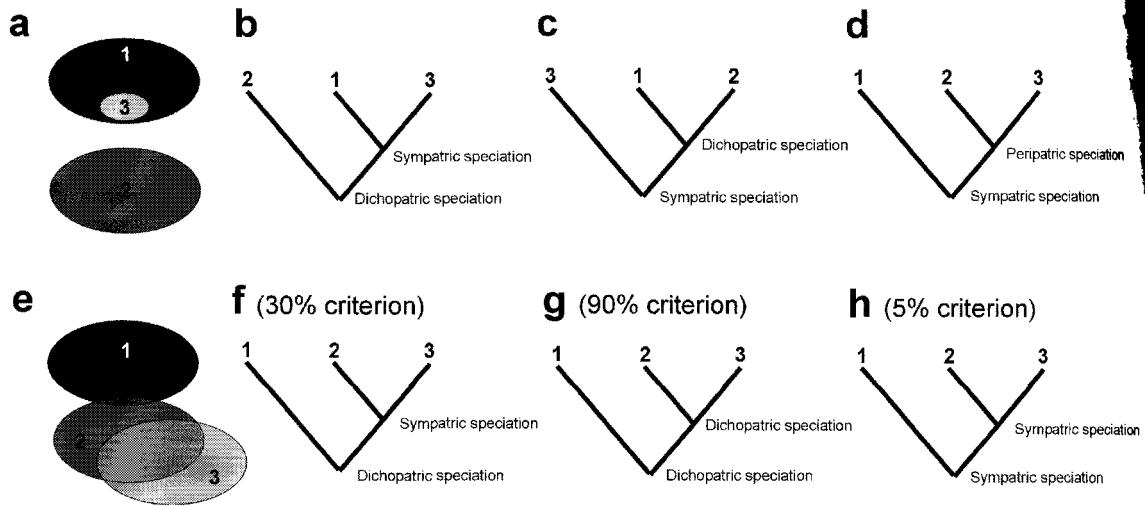
Lynch (1989) listed the assumptions that he made for vicariant species formation as (1) an ancestral cosmopolitan distribution, (2) geographic ranges of sister lineages

juxtaposed and not overlapping, and (3) sister species with ranges of equivalent sizes. Distributional gaps between sister taxa result from extinction, and sympatry requires dispersal. His peripheral isolate mode (peripatric) assumed distributions of sister lineages as well to be are juxtaposed and not overlapping, but ancestral distributions are not cosmopolitan, and the sister lineages have geographic ranges that differ markedly in size. The parental species has a large distribution. Distributional gaps are expected because the peripheral isolate resulted from dispersal. Sympatry accordingly requires more dispersal. Lynch's sympatric model assumed ancestral distributions that were not cosmopolitan and geographic ranges of sister taxa that overlap, but there were no expectations about sizes of geographic ranges, distributional gaps, or dispersal. For 66 cases analysed (three genera of frogs — *Eleutherodactylus*, *Ceratophrys*, and *Rana* — three of cyprinodontiform fishes, and one of passerine birds) his vicariant mode explained 71% of the variation, the peripheral isolate mode 15%, and the sympatric mode 6%. For the *Rana* dataset (from a resolved cladogram of 23 species in the leopard-frog complex [Hillis *et al.* 1983]), Lynch estimated that geographic-subdivision species formation accounted for 72.7% of the 22 speciation events, peripheral isolates for 4.9–9.2%, and sympatric for 4.6–18.2%. As Lynch noted, these results are surprising because of the recent emphasis on peripheral isolates and sympatric modes. The *Rana pipiens* complex has long been recognized to be especially difficult taxonomically; whether the case is unusual is difficult to determine because few amphibian clades have been studied so thoroughly (the green-frog complex of Europe is another group of *Rana* that is well studied, but it differs in its unique sexual system, see below). For *Ceratophrys* (phylogenetic hypothesis from Lynch [1982]), five of the six speciation events were attributed to vicariance, and one to peripheral isolates. For *Eleutherodactylus* (phylogenetic analysis from Miyamoto [1983]), five of eight events (62.5%) were attributed to vicariance, one to peripheral isolates, one to sympatric differentiation, and one was indeterminate. Lynch, concerned with the then-prevalent biogeographic assumption of nondispersal, concluded that dispersal was unnecessary (e.g., because of habitat alterations during the Pleistocene) and not a logical necessity.

While the analyses of Lynch are informative, they are strongly dependent on threshold values in the proportion of overlap between sister species, or on relative range sizes (Fig. 1) (Chesser and Zink 1994), and thereby on phylogenetic and distributional knowledge. Phylogenetic hypotheses are being generated at rapid rates but many of the new species being described from tropical regions are known from only a single or narrowly restricted locality. Examination of extensive phylogeographic, phylogenetic and distributional databases that currently exist suggests that the general conclusions of Lynch are valid and that the vast majority of amphibian speciation events probably were vicariant by means of geographic subdivision (see also Wiens 2004a). Considering the wealth of phylogenetic data on amphibians now available and the improved knowledge of the distribution of many species, Lynch's methodology can be applied to further datasets, and its combination with other approaches such as modelling of environmental niches (Graham *et al.* 2004) seems promising. An important perspective is also the inclusion of genetic distances, which can be used to infer the ages of nodes and thereby to use plots of range overlap through evolutionary age of nodes (Fig. 2) to make inferences about the geographic mode of species formation.

## B. Gene Flow, Dispersal and Amphibians on Islands

In the typical dichopatric mode of species formation the critical issue is cessation of gene flow, which is related to dispersal capability. Amphibian species typically show high levels of geographic variability in molecular markers (see also below, section V). Very few amphibian species give evidence of species-wide gene flow. Within direct-developing species in particular, differentiation *in situ* most probably proceeds through a combination of neutral and selective factors operating over long spans of time. This may explain why there are more than 25 species of *Thorius* in a small part of eastern Mexico, more than 140 species of *Eleutherodactylus* in Ecuador, and more than 60 species of direct-developing frogs in

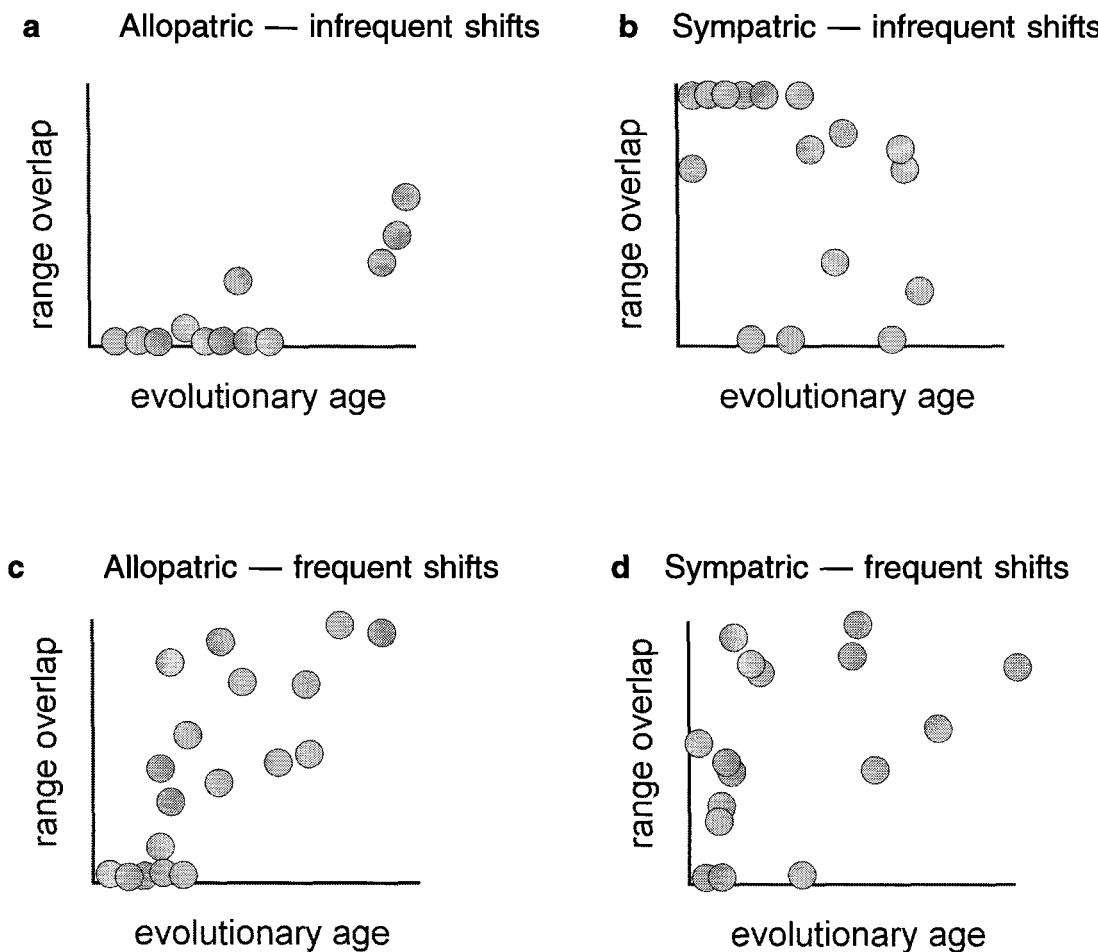


**Fig. 1.** Inferring the mechanism of speciation using interspecific phylogenies and current distributions. Partly after Lynch (1989) and Losos and Glor (2003). Given the distributional patterns in (a), the first row shows the influence of phylogeny on the assumed speciation mode. (b) Sympatric speciation is assumed for the origin of 1 and 3 because they are sister taxa and their distributions overlap. Dichopatric speciation is assumed for the split between 2 and 1-3 because the distributional area of 2 does not overlap with the merger of the distributional areas of 1 and 3. (c) Dichopatric speciation is assumed for the origin of 1 and 2 because they are sister taxa and their distributions are of similar size and do not overlap. Sympatric speciation is assumed for the split between 3 and 1-2 because the distributional area of 3 overlaps with merged areas of 1 and 2. (d) Peripatric speciation is assumed for the origin of 2 and 3 because they are sister species, their ranges do not overlap and the range of one is much smaller than the range of the other. Sympatric speciation is assumed for the split between 1 and 2-3 because the distributional area of 1 overlaps with merged areas of 2 and 3. Note, however, that there might be a contradiction in this simplistic reasoning because the overlap between 1 and 2-3 results from a presumable dispersal of 3 subsequent to the origin of 1. Given the distributional patterns in (e), the second row shows the influence of the definition of range overlap on the inferences of mode of speciation. (f) If an overlap of 30% of a species' distribution with that of a second species is defined as the cutoff, then taxa 2 and 3 are considered to have overlapping ranges and to have originated in sympatry, but the small overlap between taxon 1 and lineage 2-3 is not considered and dichopatric speciation is assumed. (g) If a very strict criterion is employed and overlap is only accepted if the ranges of two taxa coincide by 90%, then dichopatric speciation is inferred for both splits. (h) If an hypothetical relaxed criterion of 5% is employed, then even the small overlap in range between taxon 1 and lineage 2-3 would be considered and sympatric speciation inferred for both splits.

Sri Lanka (Manamendra-Arachi and Pethiyagoda 2005; Meegaskumbura *et al.* 2002; Meegaskumbura and Manamendra-Arachi 2005). What is important is spatial heterogeneity and long spans of time, because most of these species have narrowly restricted distributions and appear to be old (based on molecular divergence levels, e.g., Crawford 2003a).

European populations of *Salamandra salamandra* range over most of the continent, from Spain to Greece, and probably recolonized Central Europe after the last glaciation. The occurrence of specific east-European and west-European haplotypes as well as allozymic alleles suggests that this recolonization originated from at least two source populations, probably the Iberian peninsula and the Balkans (Steinfartz *et al.* 2000). Two divergent populations in northern Spain (*S. s. bernardezi*) and southern Italy (*S. s. gigliolii*) surprisingly were sister to each other. These probable remnants from a previous colonization event seem to have maintained their separate genetic identity even though they are not separated by geographic barriers from closely related neighbouring populations (Steinfartz *et al.* 2000). This example illustrates how gene flow and admixture, at least of mitochondrial haplotypes, can be restricted even among neighbouring and closely related amphibian populations.

Vicariant or peripatric speciation is obvious for amphibian species endemic to islands. Amphibians long have been thought to be unable to disperse over the sea but now there is compelling evidence, at least for frogs (Hedges *et al.* 1992; Vences *et al.* 2003b). Amphibians show near absence on true oceanic islands, with Mayotte, São Tomé and Príncipe (with endemic frogs, and São Tomé with a probable endemic caecilian; Measey



**Fig. 2.** Inferring the mechanism of speciation using range overlap, phylogenetic relationships and evolutionary age (as roughly equivalent to genetic differentiation). The graphs show hypothetical examples obtained by modelling under the assumption that range movements occur by large-scale shifts of entire species' ranges. Schematically redrawn from Barraclough and Vogler (2000). Note that when frequent range shifts occur the patterns produced by the two speciation modes cannot be reliably distinguished.

*et al.* 2006) being rare exceptions (Schärtti and Loumont 1992; Vences *et al.* 2003). Molecular data (e.g., Hedges *et al.* 1992; Vences *et al.* 2003b, 2004b) have revealed that overseas dispersal in frogs may be more common than previously thought, and this explanation probably applies to many of the occurrences of endemic species on Caribbean and Pacific islands (Fiji and Palau, and islands in the Sunda Region and Philippines) (see also Kaiser *et al.* 1994). Despite this change of paradigm there is little doubt that amphibians belong to the lower end of the spectrum of relative dispersal ability (Inger and Voris 2001; Brown and Guttman 2002).

Salamanders on islands are usually of the same species as on the adjacent mainland. A rare exception is *Oedipina maritima* from Isla Escudo de Veraguas (García-París and Wake 2000), an island in the Caribbean Sea off the coast of Panama that is also the home of an endemic species of sloth. Another exception is *Batrachoseps pacificus*, endemic to the northern Channel Islands in the Pacific Ocean off southern California (Jockusch *et al.* 2001). On the small Atlantic islands of Cies and Ons, off the Galician coast in northwestern Spain, separated from the mainland only for about 9 000–7 000 years, populations of *Salamandra salamandra* occur that are morphologically divergent from the mainland populations by being slightly smaller and with a reduced yellow pattern on Ons (Galán Regalado 2004). These salamanders, however, appear to be genetically rather similar to those from the

mainland (S. Steinfartz, pers. comm.) and therefore provide a good example of how rapidly different morphologies can evolve under fully allopatric conditions. A contrast to these examples is the presence of nonendemic salamanders and caecilians on Isla Gorgona, an island in the Pacific, west of Buenaventura, Colombia (Brame and Wake 1963).

The generalization that amphibians are usually poor dispersers and highly philopatric (Blaustein *et al.* 1994), with a limited osmotic tolerance (Balinsky 1981), is correct, and these conditions affect both pattern and process of species formation prevalent in this group. Their populations in most cases show a strong phylogeographic structuring (Avise 2000); this has been found in studies such as those of Alexandrino *et al.* (2000, 2002), Austin *et al.* (2002, 2004), Babik *et al.* (2004), Barber (1999a, 1999b), Bos and Sites (2001), Burns *et al.* (2004), Chiari *et al.* (2005, 2006), Church *et al.* (2003), Crawford and Smith (2005), Donnellan *et al.* (1999), García-París and Jockusch (1999), Goldberg *et al.* (2004), Green *et al.* (1996), Hoffman and Blouin (2004), Jaeger *et al.* (2005), Johansson *et al.* (2006), Masta *et al.* (2003), Macey *et al.* (2001), McGuigan *et al.* (1998), Mulcahy *et al.* (2000), Nielson *et al.* (2001), Noonan and Gaucher (2005), Riberon *et al.* (2001), Rowe *et al.* (1998), Rowe *et al.* (2005), Schneider *et al.* (1998), Shaffer *et al.* (2000, 2004), Shaffer *et al.* (2000), Steele *et al.* (2005), Symula *et al.* (2003), Vieites *et al.* (2006), Zeisset and Beebee (2001), and many others reviewed in more detail in the following sections. Slade and Moritz (1998) observed that introduced populations of *Bufo marinus* were uniform in their mitochondrial sequences, validating interpretations of phylogeographic structure as indicative of autochthonous status (Vences *et al.* 2004b). Lougheed *et al.* (1999) found that gene flow in an Amazonian poison frog, *Epipedobates femoralis*, was not relevantly hindered by major rivers but the phylogeographic structure instead reflected ancient ridges, no longer evident on the landscape. Funk *et al.* (2005) observed in the Columbian spotted frog *Rana luteiventris*, based on microsatellite data, that mountain ridges had the effect of strongly reducing gene flow among populations situated on either side of the ridge. In addition, they also found reduced gene flow among low-elevation and high-elevation populations, and a reduced genetic variation at high elevations. Their "valley-mountain" model of population structure suggests two ways in which adaptive and non-adaptive influences can interact in affecting genetic structure and differentiation at small spatial scales: (1) Differentiation (that is presumably non-adaptive) may occur following a restricted dispersal across physical barriers (ridges). (2) Adaptive differentiation may occur in frogs dispersing across an elevational gradient; their survival rates after dispersal may be lower because of missing adaptations to local ecological conditions or they may have lower rates of reproduction because of elevational differences in breeding phenology.

Newman and Squire (2001), on the basis of a literature survey on fine-scale population structures in amphibians, and their own microsatellite data on wood frog populations (*Rana sylvatica*), concluded that in general amphibian populations exhibit a high degree of spatial structure, particularly when inter-populational distances exceed several kilometres. At the finest scales (<1–2 km) populational differences were not predictable unless some barrier to dispersal were present. At this scale, the authors found similar allele frequencies suggestive of high gene flow; they emphasized the possible importance of extinction-recolonization founder events, driven, for example, by periodic drying of wetlands, in contributing to the development of genetic subdivision by increasing the rate of stochastic fluctuation in allele frequencies. Palo *et al.* (2004b) studied the geographic subdivision of populations of the widespread frog, *Rana temporaria*, using microsatellite markers. They observed a high level of substructuring even in northern Fennoscandia, a region that was presumably colonized less than 10 000 years ago following the last glaciation. They suggested that processes other than restricted dispersal capacity needed to be explored to explain the high degree of populational subdivision in amphibians. Nevertheless, compared to other areas, the genetic differentiation among these populations is rather low (as is evident from their near-identical mitochondrial haplotypes; Palo *et al.* [2004b]), and this species provides one out of many examples that amphibians are capable of very rapidly moving into newly available space. In fact, many species essentially followed the

glacial fronts northward at the end of the Pleistocene, establishing new populations of low genetic differentiation (e.g., Larson *et al.* 1984), although the mitochondrial and nuclear genetic signatures of these processes are not always fully concordant in space (see section IV below).

Despite some complications (see Marsh and Trenham 2001), amphibian spatial dynamics resemble classical metapopulation models, with subpopulations in breeding waters blinking in and out of existence, and extinction-recolonization regularly taking place through stochastic or deterministic processes. Case studies of introduced frog populations have shown that low numbers of individuals can found viable populations in which nuclear genetic variability is not significantly reduced and bottleneck effects and subsequent demographic explosions are sometimes not, or only weakly, detectable, although genetic differentiation in terms of microsatellite allele frequencies is apparent (e.g., Rowe *et al.* 1998; Estoup *et al.* 2001; Zeisset and Beebee 2003). Several other population genetic studies have shown that genetic differentiation proceeds with increasing physical barriers to gene flow (e.g., Hitchings and Beebee 1997, 1998; Gibbs 1998).

To understand the driving forces of speciation under fully vicariant (mostly peripatric) conditions in amphibians, the comparison of recent insular endemics with their closest relatives, once these have been identified, appears to be a fruitful field of study. Gene flow from insular populations, whether they originated by dispersal or vicariance, to their mainland relatives would almost certainly be interrupted, and insular populations of organisms, in general, tend to have reduced genetic diversity (Frankham 1997). They may also be subject to faster rates of molecular evolution due to their smaller population size (Johnson and Seger 2001).

### C. Examples of Vicariant Species Formation

In species with less radical geographic isolation than true of insular species, the patterns of variation are usually more complex. Determining the species-status of geographic segments of clades is a major challenge. Geographic variation and allopatry pose difficulties for all the characters typically used to determine species-status. Comparative studies of modes and patterns of species formation necessarily assume that all species are equivalent, so criteria for species recognition are important. Yet, even for well studied groups there continue to be difficulties. Important components of the debate over recognition and delineation of species are the patterns of geographic variation and their interpretation. Following are a few examples showing how different authors have dealt with this problem.

The findings from many phylogeographic studies of amphibians show that the distributions of haplotype clades are almost entirely exclusive, with relatively little geographic overlap even within a species (but see below). The studies of Mayr (1963) and many others, which led to the view that vicariance species formation is the dominant mode for terrestrial vertebrates were based on the existence of geographic variation frequently interpreted as subspecific. While the use of subspecies has been largely abandoned (but see below), many of the populational units originally so designated subsequently have been raised to the specific level, following an argument that geographic units are also genealogical units (as is the case with phylogeographic units) and are to some degree incipient species. Cracraft (1997) argued that such basal diagnosable units are effective functional equivalents of evolutionarily significant units and that they should be formally named as species. Certainly such units would have many advantages over traditionally recognized species, but the main issue is what constitutes a population-level unit. Discordances between datasets show that there is no absolute criterion for what constitutes a species. Nevertheless, the current trend is to use fine levels of diagnosability in recognizing amphibian species.

Two plethodontid salamander genera that have been studied in detail, *Batrachoseps* and *Plethodon*, are examples of vicariant species formation. *Batrachoseps* contained two species as recently as 1954 (Hendrickson 1954), but subsequently the widespread Californian taxon, *B. attenuatus*, has been extensively subdivided into the following species: *B. attenuatus*,

*B. gavilanensis*, *B. luciae*, *B. incognitus*, *B. minor*, *B. major*, *B. pacificus*, *B. nigriventris*, *B. gregarius*, *B. diabolicus*, *B. relictus*, and *B. simatus* (Jockusch and Wake 2002). In addition, new geographically isolated species have been found: *B. campi*, *B. robustus*, *B. regius*, *B. kawia*, *B. stebbinsi* and *B. gabrieli*. At least two species (*B. gregarius* and *B. major*) contain haplotypic lineages that are not sister, but in general the distribution both of species and haplotype clades are either non-overlapping or overlap only narrowly. Only *B. nigriventris*, which co-occurs with seven species, is broadly sympatric with others, and it is invariably the smallest or most attenuate member of sympatric pairs.

Species of *Plethodon* once considered to be widespread have been broken into many species, usually with minimal or no overlap, and with frequent zones of hybridization where species interact in what are presumed to be zones of secondary contact (e.g., Highton and Peabody 2000). For example, species once diagnosed by morphological differences show geographically distinct patterns of genetic differentiation, as measured using allozymes. The species known as *P. jordani*, a polytypic species with many montane isolates, now is broken into seven geographically distinct species, and *P. glutinosus* now contains 16 species. Some of these species are joined by regions of genetic exchange considered by Highton (e.g., 1998, 2000) to be hybrid zones but by others (Petraska 1998; Wake and Schneider 1998) to be regions of genetic interchange because the so-called hybrid zones are wide in relation to dispersal distances of individuals and the two parental types are not sympatric. This complicated system involves many parapatric and sympatric occurrences, but sympathy is between populations formerly included in *P. jordani* and *P. glutinosus*. There is limited sympathy between members of the more traditional species. *Plethodon glutinosus* and *P. aureolus* co-occur at one locality (and were once confused). *Plethodon kentucki* and *P. glutinosus* are sympatric over most of the range of *P. kentucki*, but some hybridization occurs. The *Plethodon cinereus* group is also complex, but in it there are a series of geographically distinct species, some of which have very narrow parapatric contacts, and one species, *P. cinereus*, that is more widely distributed and with some areas of sympathy with others. *Plethodon cinereus* may have competitively displaced other members of its group, leading to their geographic restriction.

Alternative interpretations for both *Batrachoseps* and *Plethodon* would recognize some of the parapatric assemblages of species as portions of a geographically fragmented species-complex, as is the current case with *Ensatina* (see below). Petraska (1998) offered an alternative interpretation of Highton's taxonomy of the *P. glutinosus* complex. He considered only two of Highton's 15 newly recognized species to have reached the specific level: *P. aureolus* and *P. kentucki* (sympatric with other members of the complex and with very limited or no hybridization). A third species, *P. teyahalee* (Petraska used an alternative and invalid name, *P. oconaluftee*) was tentatively recognized as a species because at one site at least it is sympatric with another form without hybridization; it does hybridize, however, with *P. glutinosus* (*sensu lato*) in many areas. Thus, Petraska considered 12 species to be subjective synonyms of *P. glutinosus*. This extreme view was ignored by Highton (2000), whose manuscript may have been prepared prior to the appearance of Petraska's (1998) book. Later, a committee of herpetologists (Crother *et al.* 2000) endorsed Highton's taxonomy. If Petraska's taxonomy were adopted, the extensive genetic interactions among members of the complex would be viewed as indications that geographic fragments of a widespread ancestral form were still capable of genetic interaction and had not crossed an irreversible genetic barrier.

Species of *Batrachoseps* display allopatric or parapatric distributions. An example is the *B. pacificus* complex, which includes genetically distinct groups of populations that Yanev (1980) considered to be "semi-species" on the basis of allozymes, but which she did not name. When Jockusch *et al.* (1998, 2001) found mtDNA to have congruent distributions, Yanev's semi-species were recognized as full species and several more species recognized. Four of these species have closely contiguous, parapatric distributions in central coastal California: *B. gavilanensis*, *B. luciae*, *B. incognitus* and *B. minor*. These morphologically similar (but not identical) forms are distinct allozymically, and have mtDNA haplotypes that are

not sister to close neighbours. Local sympatry is lacking and no hybrid individuals or hybridized populations have been identified. Two other species in the complex from southern California, *B. major* and *B. pacificus*, are allopatric with respect to each other and their more northern relatives. They were recognized as species because they are morphologically distinct (Brame and Murray 1968), but they also are differentiated with respect to allozymes and mtDNA. The current phylogenetic hypothesis (Jockusch and Wake 2002) finds *B. minor* and *B. major* to be sister taxa that are widely separated geographically; *B. incognitus* is sister to them, then sequentially to *B. pacificus* from the Channel Islands, *B. luciae*, and *B. gavilanensis*. Because none of these are strictly sympatric they could all be placed in the same species, but because they are heterogeneous in all measured characteristics, recognition of six species is most informative with respect to assessment of biodiversity. A complicating factor occurs within *B. major*, itself a species complex containing a morphologically distinct form recognized as a separate species or subspecies, *B. aridus*, as well as phylogenetically distinct haplotype groups in southern California and northern Mexico (Wake and Jockusch 2000). The haplotypes of these members of the complex are not sister to those of *B. major* (*sensu stricto*), but because *B. aridus* displays little allozymic differentiation, all the members of the complex currently are treated as being conspecific. The main theme in *Batrachoseps* has been fragmentation of ranges, partly the result of the long, complicated geological history of the region, and partly to subsequent genetic differentiation.

When populations of a taxon are readily sampled and when there is a good understanding of distributions, interpretations of phylogeographic patterns and patterns of allozymic differentiation are enhanced. This is rarely the case, however, for tropical amphibians, which constitute the vast majority of the species in the world. Increasingly, amphibian systematists are obtaining molecular information for single individuals or single populations of amphibians and then are faced with the problem of determining species status from extensive molecular data but very little information on geographic variation. Geographic and genetic fragmentation is so common in tropical amphibians that Wynn and Heyer (2001) have asked whether widespread species of tropical amphibians exist at all. Their thoughtful analysis is based on allozymes as well as direct study of the organisms in the field, laboratory morphological studies, and some recorded advertisement calls.

Wynn and Heyer (2001) studied *Leptodactylus fuscus*, a widespread tropical frog species ranging from northern Argentina and southeastern Brazil northward into Panama, generally east of the Andes. Sampling was good for a tropical species: 16 locations (most relatively small samples, but some as large as 24 individuals) from throughout this vast range. For comparison, Highton and Peabody (2000) studied salamanders from 142 locations (typically with sample sizes greater than 20) from a relatively small area in the southern Appalachian mountains of southeastern United States. Nei's genetic distances in *Leptodactylus fuscus* ranged from 0 for nearby samples (separated by only a few minutes of latitude and longitude) to as great as 0.352 (for samples from Argentina and Panama). When sample locations are few and well-separated geographically, it is difficult or impossible to determine whether divergence between samples might reflect isolation by distance, in which case denser sampling would show a gradual transition. If a large divergence is also associated with a divergence in morphology, or advertisement call, or mtDNA haplotype distribution, a border between species is inferred instead.

A "weedy" species readily colonizes new habitats and would be expected to have low levels of genetic diversity. Such a pattern was detected for *L. fuscus* in areas to the north of the Amazon, even including the Caribbean islands of Trinidad and Tobago. By contrast, to the northwest and in areas south of the Amazon, strikingly large divergences were found that did not conform to a model based on isolation by distance. From these results Wynn and Heyer inferred that more than a single species is included in *L. fuscus*, and more importantly that systematists might be grossly underestimating the amount of diversity of tropical frogs. In a subsequent examination, Heyer and Reid (2003) studied variation in advertisement call among individuals from 25 localities from throughout the distributional

range of *L. fuscus* and found that call variation was not concordant with genetic variation or geographic distance. In particular, call variation was less than that typical of differences between other closely related species in the genus. They concluded *L. fuscus* to be an example of strong genetic differentiation unaccompanied by call differences. This relatively well-studied system should serve as a caution to workers using only one kind of data to draw systematic conclusions.

The technique for making inferences about isolation by distance that Wynn and Heyer (2001) used in their study of *L. fuscus* was developed by Good and Wake (1992) and extended by de Queiroz and Good (1997). Genetic distance is plotted against geographic distance. Within a species, a straight line with a characteristic slope is expected. Statistical analysis of such a pattern is invalid because points are not independent but visual inspection can indicate whether genetic distance builds as a function of geographic distance. The technique was first used to show that abrupt transitions in patterns of genetic variation identified species borders in the species formerly known as *Rhyacotriton olympicus* (Fig. 3); three new species were identified (Good and Wake 1992). Highton (2000) endorsed the general approach, but criticized the applications, arguing, for example, that Good and Wake described too few species.

Australian frogs of the *Litoria lesueri* species group are confusingly similar in morphology and karyology, but there are some differences that suggest deeper differentiation. Allozymes were studied by Donnellan and Mahony (2004), who decided to recognize four species because they detected fixed differences in 11–19% of the loci studied. Once thus sorted, the species were found to differ in karyomorphology, with one species having one state and the other species sharing another. Each species occupies a distinctive geographic range. Despite the large allozymic differences, evidence of gene flow was detected between some of the species. This complicates the picture, but the problem is more general, because hybrid zones are frequent among Australian frogs (Littlejohn and Watson 1993). Knowles *et al.* (2004) distinguished five fully allopatric species of the myobatrachid frog genus *Philoria* in eastern Australia based on “private” (exclusive to the species recognized) alleles of each species detected by allozyme studies and by reciprocal mitochondrial monophyly. The authors stressed that the habitat of all five species is remarkably similar, that all species have terrestrial breeding (larvae developing in subterranean nests), and that advertisement calls are similar in all species except one. They argued that allozymic differences resulted from a large ancestral population fragmenting into smaller disjunct ones, with post-fragmentation divergence being sufficient to lead to speciation.

Toads from Sulawesi (*Bufo celebensis*) display strong geographic structure based on mtDNA haplotypes (Evans *et al.* 2003a). This pattern is considered to be largely the result of range fragmentation, with only one of seven isolates displaying any evidence of isolation by distance. The six major breaks in distribution correspond precisely with the range limits of macaques, currently arranged in six species (only one of the six borders fails to separate *Macaque* species). This is an especially vivid instance of genetic fragmentation and vicariance leading to the possibility of species formation, but as yet the toads are considered to be a single species because there is no evident morphological or acoustical differentiation. In contrast, fanged frogs, *Limnonectes*, on Sulawesi also are geographically differentiated, but they are not monophyletic, instead having a complex paraphyletic relationship with Philippine taxa (Evans *et al.* 2003b).

Another striking example of range fragmentation is the salamander *Salamandra luschani* (or *Lyciasalamandra luschani*, according to the taxonomic scheme of Veith and Steinfartz [2004]). This species, previously included in the genus *Mertensiella* but phylogenetically more closely related to other species of *Salamandra*, occurs in the eastern Mediterranean region (Veith *et al.* 1998; Weisrock *et al.* 2001). Populations sampled from a small part of southern Anatolia fell into six haplotypic groups, with pairwise divergences suggesting 5.9–7.9 million years of divergence between the groups, but with divergence of the haplotypic clades having taken place in a relatively short time span. These six clades included samples of eight of

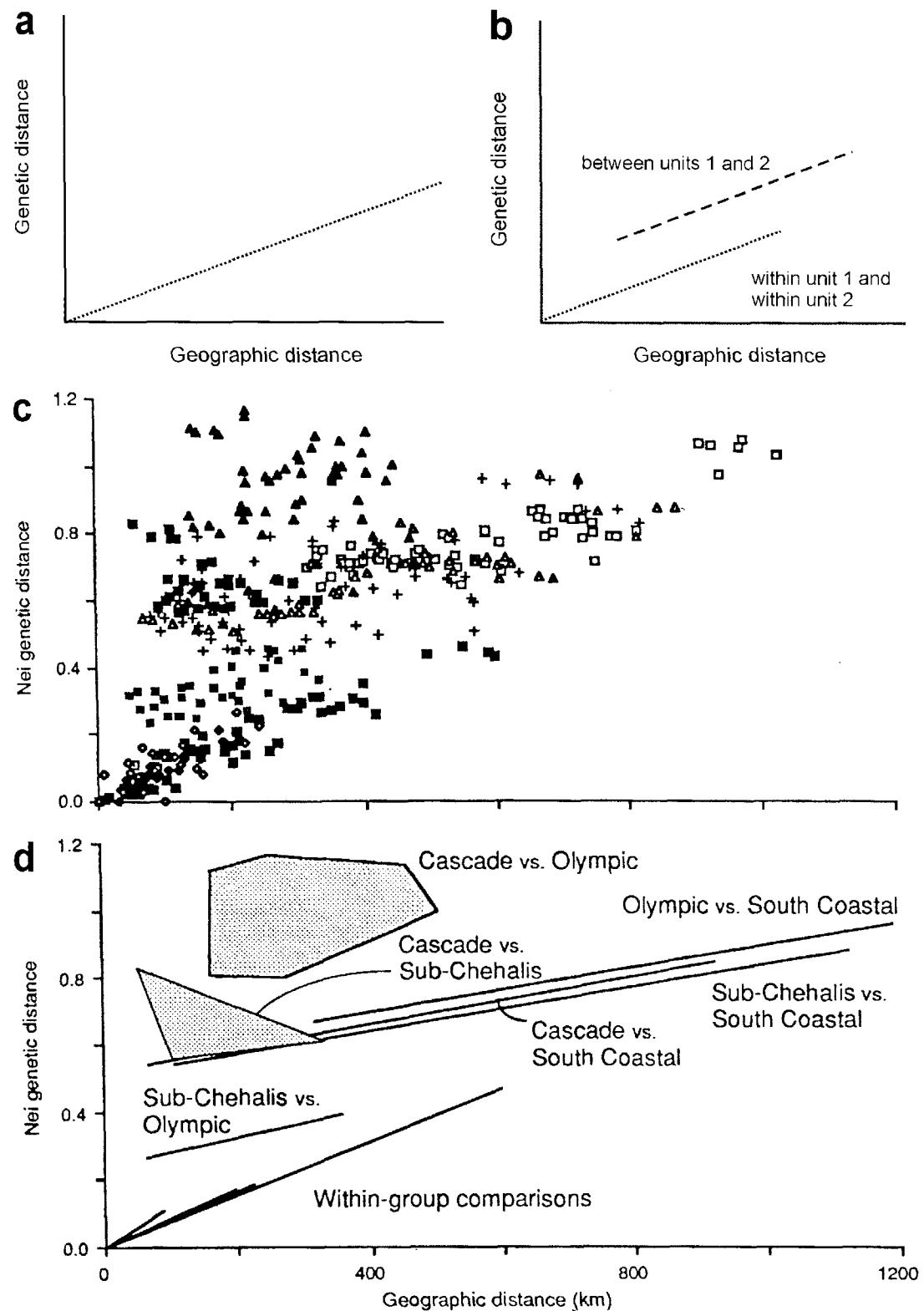


Fig. 3. Expected relationship between geographic distance and genetic distance for genetically continuous and discontinuous populations under an isolation-by-distance model: (a) within a single genetically continuous population along a linear distribution transect; (b) in the case of two units separated by a genetic discontinuity (e.g., two different species) under a vicariance model; displacement of the between-units line is proportional to the time since genetic isolation. Graphs (c) and (d) document the application of this method to the four species in the salamander genus *Rhyacotriton* (*R. cascadae*, Cascade; *R. olympicus*, Olympic; *R. kezeri*, Sub-Chehalis; *R. variegatus*, South Coastal). The original plot is shown in (c), and the comparisons explained and schematically summarized in (d). Graphs (a) and (b) redrawn after de Queiroz and Good (1997), (c) and (d) from Good and Wake (1992).

the nine subspecies (the ninth subspecies, an insular population, was not sampled), with one clade including three subspecies. Weisrock *et al.* (2001) observed that the six lineages are diagnosable on the basis of colour pattern but they made no taxonomic changes. Veith and Steinfartz (2004) subsequently added data on allozyme differentiation and divided *Salamandra luschani* into seven species.

The Sulawesi and Turkish examples highlight the role of vicariance in leading to phylogeographic patterns, as well as the taxonomic conundrum that can result. Other systematists might well have described up to six new species within the *Bufo celebensis* complex. Additional species of *Limnonectes* also will be recognized in the future. On the other hand, the genetic divergences among the four northern species of *Philoria* recognized by Knowles *et al.* (2004) are relatively low and other authors might have hesitated in describing species showing no relevant morphological or bioacoustic differences.

Amphibians have been extensively studied using allozymes, mtDNA, or both kinds of data, and documentation of geographic patterns that have been interpreted systematically is extensive. Highton (e.g., Highton and Webster 1976; Highton 1995) was one of the first to show the patchwork nature of geographic variation and to translate it into taxonomic changes (e.g., Highton 1979). Examples of other taxa in which documentation of molecular (including allozyme) geographic differentiation has led to recognition of new species include desmognathine salamanders of the southern Appalachian mountains (Tilley and Mahoney 1996; Anderson and Tilley 2003), cave and stream salamanders (*Eurycea*) from Texas (Chippindale *et al.* 2000; Hillis *et al.* 2001), minute salamanders (*Thorius*) of montane eastern Mexico (Hanken and Wake 1994, 1998), Mexican web-footed salamanders (*Bolitoglossa*; Parra-Olea *et al.* 2002), moss salamanders (*Nototriton*) of Costa Rica (Good and Wake 1993; García-París and Wake 2000), Philippine and Bornean steam frogs (*Rana signata* group) (Brown and Guttman 2002), European painted frogs (*Discoglossus*) (Lanza *et al.* 1984; Capula *et al.* 1985; Busack *et al.* 1986), treefrogs (*Hyla*) (Nascetti *et al.* 1995), midwife toads (*Alytes*) (Arntzen and Garcia-Paris 1995) and cave salamanders (*Hydromantes*) (Lanza *et al.* 1986, 1995).

While vicariant species formation is thoroughly documented for frogs and salamanders, virtually no data exist regarding the third group of amphibians, the caecilians (Gymnophiona). These poorly known, mainly fossorial species are difficult to study without special methodology (Gower and Wilkinson 2005) and few populations have been characterized for local variation (for an exception, see Wake [1980]). There is no clear understanding of what constitutes a species of caecilian. Furthermore, no extensive phylogeographic studies have been published as yet, and there are few molecular studies of any kind; but see Gower *et al.* (2002) and Bossuyt *et al.* (2005). Regional patterns suggest, however, that vicariance has been important in species formation in the group (e.g., Savage and Wake 2001). A special circumstance is the occurrence of single species on several granitic islands in the Seychelles, e.g., *Hypogeophis rostratus* and *Grandisonia alternans* (Nussbaum 1984). Whether these represent remnants of ancient, relatively widespread species (in which case one would predict very high molecular distances) or more recent dispersals is presently unknown.

There now exist many datasets for amphibians in which phylogeographic patterns either do not correspond with traditional taxonomy, e.g., the *Bufo americanus* complex (Masta *et al.* 2002), or levels of divergence are sufficiently great that current taxonomy is too conservative (reviewed by Highton 2000). Doubtless these instances will increase and will pose difficult problems for systematists in years to come. One might argue that no harm is done by maintaining conservative taxonomies when there is no evident morphological or other biological differentiation, as in the case of *Desmognathus wrighti* (Crespi *et al.* 2003). If, however, one desires high quality estimates of regional biodiversity, conservative taxonomy can seriously mislead (cf. Cracraft 1997).

Wiens (2004b) proposed that phylogenetic niche conservatism, rather than ecological divergence, contributes to the formation of geographically isolated lineages, the necessary first stage of dichopatric speciation. According to this view, four factors that are largely intrinsic to the ancestral species and to its geographically separated subpopulations, can

prevent these from dispersing across environmental barriers and thereby promote vicariant lineage splitting: lack of variability, natural selection, pleiotropy, and gene flow. In the case of amphibians, he hypothesized that for some recently diverged montane salamanders species (*Plethodon shenandoah*, *P. hubrichti*, *P. nettingi*) the geographical isolation may be caused by another related species (*P. cinereus*) that is a superior competitor in lowlands but inferior in certain physiologically stressful highland habitats. Discussing areas for future research, Wiens (2004a, 2004b) emphasized that many studies have sought the geographic barriers involved in specific speciation events but few have taken the next step of identifying the extrinsic ecological factors and intrinsic organismal traits that underlie these barriers; he considered this question to be critical for speciation research.

In addition to the question of maintenance of geographic isolation, the means whereby genetic diversification in geographically isolated units leads to speciation is poorly understood. A major question is whether this process is, or can actually be, non-adaptive (incidental) in a strict sense, or whether selection is the major factor that leads two populations to diverge genetically in allopatry after geographic disruption of gene flow (e.g., Graham *et al.* 2004). In ecologically driven adaptive speciation, some genes or gene complexes — those under selection — may show lower than average intraspecific variation and heterozygosity, and possibly even higher divergences between species. Recurrent mutations may be identified in species that independently adapt to similar environments, a phenomenon known for certain allozyme electromorphs (see Hotz and Semlitsch [2000] for amphibians). Vicariant species may show such patterns as well if their divergence has been mainly influenced by selection, but not if neutral mutations and processes of gene drift were the main driving forces. Both adaptive and neutral processes may lead either to speciation by postzygotic isolation through the divergence of “speciation genes” (Orr and Presgraves 2000), or to speciation by prezygotic isolation through ethological or ecological divergence. Although at present none of these scenarios can be systematically tested in non-model organisms, suitable tools for such studies may soon be developed in the course of the ongoing genomic and proteomic advances.

### III. ADAPTIVE SPECIES FORMATION

The possibility of adaptive species formation in amphibians has received little attention. Potential examples of adaptive geographic variation within species include the mimicry displayed by geographic segments of some species, of which *Ensatina eschscholtzii xanthoptica* (Kuchta 2005) and *Pseudotriton ruber schencki* (Howard and Brodie 1973) are examples (see below). Among the desmognathine salamanders several instances of close relatives living in sympatry or parapatry raise the possibility of adaptive species formation. The large species *Desmognathus quadramaculatus* lives in cold mountain streams in the southern Appalachian Mountains, and its close relative *D. marmoratus* is often sympatric with it but favours somewhat larger streams and is more fully aquatic (Petránka 1999). So distinctive is *D. marmoratus* in morphology that it was long placed in a separate genus, *Leurognathus*, which was thought to be the first lineage of desmognathines to diverge (Dunn 1926). Only much later was it determined that the likely order of branching starts with the fully terrestrial *Phaeognathus*, followed in a comb-like phylogeny by the small terrestrial *D. wrighti*, and then the small terrestrial *D. aeneus* (Titus and Larson 1996). Relatively late in the history of the group larvae are thought to have reappeared and streams invaded, and accordingly *D. marmoratus* represents a relatively recently derived terminal lineage. Support for this scenario is the retention by *D. marmoratus* of terrestrial feeding behaviour, i.e., using the tongue to apprehend prey, even under water (Schwenk and Wake 1988). Other members of the species-rich *Desmognathus* in the southern Appalachian Mountains may also have evolved by adaptive means associated with the terrestrial to aquatic gradient and with the elevational segregation of taxa.

Weitere *et al.* (2004) found compelling evidence for a rapid ecological diversification of salamanders in Germany. The populations of *Salamandra salamandra* inhabiting areas around Bonn were demonstrated to be a monophyletic group, descended from a postglacial

colonization. Although usually depositing their larvae in brooks, some individuals of these live-bearing animals deposit them in ponds, where they show a different metamorphic behaviour, i.e., they leave the water earlier at the cost of a lower body mass. Common-environment experiments with larva from ponds and streams indicated that metamorphic behaviour of pond-larvae under limited-food conditions may be determined genetically instead of being simply a plastic response to differing habitat conditions. Whether such ecological diversification is seen as a first step towards speciation or not, the example of Weitere *et al.* (2004) shows that rapid evolution of genetically determined adaptations is possible when new habitats are exploited, even under sympatric conditions.

#### A. Ecological Diversification along Clines and Transects

Elevational segregation of taxa has been studied by several research groups (Wake and Lynch [1976] and Wake *et al.* [1992] for salamanders; Duellman [2001] for frogs), especially in tropical regions where the phenomenon is pronounced. From 14 to 18 or even more species of plethodontid salamanders occur along elevational transects of modest length (a few kilometers in many cases) at different tropical latitudes. Tropical transects offer many opportunities for studying species formation. Amphibians are often concentrated in montane cloud forests (e.g., Wake 1987) and these are subject to fragmentation and isolation, especially in such tectonically active regions as Middle America. As new volcanoes arise and are vegetated, new cloud forests form, offering opportunities for *in situ* species formation because invasion of such habitats by other cloud-forest species is unlikely unless a corridor exists. Of especial interest are those ancient, isolated, upland sites that are occupied by sympatric sister taxa or complexes of close phylogenetic relatives, because this raises the possibility of some kind of parapatric (or even microparapatric to sympatric), adaptive species formation.

An intriguing case of possible adaptive species formation involves Mexican species of the plethodontid genera *Lineatriton* and *Pseudoeurycea* (Parra-Olea and Wake 2001). *Lineatriton lineola* long was considered to be a narrowly endemic species with no close relatives because it differs strikingly from sympatric and parapatric species in morphology and ecology. *Lineatriton* occurs at elevations of about 1 000 m and below; these salamanders are elongated, fossorial animals with very short limbs and very long tails. They differ dramatically from the terrestrial to semi-arboreal species of *Pseudoeurycea*, but analysis of mtDNA sequences of several individuals for each species supported the diphyletic origin of the genus (see also Parra-Olea 2002). One species, *L. lineola*, is a sister taxon of a clade including *Pseudoeurycea leprosa* and *P. lynchii*, parapatric species that occur above 2 000 m in the vicinity of Cofre de Perote and Pico de Orizaba, Veracruz. In contrast, the other species, *L. orchimelas*, is a sister taxon of *P. werleri*, and the two are found in the isolated Los Tuxtlas area in coastal Veracruz where they are narrowly sympatric, with *Lineatriton* occurring at lower elevations. If this phylogeny is correct the different forms assigned to *Lineatriton* evolved independently in the context of relatively diverse salamander faunas, and in both instances they evolved into an unoccupied fossorial niche.

On Cerro de la Muerte in the Cordillera de Talamanca, Costa Rica, closely related species of *Bolitoglossa* replace each other in a parapatric manner along a steep elevational gradient between 2 000 and 3 000 m elevation (García-París *et al.* 2000). One of the species, *B. pesrubra*, shows remarkable levels of local genetic divergence (Nei's D to 0.18 across a few km) corresponding to ecological and elevational changes. This is in accordance with the well-known phenomenon of narrowed climatic zonation along slopes in tropical zones, as contrasted with temperate ones. One would expect range restriction and local adaptation along tropical elevational transects, and that is apparent in this example.

On Cofre de Perote, Veracruz, Mexico, 18 species of salamanders occur on a transect extending between 3 800 m and about 1 000 m. Several instances of sister taxa that differ in elevation exist: the relatively widespread *P. gigantea* and its more upland, narrowly endemic sister-species *P. naucampateptl*, the geographically widespread *Pseudoeurycea leprosa*

and its more narrowly endemic and lower-elevation sister-species *P. lynchii*, and the geographically widespread *Chiropterotriton chiropterus* and its lower-elevation and narrowly endemic *C. lavae* (Parra-Olea *et al.* 2001; Parra-Olea 2002, 2004). Several additional examples that are less well understood may occur along this transect. These instances contrast with another pattern found among transects, that is, sister taxa that most likely have arisen from vicariant species formation on neighbouring mountains. An example is *Pseudoeurycea gadovii*, known from Pico de Orizaba and Cerro La Malinche, very high peaks located to the south and west of Cofre de Perote, and its sister-species *P. melanomolge*, known only from elevations above 2 800 m on Cofre de Perote (Parra-Olea 2002). There are no present-day dispersal corridors available to this highland species, but doubtless such routes existed in the past.

In the region south of Pico de Orizaba, on the heights about Acultzingo in Veracruz and Puebla, Mexico, three minute salamanders of the genus *Thorius* (*T. troglodytes*, *T. dubitus*, and *T. magnipes*) are locally sympatric, and all are endemic to a very small region (Hanken and Wake 1998). The first two are sister taxa while *T. magnipes* has a sister taxon that is a geographic vicariant to the south (G. Parra-Olea and co-workers, unpubl. data). The three species are ecologically similar, but *T. troglodytes* is the most generalized. It is a terrestrial species found under rocks and logs and under bark of decaying logs, whereas *T. dubitus* favours pine-needle litter and *T. magnipes* climbs and can be found in arboreal bromeliads, suggesting the possibility of adaptive divergence. These species occur on mountain tops and they have very small geographic ranges so it is probable that they evolved more or less *in situ*, although it is equally possible that *T. magnipes* (a) dispersed from the south, or (b) gave rise to its more southern sister-species. Other complexes of species of *Thorius* occur further south and all are narrowly endemic (Hanken and Wake 1994), suggesting more *in situ* species formation.

It is not only on tropical transects that parapatric, adaptively differentiated species of amphibians are found. The sister taxa *Ambystoma texanum* and *A. barbouri* from eastern North America represent contrasting life histories. The widespread *A. texanum* has aquatic larvae that live in ponds, whereas the more narrowly distributed *A. barbouri* breeds in streams and has stream-adapted larvae (Petraska 1998). These species are difficult to distinguish on molecular and morphological grounds (although some unpublished data suggests they are not as closely related as once thought; see Bogart [2003]), yet they differ in preferred breeding habitat, mode of egg deposition, egg size, clutch size, larval morphology, larval behaviour and in the shape of the teeth and premaxillary bones. The two replace one another geographically, and in the few zones of contact they occasionally hybridize. There are numerous other potential examples, including the complex pattern in the eastern North American plethodontid salamanders *Desmognathus* (Rissler and Taylor 2003).

Stebbins (1949) proposed that the genus *Ensatina*, previously with four species, is, in fact, a single polytypic species distributed in a ring-like pattern surrounding the Central Valley of California (Fig. 4). Along the coast, more or less uniformly coloured animals (unblotted) are found, whereas in internal montane regions these salamanders are spotted, blotched and banded with boldly contrasting colours. Stebbins postulated clinal variation with broad areas of intergradation. During Pleistocene times the central valley barrier was breached in the middle, where brightly coloured, unblotted coastal forms (aposematic mimics of poisonous newts, *Taricha*; Kuchta [2005]) penetrated inland and met the more upland and cryptic blotched forms. Later studies showed that the two forms narrowly hybridized in an ecotone without introgression (Wake *et al.* 1989), and this result has been confirmed and extended by a study that showed that the hybrid zone between these two adaptively divergent groups has been stable for at least 20 years and that in the zone where both parental types are found, there is strong selection against hybrids (Alexandrino *et al.* 2005). In this region two biological species are present, separated by different colour patterns, habitat, and elevation. Backtracking through the ring, however, one finds continuity, with some areas of reduced genetic interaction where historical units (defined

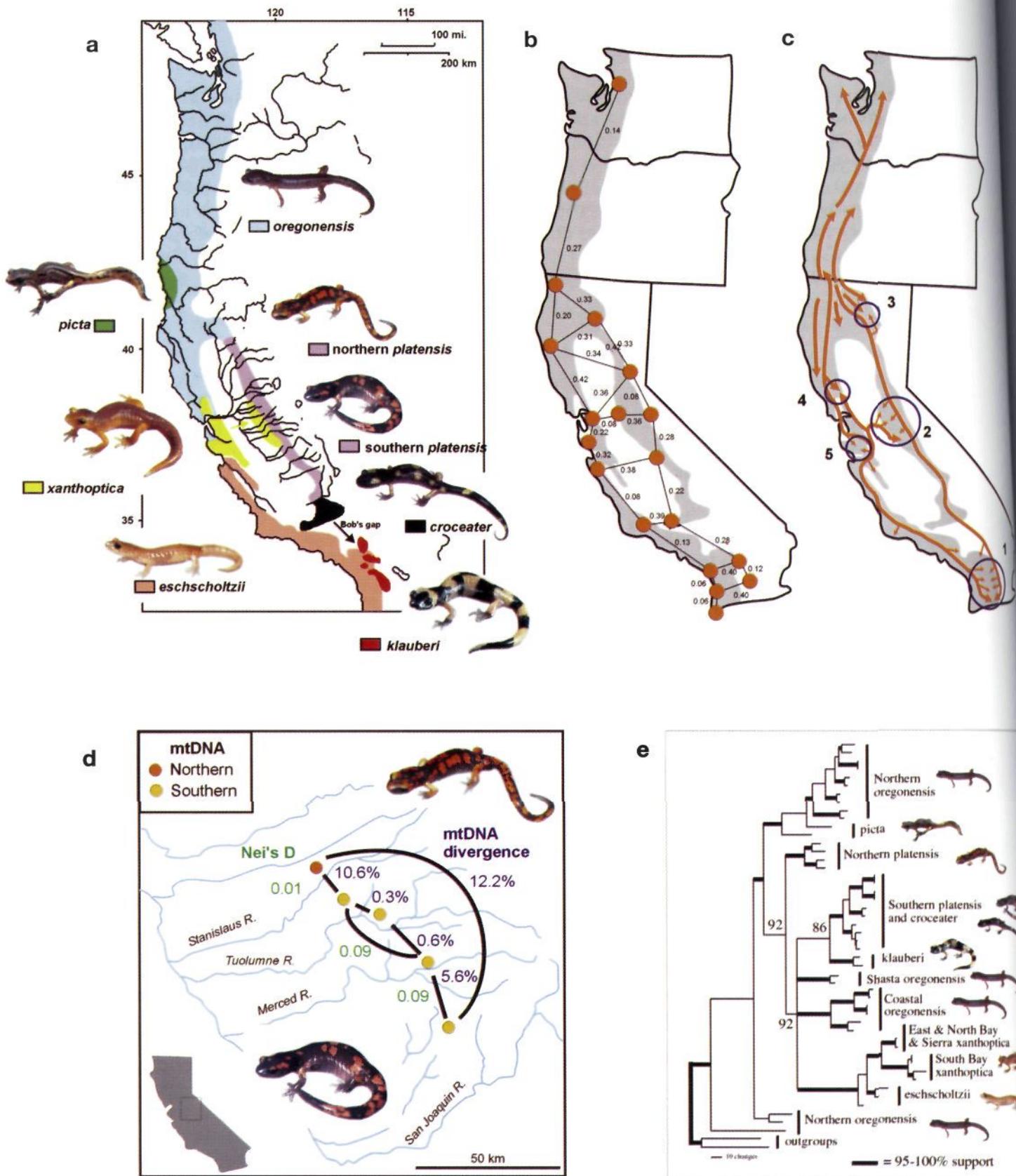
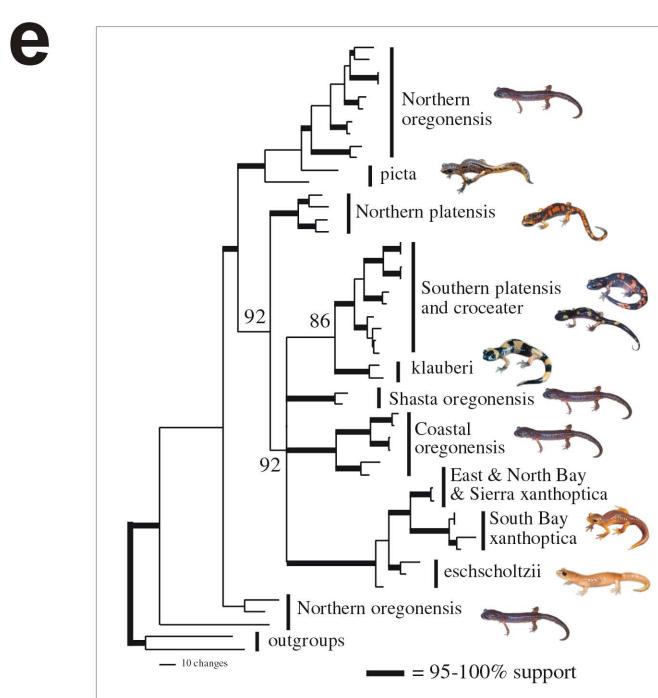
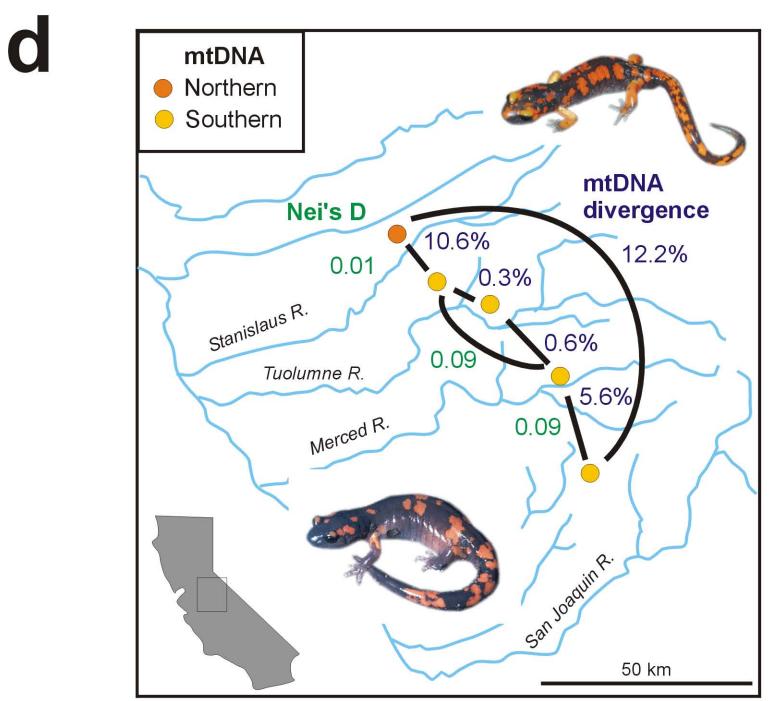
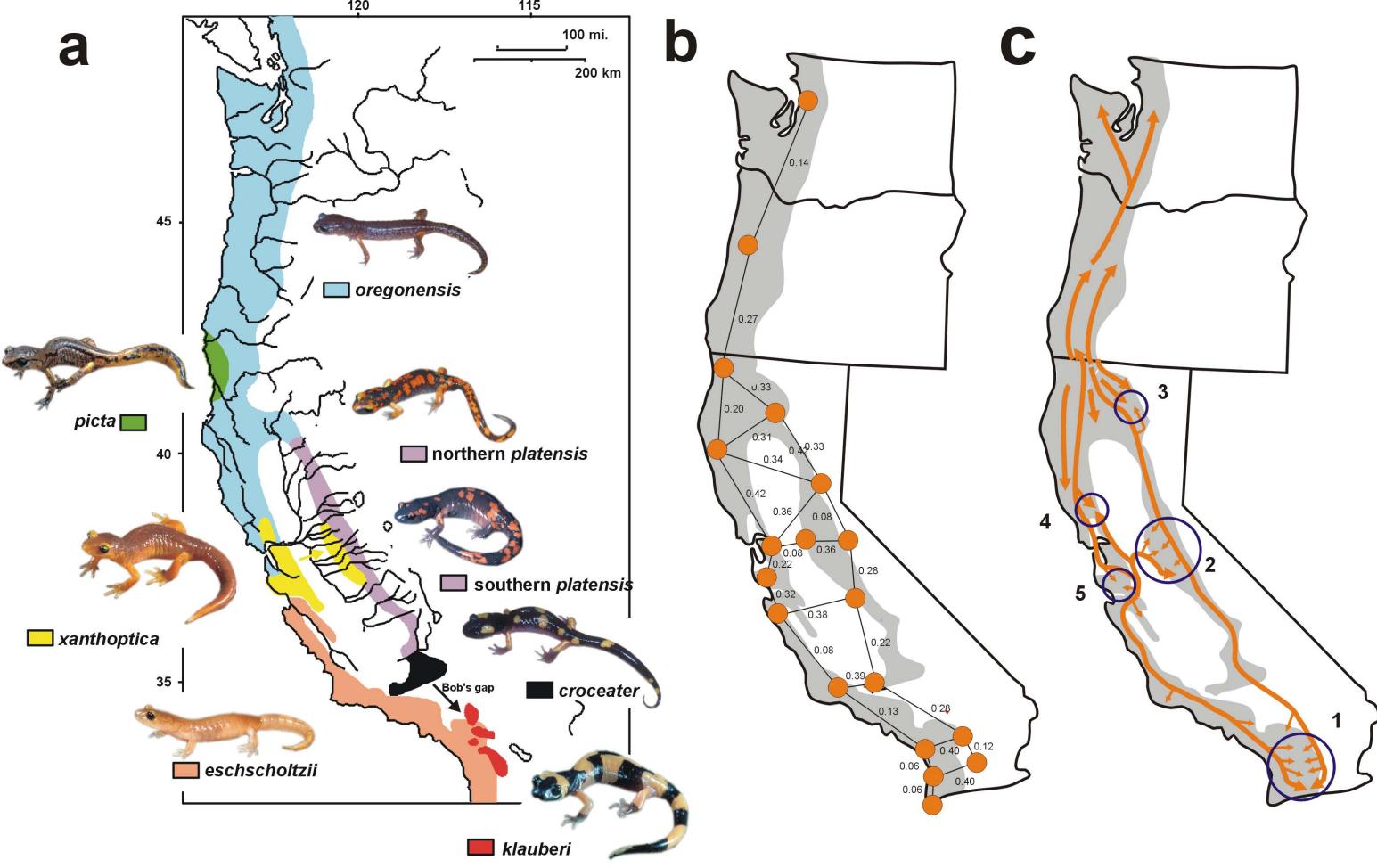


Fig. 4. Distribution and incipient species-formation in the salamander *Ensatina escholtzii*. The first map (a) shows the west coast of North America and the distribution of the different units recognized as subspecies of *E. escholtzii*. Arrows indicate assumed past dispersal or connection of populations that at present are separated by unsuitable habitat. The second map (b) shows Nei's genetic distances among populations, based on the allozyme study by Wake and Yanev (1986). The third map (c) shows the hypothetical routes of colonization of current *Ensatina*, with locations of hybridization (1 and 2) and secondary contacts with localized gene flow (3, 4 and 5), after Wake (1997). Figure (d) shows an example of a mitochondrial haplotype boundary (between northern and southern *platensis*) that is not accompanied by a significant allozyme boundary or by phenotypic (colour pattern) changes (from Wake and Schneider 1998). The tree (e) is an unpublished (Kuchta *et al.*) phylogram based on mitochondrial DNA sequences.



on the basis of distribution of mtDNA haplotypes) meet. Although contested by Highton (1998), this complex does, indeed, appear to be a ring species, but not one in which there is continuous gene flow. The overall pattern is in the geographic form of a ring, and the existence of two adaptive morphs is unquestioned. Within each of the two main morphs, however, there have been periods of geographic fragmentation, genetic divergence, and subsequent recontact with genetic merger, both in the Sierra Nevada (Wake and Schneider 1998) and in the vicinity of San Francisco Bay (Wake 1997). The most intriguing of these transitional zones is in the central Sierra Nevada, where the subspecies *Ensatina escholtzii platensis* occurs. Northern and southern populations of this taxon have mitochondrial haplotypes that are not sisters in phylogenetic analyses (Moritz *et al.* 1992) and the populational units are also differentiated by proteins, with northern populations being more closely related to neighbouring *E. e. oregonensis* than to southern populations of its own taxon (Jackman and Wake 1994). The northern *platensis* populations are geneologically *oregonensis* and a zone of secondary contact in the central Sierra Nevada is marked by sharp transitions in mtDNA and proteins (Wake and Schneider 1998). The blotched colour pattern is an effective camouflage (disruptive coloration) in the wooded uplands, and is postulated to have been adaptively superior to the ancestral *oregonensis* pattern, which it has replaced in an apparent adaptive sweep. In contrast, proteins and mtDNA have been governed largely by stochastic and demographic effects and have moved relatively little from the postulated site of initial contact. The *Ensatina* example illustrates the interplay between adaptive divergence and vicariance in species formation. Adaptive divergence produces differences that are sufficiently great that when blotched and unblotched populations meet in secondary contacts they behave as species, usually displaying sharply delimited, narrow zones of sympatry and hybridization. At the southernmost site of sympatry there is no evidence of present or past hybridization (Wake *et al.* 1986); clearly, two biological species are present. Within either the blotched or unblotched groups, however, there is evidence of fragmentation and recontacts that result in genetic merger between adaptively similar groups. At the northern end of the central valley blotched and unblotched forms merge into a paraphyletic complex labelled *E. e. oregonensis*. The *Ensatina* complex as a whole includes strongly differentiated groups that appear to be species, but the existence of intermediates creates a taxonomic conundrum. In Peruvian poison frogs of the genus *Epipedobates*, phylogeographic evidence supports a scenario of initial colonization of mountains by ancestors living at lower elevations, with a subsequent reinvasion of lowlands by one species, *E. trivittatus* (Roberts *et al.* 2006). Extensive speciation took place in the highlands. By comparative analysis of genetic and chromatic divergences, Roberts *et al.* (2006) found an acceleration of divergence in colour among populations and species in the highlands, and suggest that divergent selection regimes, probably largely in allopatry, may have triggered the diversification in highlands.

A large degree of elevational morphological differentiation has been reported in Palearctic brown frogs of the genus *Rana*. One of these, the common frog *Rana temporaria*, occurs over a very wide elevational and latitudinal range in Europe. In the Pyrenean mountains, but probably also in the Alps, the montane populations occurring above the treeline are smaller, have shorter hindlimbs, a different coloration, and several other morphological differences relative to low-elevation populations (Dubois 1982; Vences *et al.* 1999a). Furthermore their behaviour is more diurnal and aquatic (Vences *et al.* 2000). Indeed, these differences are so large that experienced batrachologists initially suspected the presence of different species subsumed under the name *Rana temporaria* (e.g., Dubois 1982). Allozyme data, however, did not reveal any relevant differentiation between these high-elevation and low-elevation populations, and results of analyses of mtDNA are similar (Veith *et al.* 2002). Differentiation of these frogs might have evolved very rapidly along an elevational gradient after the re-colonization of the mountains following the last glaciation (Veith *et al.* 2003a). Habitat specialization in pond-breeding anurans is known to evolve quickly (van Buskirk 2003). However, in frogs, several non-genetic factors such as ovum size, average temperature and temperature variance during early development can affect body sizes and shapes, as well as locomotor performance (Kaplan and Phillips 2006), and

the possibility of such maternal and environmental effects need to be considered before morphological differences along clines are interpreted as adaptive and heritable.

Similar phenomena seem to govern the evolutionary history of brown frogs from Anatolia (Veith *et al.* 2003b), and especially from the Caucasus region, where brown frogs have been intensively studied by Tarkhnishvili and Gokhelashvili (1996) and by Tarkhnishvili *et al.* (1999a, 1999b, 2001). Two forms, or species, of frogs are assumed to occur in this area: *Rana camerani* and *R. macrocnemis*. In the western Caucasus, these forms occur more or less adjacent to each other and are morphologically and genetically distinct. Nominal *macrocnemis* from humid forests of Colchis differs from nominal *camerani* from Georgia in many morphological and genetic characters — including body shape, coloration, skin structure, the distribution of alleles at allozymic and microsatellite DNA loci. Frogs from the rest of the Near East, however, are rather intermediate, at least morphologically. Distribution of microsatellite genotypes in populations from the centre of the cline significantly deviates from Hardy-Weinberg equilibrium and linkage equilibrium, with a significant deficit of heterozygotes. In fact, in the centre of the cline the two forms coexist and hybridization is limited. There is some evidence that the differences between *macrocnemis* and *camerani* on opposite sides of the Lesser Caucasus are maintained by differential landscape-dependent selection. Under the same ecological conditions, *camerani* grows more rapidly than does *macrocnemis* and produces more offspring per unit time. This provides a selective advantage under the severe climatic conditions of the Javakheti plateau. In other parts of the range, the gradients of ecological conditions are not so sharp. Tarkhnishvili concluded that *camerani* and *macrocnemis* are two clearly different subspecies or semi-species, but neither of the two forms can be clearly delimited in geographic space or by genetic and morphological traits. He compared the situation with that of ring species, but in these frogs he assumed that the “extreme” populations have been in contact at all stages of divergence, whereas in typical ring species the extreme populations usually have a long history of independent evolution.

Graham *et al.* (2004) integrated phylogenetics and environmental niche modelling to explore the role of adaptive divergence in speciation of Ecuadorian poison frogs (Dendrobatidae). They found a general trend of environmental divergence among sister groups, irrespective of geography, and no cases suggesting incidental divergence in isolation. Only seven comparisons among lineages, however, were possible from their dataset, and only three of these were strict comparisons among sister species. This approach promises to be informative in terms of understanding the importance of adaptational divergence under allopatric and sympatric conditions when applied comprehensively across diverse genera. The method requires dense point-distribution data and complete, extensively sampled and well-resolved phylogenies.

An ideal system for fully understanding amphibian speciation along environmental gradients would consist of a small local radiation in a habitat island where species sister to each other occupy different portions of a gradient. Groups that reproduce independently from bodies of water and are therefore continuously distributed over areas of suitable habitat probably are most suitable for research. On isolated volcanos in the Philippines, at least two instances of locally endemic and genetically similar frogs of the direct-developing genus *Platymantis* have been identified that are restricted to sharply defined elevational intervals (R. Brown, pers. comm.). These and similar examples of Middle American salamanders (see above) deserve further study.

## B. Sexual Selection: Colour, Crests and Calls

In several animal groups, sexual selection is known to be a major evolutionary factor shaping colour patterns, especially of males. In some groups of cichlid fishes it is likely that sexual selection of colour is the driving force of speciation (e.g., Wilson *et al.* 2000; van Alphen and Seehausen 2001). The process starts with small differences in preference by females for male coloration, and results in two incipient species in which the males have different nuptial colours and the females have a preference for males of either colour.

Such mechanisms have proven to be powerful triggers of sympatric speciation in simulation studies (Gavrilets 2003).

A high degree of polychromatism is known within and among populations of amphibians. The literature on this subject is extensive and has recently been reviewed by Hoffmann and Blouin (2000) for frogs. In many cases the different colour morphs are well-defined and follow simple Mendelian genetics. This also applies to salamanders (see Matsui *et al.* 2003). The different phenotypes of several species are known to be adaptive in terms of substrate-dependent differential predation and linked physiological characteristics. Most amphibians are nocturnal, but optical communication is well known in many taxa, especially diurnal ones (Duellman and Trueb 1986). Also, in nocturnal frogs the vocal sacs of male frogs are often coloured conspicuous white or black, sometimes in combination with a strongly contrasting nuptial body coloration. It is obvious that this pattern serves as a visual stimulus that attracts females (e.g., the Malagasy frog, *Mantidactylus liber*; see plate 66 in Glaw and Vences [1994]). Nevertheless, sexual dichromatism is uncommon and has been recorded in only 32 out of 225 polychromatic frogs (Hoffmann and Blouin 2000). In these examples, the brightest colour and most striking pattern does not always occur in the males. While in the probably extinct golden toad, *Bufo periglenes*, the males were bright uniform orange and the females more cryptic, the inverse situation is found in at least one hyperoliid treefrog (*Heterixalus variabilis*) in which females have the more distinct and colourful pattern (Glaw and Vences 1994; Hoffmann and Blouin 2000). Remarkable colour polymorphism in *Hyperolius argus* was studied by Hayes and Melendez (1999), who discovered a role for steroids in both primary and secondary sexual differentiation. Males of this species are uniformly green dorsally but females are reddish-brown with large white spots. Both sexes are completely green without spots at metamorphosis. After about 2 months females transform to their typical adult colour pattern, but males retain the juvenile pattern. Experimental treatments using testosterone and an estrogen gave divergent results. Both hormonal treatments induced secondary sexual characteristics (vocal sac development and dorsal colour change, respectively) but the estrogen also produced hermaphroditic gonads, whereas testosterone induced complete sexual reversal.

An intriguing issue is the function of eye coloration in frogs and, to a lesser degree, also in salamanders. The pigments of the iris are often very distinct from those of the body skin in vertebrates. In arboreal frogs, especially, the eye colour is constant and typical within a species, even in cases in which the colour and pattern of the body is highly variable (Glaw and Vences 1997b). Some treefrogs, of which *Agalychnis callidryas* is certainly the most prominent example, have a very bright and conspicuous iris, while the body colour is cryptically green or brown. These bright eye colours may play a role either in intraspecific communication or in predator deterrence (Glaw and Vences 1997b); if the former is true, then eye pigments may be under sexual selection and could be involved in species formation. Bright iris colour is also known from other groups of vertebrates and is especially common in birds, where, however, it does not seem to be consistently correlated with any trait of biology or social behaviour (Craig and Hulley 2004).

Abrupt evolution of mimetic colour patterns occurs rarely in species of aposematic amphibians such as dendrobatids or *Mantella* (Symula *et al.* 2001; Schaefer *et al.* 2002; Chiari *et al.* 2004; Darst and Cummings 2006). Should this phenomenon be linked to assortative mating among colour morphs it could lead to speciation (Mallet and Joron 1999), and in some cases sexual selection might be responsible for the evolution of coloration in frogs (Glaw and Vences 1997b; Summers *et al.* 2003). Aposematic behaviour and coloration of poisonous amphibians has been mimicked in different salamanders. The dangerously toxic species of *Taricha* are mimicked by populations of the relatively harmless *Ensatina* (Kuchta 2005), and the toxic, bright red, eft stage of *Notophthalmus viridescens* is mimicked by populations of *Pseudotriton ruber*, which are also toxic, especially as juveniles, at which stage they are similar to efts in size (Brandon and Huheey 1981). *Desmognathus imitator* was named because of its mimetic resemblance to *Plethodon jordani*. All the salamander mimics are

members of taxa that have nearby close relatives, and mimicry may have been involved in local differentiation, as in the case of the geographically restricted red phase of *Plethodon cinereus* that mimics efts (Brodie and Brodie 1980) and, in the case of *D. imitator*, speciation. Such mechanisms, however, are unlikely to be common means of speciation in amphibians.

Mating calls of frogs are paramount for premating isolation. In birds, at least under special circumstances, call divergences can lead to sympatric speciation (Sorenson *et al.* 2003; Kingston and Rossiter 2004; but see Slabbekoorn and Smith 2002). Many species of frogs can co-occur, and experienced naturalists can recognize the calls of different species. These calls can be quantitatively analysed, showing that they are usually species-specific. Strong local sexual selection appears to be a dominant theme, and there are many associated factors, such as size, environmental effects or predation (Ryan 1988). Geographic intraspecific variation in frog calls has been reported (e.g., papers cited by Foster and Endler 1999). It, however, usually affects details of the temporal or spectral call-parameters but not the general call-structure itself. The most common type of variation within species or among very closely related species is in temporal features such as pulse rate (e.g., Giacoma and Castellano 2001; Schneider and Sinsch, Chapter 8, this volume), but sometimes there are slight shifts in fundamental frequency (Knowles *et al.* 2004) or more distinct differences in frequency are encountered (e.g., in *Tomopterna*; Channing [2001]).

In contrast to the situation in birds, call patterns in frogs are genetically determined and no ontogenetic learning takes place, although some species are flexible in adjusting their call characteristics in order to best exploit the acoustic environment (Lardner and bin Lakim 2002). Acoustic partitioning in sympatric anuran assemblages has often been described but alternatives to active evolutionary processes were usually not tested (Gerhardt and Schwartz 1995). Chek *et al.* (2003b) provided a null model test of signal partitioning and applied it to data from the literature, finding evidence of structuring in three out of 11 multi-species frog assemblages. Although noticing that the result was not significant after Bonferroni correction over multiple comparisons, these authors nevertheless inferred that there were strong indications of structure within some of the frog assemblages studied. It is unclear whether such partitioning reflects merely a sorting of species into assemblages in which acoustic overlap is minimized, or indicates an actual evolutionary adjustment of the calls. The latter hypothesis would imply a significant importance of selection by the acoustic environment in the speciation process (Chek *et al.* 2003b).

Extensive studies of the leptodactylid frog *Physalaemus* (recently reclassified as *Engystomops*) in southern Central America and in South America have shown that allopatric sister taxa in this genus often differ only slightly in call characteristics, although these differences can be sufficiently important that females prefer conspecific over heterospecific calls (Ryan and Rand 1993). The most unequivocal discrimination against heterospecific calls occurs under conditions of sympatry (Ryan *et al.* 2003). Indeed, the calls of presumably closely related frogs are usually more different when such species occur in sympatry than in allopatry (e.g., Duellman and Pyles 1983). Nevertheless, cases of bioacoustic character-displacement involving the development of species-specific codes seem to be rare, and in most of the species-pairs with divergent calls that have been studied, acoustic signals were just as distinctive in allopatry as in sympatry (Blair 1974; Littlejohn 1977). Nevo and Capranica (1985) concluded that call differences in the genus *Acris* were largely explained by environmental factors independent of sympatry, and rejected the hypothesis of sympatric origin of premating reproductive isolation in these frogs. Vences *et al.* (2002a) compared levels of genetic divergence among pairs of species of Malagasy treefrogs (*Boophis*) that are morphologically very similar and phylogenetically sister to each other. They found that sympatric species always had strongly different calls and high degrees of genetic differentiation (and thus had diverged long ago), while the lowest genetic differentiation (and thus more recent divergence) was found between allopatric species with low call differences. Further analysis of species-pairs of Malagasy frogs (Mantellidae) corroborated this pattern (M. Vences, unpubl. data). These results seem to suggest that bioacoustic differentiation in frogs is usually not the primary event leading to reproductive isolation of incipient species.

On the other hand, Gerhardt and Schwartz (1995) listed three examples of reproductive character displacement involving frog advertisement calls, namely in the species-pairs *Litoria ewingi* and *L. verreauxii*, *Pseudacris nigrita* and *P. feriarum*, and *Gastrophryne carolinensis* and *G. oliveacea*. Given the high levels of genetic heterogeneity and the low levels of gene flow characteristic of amphibian species, especially fully terrestrial ones, there would be little opportunity for a particular call-type to spread if it were specific to a particular sound environment. Rapid evolution of calls through character displacement in a secondary contact zone of two species could lead to new calls, different from calls of those of geographically distant populations, which were not subject to the selective pressures of secondary sympatry. For this reason, these originally conspecific populations could remain reproductively isolated — becoming separate species — through a combination of (1) non-adaptive processes, such as restricted gene flow and genetic differentiation between geographically distant, discontinuously distributed, conspecific populations, and (2) adaptive processes, including rapid evolution of calls in one of the populations in an area of secondary contact with another species, or in an otherwise new, strongly selective acoustic environment, e.g., open versus forest habitat (Ryan and Wilczynski 1991).

Sound is unimportant in salamander courtship but both vision and olfaction are very important (reviewed by Houck and Arnold 2003). European newts of the genus *Triturus* are well known for their colourful visual displays which are used by males to attract the attention of females. Male newts develop elaborated crests during their aquatic phase and use these for complex optical displays. These displays may serve for prezygotic isolation among sympatric species (Halliday 1977), although experimental evidence for this is still lacking. However, the displays are similar among phylogenetically related incipient species such as those of the *Triturus cristatus* superspecies (Arntzen and Sparreboom 1989). These taxa are distributed allopatrically but hybridize in contact zones (Arntzen and Wallis 1999). This makes it unlikely that differences in nuptial display contribute to the speciation process in these newts, but *Triturus* is relatively species-poor and the opportunities for quantitative comparisons of pairs of sibling species are therefore limited. Visual signalling is also widespread in frogs but its importance as prezygotic isolation mechanism has been little studied (Hödl and Amézquita 2001). The same is true for vibrational communication that appears to be prevalent in various anuran species (Lewis *et al.* 2001).

One of the most convincing cases for species formation by reinforcement of advertisement call differentiation has been made by Hoskin *et al.* (2006) (see also Littlejohn and Loftus-Hills 1968). In the Eastern Australian wet tropics, the green-eyed tree frog *Litoria genimaculata* consists of two highly divergent lineages of northern (N) and southern (S) distribution, respectively. One isolated group of populations of the southern lineage (iS) occurs within the distribution area of the northern lineage, and is characterized by a smaller body size of males compared to S and N populations. Genetically, iS haplotypes are only weakly divergent from S haplotypes. Artificial crosses between S and N lineages showed that crosses with S or iS females are not viable and reciprocal crosses, too, have a slower larval development. Absence of introgression of S or iS mtDNA into N populations, and relative rareness of introgression of N mtDNA into S populations indicated selection against hybridization. Call divergence between N and iS was higher than between N and S, indicating that the relatively small iS population has responded by reinforcement of call differences to the selection against hybridization at the contact zone with populations of the N lineage. Since many call patterns, especially frequency, in frogs are size related, the smaller iS male size may be a by-product of this call reinforcement. Incidentally, the call of iS is also significantly different from those of the remainder of the S lineage, and the premating isolation between these two lineages, as suggested by female call preference experiments, was complete, females always choosing the calls of their own group. Hence, call differentiation by reinforcement in the isolated group of populations of the southern lineage has incidentally also caused call divergence from the main group of populations in this same lineage, and hence led to speciation. These results suggest that N, S and iS lineages may be considered to represent three different species. As pointed out by Hoskin

*et al.* (2006), however, the prevalence of such speciation in nature may be limited by the probability of extinction of small isolated populations on initial contact with other lineages.

Once courtship begins, pheromones play an important role in salamanders. Biomechanical details of courtship differ greatly among taxa, and these are coupled with differences in the ways in which pheromones are delivered. Male salamanders are supplied with diverse glands that produce pheromones. In plethodontids the most prominent ones are the large mental glands. Pheromone delivery systems were studied in detail in the eastern clade of *Plethodon* and two main modes were found (Watts *et al.* 2004). The members of the *cinereus* clade retain a presumed ancestral state in which pheromones are “vaccinated” into the skin of the female by the enlarged premaxillary teeth of the male. In contrast, members of the *glutinosus* clade have evolved a unique behaviour (termed olfactory) in which the male slaps his mental gland on the external nares of the female. While these behavioural patterns are relatively conservative and static within clades, Watts *et al.* (2004) reported positive selection on the pheromonal signal that is mediated by receptors, and the outcome is premating isolation among sister taxa.

We here point to two major fields of research that have the potential to lead to an understanding of the impact of sexual selection on amphibian speciation. On the theoretical level, the persistence of species-specificity of frog calls, despite the often restricted gene flow among amphibian populations, requires more attention. In some cases, the lack of major intraspecific variation in advertisement calls may be due to circular reasoning — populations with divergent calls would be considered as distinct species by taxonomists, thereby eliminating the variation. In general, however, the available data indicate that evolution of calls proceeds much more slowly than does evolution of morphology, coloration and ecology, although different sound environments, the possibly small effective population sizes (but see Crawford [2003a] and our discussion of effective population sizes in section IV), and the genetic isolation of many frog populations would offer ideal conditions for rapid change. Related to this question, if speciation by sexual selection is to be demonstrated in amphibians, a crucial step would be to identify compelling examples of species that are (a) sympatric, (b) phylogenetically sister to each other, (c) morphologically and ecologically similar, (d) genetically not strongly divergent and therefore likely to have originated by a recent speciation event, and (e) differing in any sexually selected character such as size, call, pheromones or nuptial coloration.

### C. Temporal Processes: Allochrony and Heterochrony

Shifts in life cycles can strongly reduce gene flow between populations, and thereby promote speciation (Miyatake and Shimuzu 1999; Marshall and Cooley 2000). In the natterjack toad, *Bufo calamita*, a species with an extended breeding period during spring and summer, evidence from mark-recapture experiments (Sinsch 1992; Sinsch and Seidel 1995) indicated the existence of two to three successive immigrations of males to the breeding ponds. These temporal subpopulations appear to show a distinct genetic differentiation, higher than that among local subpopulations, suggesting reduced gene flow (Sinsch 1992; Schröer 1993).

Among tropical amphibians, there are some examples of species deviating from the common pattern of reproducing during the wet season. The Malagasy frog, *Boophis burgeri*, breeds during the dry season, while several sympatric species belonging to the same group (e.g., *B. boehmei*, *B. reticulatus*, *B. rufioculis*) appear to have their peaks of reproductive activity during the rainy season (Glaw and Vences 1997a). Such seasonal disjunction of breeding seasons is known in many sympatric species (Gerhardt and Schwartz 1995). There does not seem, however, to be any thorough evidence for a sympatric pair of sister-species that distinctly differ in their breeding seasons. Grohovaz *et al.* (1996) hypothesized major phylogeographic subdivisions of *Xenopus laevis* populations in southern Africa to be related to a wet and dry season inversion that separates the distribution areas of the two lineages and may constitute a barrier to gene flow. Hillis (1981) found a segregation in times of breeding to be a major premating isolating mechanism among three species of the *Rana*

*pipiens* complex. Of these three species, the most divergent breeding period in sympatry is that of *Rana berlandieri*, while the genetically closest (but not sister) species *R. blairi* and *R. sphenocephala* (see Hillis *et al.* 1983) have partly overlapping breeding periods.

Ambystomatid salamanders in eastern North America also differ in their breeding phenologies. Most species breed in early spring, but *A. maculatum* is typically the first to breed in the north-east. In contrast, *A. opacum* lays eggs on land in late fall. Winter rains fill adjacent ponds, flooding the eggs, which hatch before other species have bred. In late spring several size classes of larvae are present in ponds, representing species that have bred at different times (Worthington 1968, 1969).

Along the northern coast of California three closely related species of newts, *Taricha*, are syntopic, occupying adjacent habitats and breeding at different times: *T. granulosa*, a pond-breeder, *T. torosa*, which uses ponds and slow-moving streams, and *T. rivularis*, which uses fast-moving streams (Twitty 1966). Those in ponds breed first, and then fast and finally slow streams are used in that temporal sequence. *Taricha torosa* breeds in streams in late spring when flow is diminishing. Natural ponds are rare, but when present *T. torosa* uses them, typically breeding as much as two months earlier. Agricultural development has led to creation of stock tanks, many of which are used by newts who breed earlier than nearby stream breeders. While there is no evidence of genetic differentiation of populations showing different breeding phenologies, such circumstances set the stage for allochronic divergence. This example raises the possibility of local adaptive divergence coupled with offsets in breeding periods.

Although these observations suggest that allochronic specialization could sporadically influence speciation in amphibians, such mechanisms are unlikely to be a major factor because amphibian reproduction is usually linked to the onset of rainfall, occurs every year, and non-overlapping reproductive periods among closely related species are rarely encountered. An apparently minor role in speciation can also be attributed to the phenomenon of facultative paedomorphosis that is relatively widespread among salamanders: some individuals or populations do not metamorphose but become sexually mature as fully aquatic organisms, retaining many larval features such as external gills, often triggered by environmental influences (Whiteman 1994). Several species, especially cave-dwelling forms in the Plethodontidae, are fully neotenic and some, although bizarrely specialized in morphology, are close relatives of less specialized, but nonetheless permanently larval, surface-dwelling taxa (Chippindale *et al.* 2000). Denoël (2003 and references therein) tested whether the morphological dimorphism of paedomorphic and metamorphic newts (*Triturus alpestris*) is correlated with ethological or ecological differences that could lead to assortative mating. He found evidence for a high degree of intermorph gene flow and therefore rejected the hypothesis that the heterochronic shift could be a mechanism leading to sympatric speciation. Mexican species of *Ambystoma* are all very close relatives that are believed to have diverged recently (Shaffer and McKnight 1996). Some of these always metamorphose, some never metamorphose, and some show plasticity. On the Edwards Plateau of Texas members of the plethodontid genus *Eurycea* are permanently larval (in some species there are rare examples of metamorphosis) and this phenomenon has led to species formation because in these relatively dry environments streams become easily isolated from one another. Several of the species occupy underground aquifers. As major rivers erode into the substrate, the aquifers become isolated from each other, thus promoting species formation (Chippindale *et al.* 2000). A complex situation exists in the Ozark and Ouachita mountain region of southcentral United States, where a species complex of *Eurycea* shows lability in metamorphic development (Bonett and Chippindale 2004). Metamorphic surface-dwelling, paedomorphic surface-dwelling and metamorphic subterranean salamanders occur in the same region. The subterranean population (previously known as *Typholotriton spelaeus*) is sister to a clade that contains the other species, but paedomorphic and transformed salamanders do not form reciprocally monophyletic groups and many share identical mtDNA haplotypes. Paedomorphosis in this complex may have arisen independently a minimum of three and a maximum of nine times. Allozyme

studies on the paedomorphic European salamander, *Proteus anguinus*, revealed that the pigmented and not strictly cave-dwelling subspecies *P. a. parkeli* is nested among troglomorphic lineages without pigmentation, reduced eyes, and other morphological characteristics (Sket and Arntzen 1994). A parallel invasion of caves with subsequent convergent adaptation to this environment (Sket 1997) is a possibility, although a reversal to life outside caves in *parkeli* is theoretically also conceivable and would be more parsimonious. In the Dicamptodontidae the permanently larval *Dicamptodon copei* is found on the Olympic Peninsula in the state of Washington, where it occurs alone, but it is sympatric with its close relative *D. tenebrosus* further to the south and east (Nussbaum 1976). This example could be a case of paedomorphic speciation, although *D. tenebrosus* and *D. copei* may not be direct sister-species (Steele *et al.* 2005).

#### D. Chromosome Rearrangements and Polyploidy

Recent discussions usually deal with the relative frequency of the various types of gradual speciation, while instant speciation events through karyotypic rearrangements attract much less attention. Chromosomal evolution of amphibians has been the subject of intensive research in the past (e.g., Bogart 1980; review by Green and Sessions, Chapter 6 this volume). Amphibian karyotypes are characterized by (1) a tendency toward genome hypertrophy, (2) a high degree of DNA spiralization, and (3) a large variation among species in the amount of nuclear DNA (Duellman and Trueb 1986), with extremely large genome sizes in obligate neotenic salamanders and some terrestrial species (Olmo and Morescalchi 1978). General evolutionary trends in all three amphibian orders, especially in frogs, are towards a reduction of chromosome number and elimination of microchromosomes (Duellman and Trueb 1986). Increases as well as reductions in chromosome numbers occur in many frog lineages and may be the result of different mechanisms. Comprehensive lists of amphibian karyotypes were published by King (1990) and Kuramoto (1990), and evolutionary aspects of amphibian cytogenetics have been discussed in a book edited by Green and Sessions (1991). Correlations may occur between karyotypic diversity and life history (Bogart 1991; Bogart and Hedges 1995), with species that live in small isolated demes showing much karyotypic variation and panmixis-stabilizing karyotypes, but these tendencies require further testing (see section V below). Sex chromosomes occur in at least some amphibians, and at least some cases of male heterogamy as well as of female heterogamy are known. Modern banding techniques have led to more detailed insights into the value of chromosomal characters for amphibian systematics and phylogeny. Besides chromosome number, centromere index and relative size, also the position of the nucleolar organizer region shows a suitable amount of variation to serve as a phylogenetic marker among closely related lineages (e.g., Baldissera *et al.* 1999; Andreone *et al.* 2003). Heterochromatin distribution as revealed by various banding techniques is highly variable among species, but within-species variation has not yet been sufficiently surveyed.

Karyotypic rearrangements, especially increases or decreases in karyotype numbers, have been assumed to be the initial step towards speciation in some groups of animals, e.g., in mole rats (Nevo *et al.* 1987). Bogart and Hedges (1995) observed that rates of karyotypic evolution among Jamaican *Eleutherodactylus* are much faster than previous published rates for frogs, and are best explained by chromosome fission, fusion, translocations and inversions arising in isolated demes and fixed through inbreeding and genetic drift. They concluded that karyotypic evolution appears to be dictated by behavioural factors and effective population sizes irrespective of taxonomic groupings. In amphibians, however, an example has yet to be documented in which such chromosomal rearrangements actually constitute the major effective postzygotic isolation mechanism, thereby constituting a trigger for speciation, rather than being a secondary consequence of accumulation of changes in two reproductively separated lineages.

The situation is different regarding a special kind of karyological rearrangement: polyploidization. Two basic types of polyploids are recognized: autopolyploids, which contain more than two genomes derived from the same taxon, and allopolyploids which combine

genomes from more than one ancestral species. The origin of new species through polyploidization is a well-known phenomenon in plants (Ramsey and Schemske 1998; Soltis and Soltis 1999) and it is also widespread among animals. The prevalent mechanism in vertebrates is allopolyploidization (Simon *et al.* 2003), although Evans *et al.* (2004) hypothesized that autoploidization may be the most common mode in frogs. Polyploid species are known from a variety of amphibian genera and families (Bogart 1980; Kawamura 1984; Table 1). In most of these cases the data available are insufficient to identify the mechanism by which genome duplication arose. Vences *et al.* (2002b) provided evidence that *Scaphiophryne gottlebei* is an allotetraploid, and the same is true for all polyploid *Xenopus* and *Silurana* (Evans *et al.* 2004, 2005). Duellman and Trueb (1986) even remarked that presumably most, if not all, polyploid species of amphibians are allopolyploids, although Schmid *et al.* (1985) hypothesized an autoploid origin for the polyploid populations and species of *Odontophrynus* and *Ceratophrys* (see also Beçak and Kobashi 2004). Mitochondrial DNA of multiple sources has been found in several polyploid amphibians, and this increases polyploid diversity following its origin (Ptacek *et al.* 1994; Mable and Roberts 1997; Stöck *et al.* 2005). Complicated scenarios with multiple origins, followed by further differentiation, is a possibility in polyploid anurans (Wendel 2000), and has been deciphered in detail in the *Hyla versicolor* complex as well as in *Xenopus* (Evens *et al.* 2004, 2005; Holloway *et al.* 2006). In the *Hyla versicolor* complex, one tetraploid species has arisen from multiple events of allopolyploidization in at least three diploid ancestors. These independent tetraploid lineages then merged through interbreeding to result in a single species (Holloway *et al.* 2006).

Polyplid speciation differs from the other modes of species formation discussed here by not being gradual. In Mayr's (1963) classification of speciation modes, polyplid speciation belongs to the instant speciation category: one hybridizing pair of individuals, or one single event such as incomplete meiosis in one individual, can give rise to a new

**Table 1.** Species of polyploid amphibians, their ploidy level and reproductive mode. Only species or kleptons that are constantly polyploid, or that contain constantly or regularly polyploid populations are listed. Sirenid salamanders and *Eleutherodactylus binotatus*, for which only indirect evidence for polyploidy is available, are excluded. In several cases triploid hybrids (3x) between 2x and 4x have also been reported. The term "pond-breeder" is used to refer to species that generally reproduce in lentic water (which occasionally can also include slow-moving streams and similar environments). Karyological data are from Bogart and Wasserman (1972), Beçak and Beçak (1974b), Barrio (1976), Bogart and Tandy (1976), Mahony and Robinson (1980), Tandy *et al.* (1982), Kawamura (1984), Schmid *et al.* (1985), Kobel and Pasquier (1986), Mahony and Roberts (1986), Kuramoto and Allison (1989), King (1990), Tymowska (1991), Haddad *et al.* (1994), Ptacek *et al.* (1994), Bogart and Klemens (1997), Kasahara and Haddad (1997), Roberts (1997), Martino and Sinsch (2002), Stöck *et al.* (2001a, 2002) and Bogart (2003).

Species	Family	Ploidy level	Breeding habitat and mode
<b>Caudata</b>			
<i>Ambystoma</i> spp.	Ambystomatidae	2x / 3x / 4x/5x	Pond-breeders
<b>Anura</b>			
<i>Silurana</i> spp.	Pipidae	4x	Pond-breeder
<i>Xenopus</i> spp.	Pipidae	2x / 4x / 8x / 12x	Pond-breeders
<i>Bufo asmariae</i>	Bufonidae	4x	Pond-breeder (?)
<i>Bufo viridis</i> subgroup	Bufonidae	2x / 4x / 3x	Pond to partly stream breeder
<i>Hyla versicolor</i> complex	Hylidae	4x	Pond-breeder
<i>Phyllomedusa</i> spp.	Hylidae	4x	Pond-breeder
<i>Ceratophrys aurita</i>	Leptodactylidae	8x	Pond-breeder
<i>Ceratophrys ornata</i>	Leptodactylidae	2x / 8x	Pond-breeder
<i>Odontophrynus</i> spp.	Leptodactylidae	2x / 4x	Pond-breeder
<i>Pleurodema bibrornii</i>	Leptodactylidae	4x	Pond-breeder
<i>Pleurodema kriegi</i>	Leptodactylidae	4x	Probably pond-breeder
<i>Neobatrachus</i> spp.	Myobatrachidae	2x / 4x	Explosive pond-breeder
<i>Hoplobatrachus</i> spp.	Ranidae	2x / 4x	Pond-breeder
<i>Rana (Pelophylax)</i> spp.	Ranidae	2x / 3x	Pond-breeders
<i>Tomopterna</i> spp.	Ranidae	2x / 4x	Explosive pond-breeder
<i>Aphantophryne</i> <i>pansa</i>	Microhylidae	4x	Direct development
<i>Chiasmocleis leucosticta</i>	Microhylidae	4x	Pond-breeder
<i>Scaphiophryne</i> <i>gottlebei</i>	Microhylidae	4x	Explosive pond-breeder

polyploid species under fully sympatric conditions. The inclusion of this category in the present section on adaptive species formation is therefore debatable. This exemplifies the difficulty of dividing modes of speciation into static categories and reflects that a mix of various factors — adaptive and non-adaptive — is certainly involved in many cases of species formation. In the case of polyploidization, the new polyploid species faces the problem of competition with the two syntopic parental species. The new species may have a great initial advantage by being immediately reproductively isolated from its parental species because, in the case of a tetraploid species, backcrossing into a diploid parental species results in hybrids (typically triploids) that are likely to be sterile. In addition, multiple alleles of polyploids provide an enormous reservoir of evolutionary innovations, rearrangements of chromosomes and genes, and new gene functions. As summarized by Evans *et al.* (2004) for the example of clawed frogs, allopolyploid species could benefit from a variety of features such as (1) novel protein functions when one duplicated gene-copy is released from selection, (2) increased levels of gene expression, or (3) resistance to parasites to which either parental species has evolved resistance. The hybridogenetic and sometimes triploid Palearctic water frog *Rana kl. esculenta* is known to be ecologically intermediate to its parental species, *R. lessonae* and *R. ridibunda*, thereby probably being more generalistic and able to occupying intermediate niches (e.g., Pagano *et al.* 2001). These examples show that adaptation may play a major role in some cases of chromosome speciation, not driving the event of species formation itself but enabling the newly formed species to persist.

Hybridogenetic Palearctic water frogs (genus *Rana*, subgenus *Pelophylax*) and gynogenetic salamanders of the genus *Ambystoma* are special polyploid systems. They involve triploid forms that, with high probability, arose by hybridization (Simon *et al.* 2003). An exceptional example from green toads also demonstrates the possibility of all-triploid, bisexual reproducing populations (Stöck *et al.* 2002).

*Rana (Pelophylax)* displays multiple occurrences of hybridogenetic taxa. These aquatic frogs have a hemiclonal mode of reproduction, with sperm and egg nuclei fusing and paternal genes being expressed in the offspring, but only the maternal genome being transmitted to the next generation (Simon *et al.* 2003). These hemiclonal taxa can be diploid or triploid, but always originally evolved through hybridization of two diploid parental species. To emphasize the obvious differences from species with biparental reproduction, Dubois and Günther (1982) proposed the term “klepton” for the hybridogenetic and gynogenetic forms, with the abbreviation “kl.” between the generic and specific epithets (e.g., *Rana kl. esculenta*). This suggestion has since been largely followed for water frogs. The diploid-triploid members of this complex, and the all-triploid *Bufo pseudoraddei baturae* (see below), are the only known reproductive systems in anurans consisting entirely of hybrids (overview by Günther [1990] and Ragghianti *et al.* [1999]).

Currently, the Palearctic *Bufo viridis* subgroup (Stöck *et al.* 2001a) is the only known amphibian species complex that comprises diploid, triploid and tetraploid bisexual reproducing taxa. In Asia cryptic diploid, tetraploid (Mazik *et al.* 1976) and even triploid bisexual taxa (Stöck *et al.* 1999, 2002) occur, and complicated mixed breeding systems (e.g., 2n/3n or 3n/4n) may exist in northern Central Asia (Borkin *et al.* 2001). Using cytogenetic and molecular techniques on Eurasian diploid and tetraploid taxa, Stöck (2002) and Stöck *et al.* (2005) showed that Asian tetraploid green toads have evolved independently at least twice. They also reported on the discovery of a zone of common ancestry or hybridization of diploid and tetraploid taxa of these toads in northeastern Iran. The polyploids appear to be more resistant and dominate in the climatically extreme continental high mountains and deserts of Central Asia (reviewed in Stöck *et al.* 2001b), where they are practically the only amphibian present; they may breed in streams if ponds are unavailable.

The mole salamanders (*Ambystoma*) in northeastern North America resemble the *Rana* example, and much early literature referred to triploid species. The situation is very complex, however, and appears to be unique (Bogart 2003). Populations typically involve

diploid sexual members of any of four species of *Ambystoma* (*A. jeffersonianum*, *A. laterale*, *A. texanum*, *A. tigrinum*) as well as hybridized females that usually are triploid but can be either diploid or tetraploid. The unisexual forms were thought to produce unreduced triploid ova, activated in nature by the sperm of one of the “parental” species, rejecting the sperm itself (gynogenesis), and this may be correct in many areas. On some islands in Lake Erie, however, populations containing only hybrids are present and no males have been found. Elsewhere, rare hybridized males, usually triploid, are found. Typically unisexuals greatly outnumber the “parasitized” sexual species in local populations. Individuals in hybrid zones may contain chromosomes derived from one, two, three or even four species. Although allozymes of hybridized individuals can be used to identify parental species, mtDNA gives a different story. The hybrid forms cluster together and are most closely related to *A. barbouri*, a close relative of *A. texanum* (see above), a species whose allozymes are thought to be involved in many hybrid populations. Bogart (2003) postulated that an *A. barbouri*-like ancestral female initially hybridized with *A. laterale* (all 20 different hybrid combinations include at least one *A. laterale* genome). This event must have occurred long ago, perhaps a million years or more (summarized by Bogart 2003). At one time three hybrid species were recognized, but the situation is too complex to justify recognition of just a few species (Lowcock *et al.* 1987). Bogart (2003) noted that it is unsatisfying to be unable to assign common and widespread salamanders to a recognized taxon. Unisexuals could be placed in separate species but then at least twenty would have to be recognized. These may be ephemeral because of genome swapping and ploidy changes, so instead of formal taxonomy a symbolic designation based on nuclear genotypes is used (Lowcock *et al.* 1987). For instance, the former *A. platineum* is designated JJL (two *jeffersonianum*, one *laterale* genomes), and a combination of *A. laterale*, *A. texanum* and *A. tigrinum* is LTTi. The term klepton is not used by researchers of this complex.

Most other cases of polyploidy in amphibians refer to complete duplications (sometimes multiple duplications) of the chromosome complement, leading to tetraploid or octoploid species that are reproductively isolated from their diploid relatives (e.g., Kobel and du Pasquier 1986). Vences *et al.* (2002b) noted a possible correlation in frogs of occurrence of polyploidy and reproductive mode. They recorded tetraploidy in *Scaphiophryne gottlebei*, which is the only instance of polyploidy in a Malagasy anuran. *Scaphiophryne* are conspicuous by being specialized pond-breeders with an explosive phenology. Mating is triggered by heavy rainfall and is completed after a few days, typically in temporary waters. Additionally, the advertisement calls of several sympatric *Scaphiophryne* are known to be virtually identical (Vences *et al.* 2003a). Different species with explosive breeding usually gather simultaneously at breeding ponds after the onset of rain. Males of such species are known to be unselective regarding the conspecificity of mates (Sullivan *et al.* 1995). In many explosive breeders the active searching by males for females dominates breeding activity more than does defence of calling sites and attraction of females to individual males (Wells 1977). These factors are likely to favour interspecific mating and therefore hybridization.

An overview of the reproductive modes of polyploid anurans, including triploid species, shows that most of them are generalized pond-breeders (Table 1). Many, though not all of them, occur in seasonal environments, show a rather explosive breeding behaviour and have calls with limited structural differences. Besides *Scaphiophryne*, this clearly applies to *Neobatrachus* and *Tomopterna* (Channing and Bogart 1996; Roberts 1997). Despite intensive karyotyping of speciose lineages characterized by brook-breeding or specialized reproductive modes (King 1990), the only non-pond-breeding polyploid frog is *Aphantophryne pansa*, a Papuan microhylid with direct development (Kuramoto and Allison 1989). Another record of polyploidy in a direct-developing frog, *Eleutherodactylus binotatus* (Beçak and Beçak 1974a), is based on indirect evidence of large chromosome sizes in a diploid chromosome set and could also be explained by alternative hypotheses (M. Schmid, pers. comm. in 2003). Whether the higher frequency of polyploids in pond-breeding amphibians is caused by an increased frequency of hybridization or by environmental factors favouring spontaneous polyploidization cannot be clarified without more detailed studies.

The general frequency of polyploid species is low: out of more than 800 frog species for which King (1990) listed karyotypes, less than 30 (about 3%) were polyploid. No salamanders except the gynogenetic *Ambystoma* are known to be polyploid, and no records exist for the caecilians. In some frog genera polyploidy is a frequent phenomenon. This especially applies to clawed frogs of the genera *Xenopus* and *Silurana*, for which Evans *et al.* (2004, 2005) estimated that allopolyploidization occurred at least six times, possibly nine times, independently. In no other vertebrate class are polyploid taxa as frequent as in amphibians (Schmid 1980). Nevertheless, as a general conclusion, polyploidization is certainly not a frequent mode of speciation in amphibians. To understand which factors promote this mode, there remains the major challenge of overcoming the uncertainty of whether allopolyploidization or autoploidization is most common in amphibians. A second field of fruitful studies would be to test whether chromosomal rearrangements other than polyploidization, i.e., changes in chromosome number or structure, may in some cases lead to an instant reproductive isolation among amphibian populations and thereby be a major, maybe even initial, step towards species formation. Finally, most polyploid amphibians were discovered in the era of cytogenetics and these organisms have not been evaluated using the methods of molecular genetics and genomics. Such studies may elucidate factors responsible for stabilization of the hybrids and be a point of departure for studies of the diverse genetic interactions that are possible because of polyploidization.

#### IV. GENETIC CRITERIA FOR SPECIES RECOGNITION

##### A. Allozyme Threshold Values

Intensive discussions have tackled the problem of whether there are threshold values of various kinds of genetic distances that can define distinct species. Thorpe (1982) reviewed 100 allozyme studies in non-avian vertebrates and found that 98% of the between-species values of Nei's genetic distance were  $>0.15$ . Sasa *et al.* (1998) studied the evolution of postzygotic isolation among frog species as correlated with genetic (allozyme) distance and therefore, presumably, time. Reviewing data from the literature and correcting them for phylogenetic influences, they found a positive correlation between genetic distance and degree of postzygotic isolation, with hybrid sterility evolving more quickly than hybrid inviability, thereby conforming with conclusions of studies on insects. Using different indices of postzygotic isolation that take both fertility and viability of hybrids into account, they found that, on average, at Nei's genetic distances of 0.25 a certain degree of sterility of offspring is present (their postzygotic isolation index IPO1 = 0.25, inviable or sterile offspring of at least one sex out of two reciprocal crosses), whereas at genetic distances above 0.8, viable and fertile offspring are rare exceptions (IPO = 0.75 and 1). Although certainly not universally applicable to amphibians, these data indicate that genetic distances of 0.25 or higher can in some cases be good indicators for postzygotic isolation and, hence, for differentiation at the species level in frogs.

Highton (e.g., 2000) has long studied salamanders in eastern North America that show much geographic differentiation in allozyme markers, but little in morphology. He termed these cryptic species, and the central issue for him is that there are many more species than have been, or even could have been, recognized using traditional morphological characters. Even when populations are sorted using genetic criteria there is often a failure to discover significant numbers of morphological characters, if any, between genetically distinct, geographic segments of populations. Such observations on an enormous database led Highton to conclude that once a particular level of genetic divergence has been attained (Thorpe's value of 0.15 Nei's Genetic Distance), it is probable that the divergent units will not rejoin or merge upon recontact, but will become parapatric, with one form replacing the other geographically and with hybridization occurring at their borders — or they will become sympatric. A Nei's D of  $>0.15$  corresponds to a genetic isolation of approximately 2 mya (Maxson and Maxson 1979), and Highton (2000) hypothesized that populations

isolated for <2 mya may often merge upon contact, because otherwise many good species of amphibians would differ from each other by lower genetic distances. Apparently, those isolated for longer periods usually complete the process of speciation. At issue is the question of what constitutes hybridization, and whether hybridization is a prelude to merging again or an indication of species status (from which it could be inferred that divergence is irreversible).

Using his criteria Highton has undertaken a radical taxonomic revision, dividing once widespread species into numerous taxa (e.g., Highton 1989; Highton and Peabody 2000). The resulting patterns are like a patchwork quilt in appearance, with some patches abruptly replacing one another at a minor geographic barrier (extremely tight stitching), others showing hybridization over very narrow (moderately tight stitching) to broad (loose stitching) zones, with other patches overlapping each other, sometimes completely. For example, members of the former species *P. glutinosus* generally occur at lower elevations in the southern Appalachian mountains of North America, whereas members of the former species *P. jordani* are generally more upland in distribution. There are hybrid interactions where taxa from within one or other of the former species meet, as well as where taxa from two different former species meet. Highton's sampling is thorough, and this system is exceptionally well understood. The allozyme database has recently been supplemented by an mtDNA dataset, still unpublished (Weisrock *et al.* 2005), which shows that while some allozyme borders match those of haplotype clades, others do not, although overall there is statistically significant congruence.

What constitutes genetic merger upon recontact as opposed to hybridization is the central issue in the debate between Highton (1998, 2000) and Wake (1997; Wake and Schneider 1998) over what criteria one uses to delimit species. There is no debate that genetic divergence has taken place, nor that the diverged populations are exchanging genes, but whether the amount of genetic interchange is sufficient to assure that the gene flow will only be local and that the two diverged units will continue along independent evolutionary trajectories. At one extreme is true hybridization, in which both parental types are syntopic, there are numbers of fixed allozymic differences (on the order of 8–10 for the cases cited), hybrid individuals can be identified and there is little or no introgression (e.g., where blotched and unblotched *Ensatina* meet, Wake *et al.* [1989], Alexandrino *et al.* [2005]). Such hybridization occurs on the scale of hundreds of meters, or a few individual dispersal distances. At the other extreme are zones of gene flow that extend over tens of kilometers, and in which there are no fixed allozymic differences between the parental forms (many examples in *Pllethodon*; e.g., Duncan and Highton [1979], Highton and Peabody [2000]). In the first instance nearly all species criteria would recognize the interactors as distinct species (complicated in the case of *Ensatina* by evidence of gene flow "backward" through the ring; see above), but in the second only certain biologists emphasizing phylogenetic criteria might accept the interactors as species. Among those likely not to accept such species are population geneticists: "We do not consider taxa having substantial gene flow despite morphological distinctness to be species." [Coyne and Orr 2004:34] and evolutionary philosophers: "Biological species are populations within which there is, but between which this is not, sufficient cohesive capacity to preclude indefinite divergence" [Ghiselin 1997:99]. Of course what constitutes substantial gene flow is open to interpretation, but this matter has been dealt with by many researchers (e.g., Barton and Gale 1993).

## B. Mitochondrial Threshold Values

As genetic markers, DNA sequences fundamentally differ from allozymes in two aspects. One of these is a clear advantage: while allozyme studies are not fully comparable, and alleles will be scored differently by different researchers, dependent on the specific electrophoretic conditions used, the basically digital nature of DNA allows direct comparison of newly determined sequences, which add to the ever-increasing database of homologous sequences obtained by other studies.

The second difference is the level at which analyses are carried out. Amphibian mtDNA typically occurs as a single haplotype per individual. Thus, in comparison to allozymes, it affords a fundamentally different means for analysing genetic differentiation. Allozyme differentiation is usually studied as a population phenomenon, and sometimes only differences in allele frequencies characterize sister species. Allozymes, as nuclear markers, are more comparable to microsatellites (although they generally evolve more slowly) than to mtDNA. While mtDNA sequences can also be used to compute differences among populations (using measures such as average nucleotide difference), more frequently genetic divergence is calculated between DNA sequences of two individuals. If these belong to different species, their sequence divergence is often considered equivalent to the sequence divergence between the two species. Although this approach is mostly valid, it is important to be aware of the many underlying basic assumptions: (1) that intraspecific variation is negligible as compared to interspecific variation (justified by the much shorter coalescence time for haploid markers), and (2) that each individual of the first species has a DNA sequence of roughly similar divergence to each individual of the second species (which includes the assumptions of species monophyly and equal substitution rates within each species). It will be shown below that these assumptions are often violated (see Funk and Omland 2003). Nevertheless, the ease and rapidity of obtaining individual mtDNA sequences and their unambiguous comparison to those of other individuals enable large-scale genetic screening, such as DNA barcoding.

Bradley and Baker (2001) attempted to define a mtDNA threshold for species recognition, using published data on cytochrome *b* sequence differentiation in mammals. Based on a large number of interspecific and intraspecific comparisons they argued that a pairwise sequence differentiation of >13% in the cytochrome *b* gene is diagnostic for differentiation of mammals at the species level, and advocated a genetic species concept in order to exploit such datasets for taxonomic conclusions.

Veith (1996) emphasized that invoking such “magical limits”, while appealing, is problematic in that interspecific genetic distances are certainly in part taxon-specific. Considering the severe problems both with allozyme distances, which can reflect mere differences of allele frequencies rather than fixed differences, and with mitochondrial sequences, which can be misleading due to introgression or incomplete lineage sorting, an uncritical recognition of species based on such arguments alone is not advisable.

Any given marker must be used cautiously. With respect to allozymes, Wake and Schneider (1998) listed amphibian taxa in which intraspecific differences of Nei's *D* > 0.15 had been observed. Highton (2000) appropriately observed that several of these instances reflect the existence of unrecognized cryptic species. Nevertheless, there are sufficient instances of discordance among mtDNA and allozyme genetic distance (see below). Such discordances can mislead mitochondrial evidence for species distinction, and should serve as a general caution.

### C. Discordance among Datasets

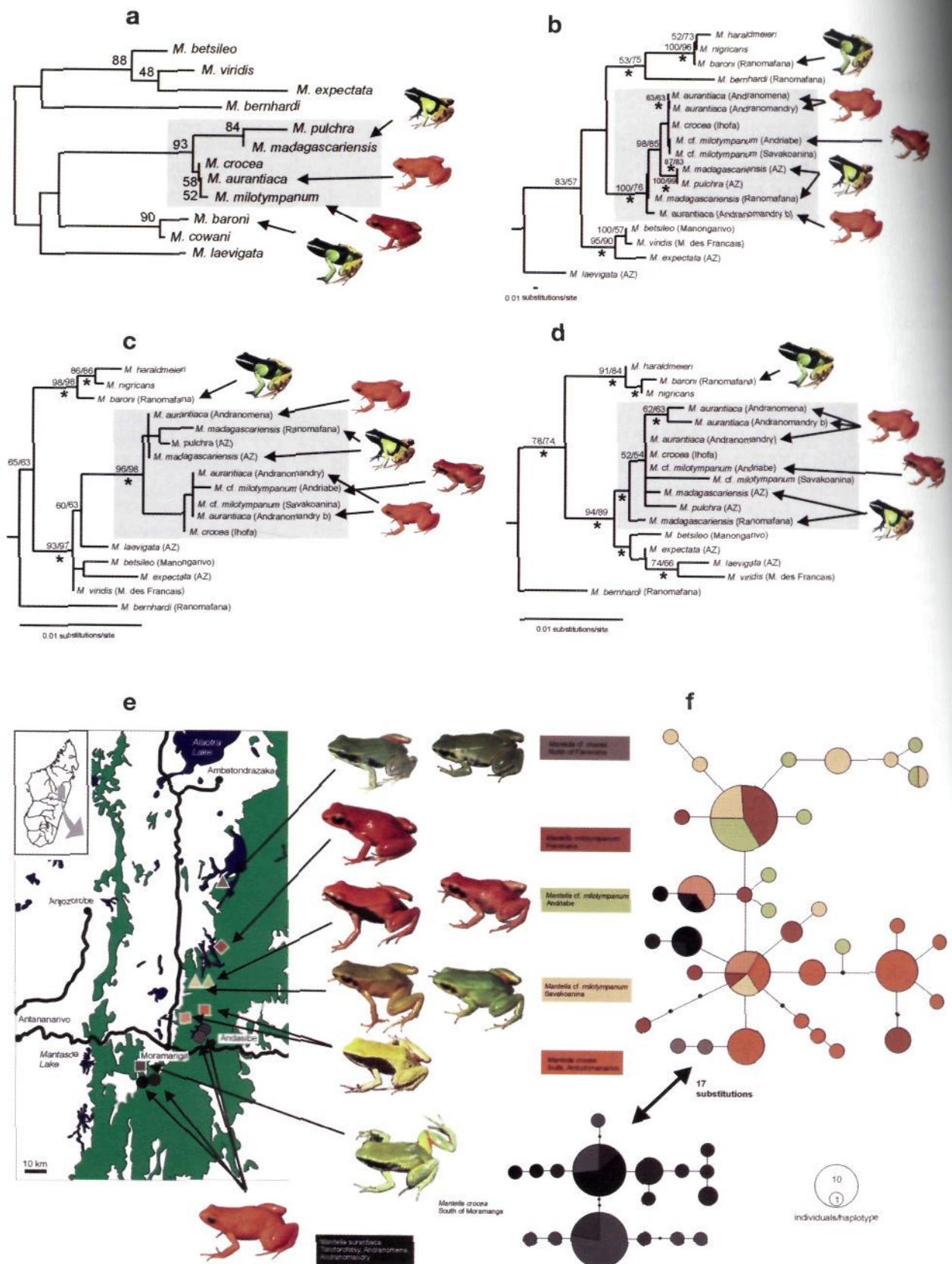
As yet there have been relatively few studies using comprehensive databases comprising morphology, acoustics (for anurans), mtDNA haplotype information, and some nuclear gene information (such as allozymes, nuclear DNA sequences, and microsatellites). Nevertheless, the known examples of nonconcordance of datasets serve as a warning to take care in making systematic decisions from single datasets. Sharing of haplotypes among species is known (Vences *et al.* 2004a; see below), and there are also instances of the presence of non-sister haplotype clades within what are recognized as single species based on other datasets (e.g., Wake and Jockusch 2000; see below). The high frequency of hybridization among anuran species (e.g., Littlejohn and Watson 1993; Vines *et al.* 2003) predicts that mtDNA alone can be misleading due to introgression, and this is confirmed by the many instances of paraphyletic species that are being found in mtDNA studies (Funk and Omland 2003), and by various case studies in amphibians (e.g., Babik *et al.* 2005; Vörös *et al.* 2005; Weisrock *et al.* 2005).

Malagasy poison frogs of the *Mantella madagascariensis* group are closely related to each other, and are distributed parapatrically and allopatrically (Fig. 5). The five taxa in this group (*M. aurantiaca*, *M. crocea*, *M. milotympanum*, *M. madagascariensis*, *M. pulchra*) differ in colour pattern, (which seems to be stable in most, although not all, populations), habitat choice (swamp forest versus rainforest), and ecology (pronounced decrease of activity during estivation versus virtually continuous activity). Hybridization among these forms has been successfully achieved in captivity. Their status as separate species is debatable because genetic admixture certainly occurs, at least between *M. milotympanum* and *M. crocea*, but changes in taxonomy are resisted for practical reasons, such as more efficient control of the pet trade in these animals (Vences *et al.* 1999b). As can be expected in such a group with incipient speciation, haplotype sharing among taxa has been observed, namely among *Mantella crocea* and *M. aurantiaca* (Vences *et al.* 2004a), and among *M. madagascariensis* and *M. aurantiaca* (Chiari *et al.* 2004). Interestingly, allozyme trees grouped the five taxa into two clusters that agree with ecological and chromatic characters: (1) *M. madagascariensis* and *M. pulchra*, rainforest species with yellow-black colour and (2) *M. aurantiaca*, *M. crocea* and *M. milotympanum*, swamp-forest species (Vences *et al.* 1998), whereas mitochondrial sequences suggested other phylogenetic relationships (Vences *et al.* 2004a).

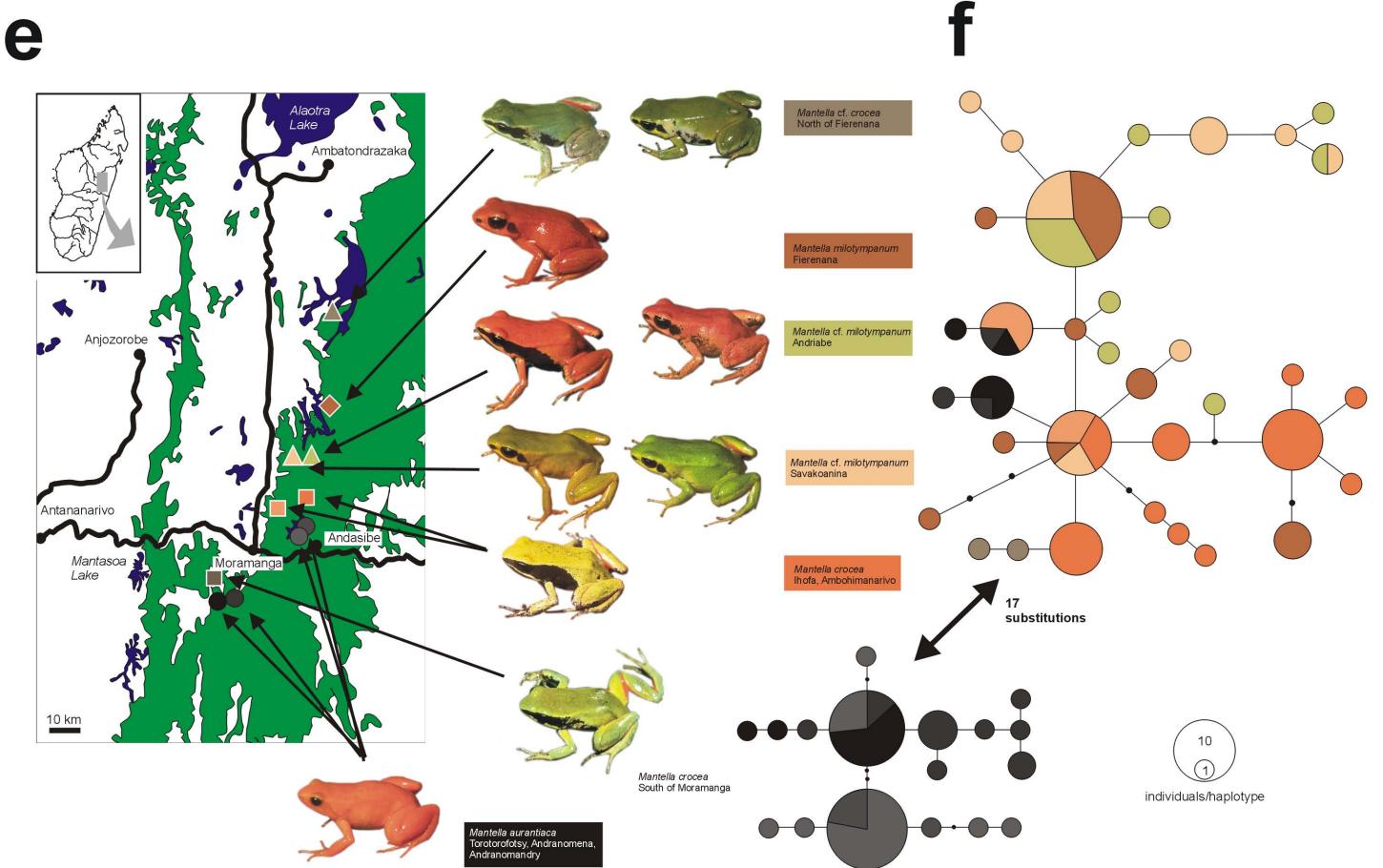
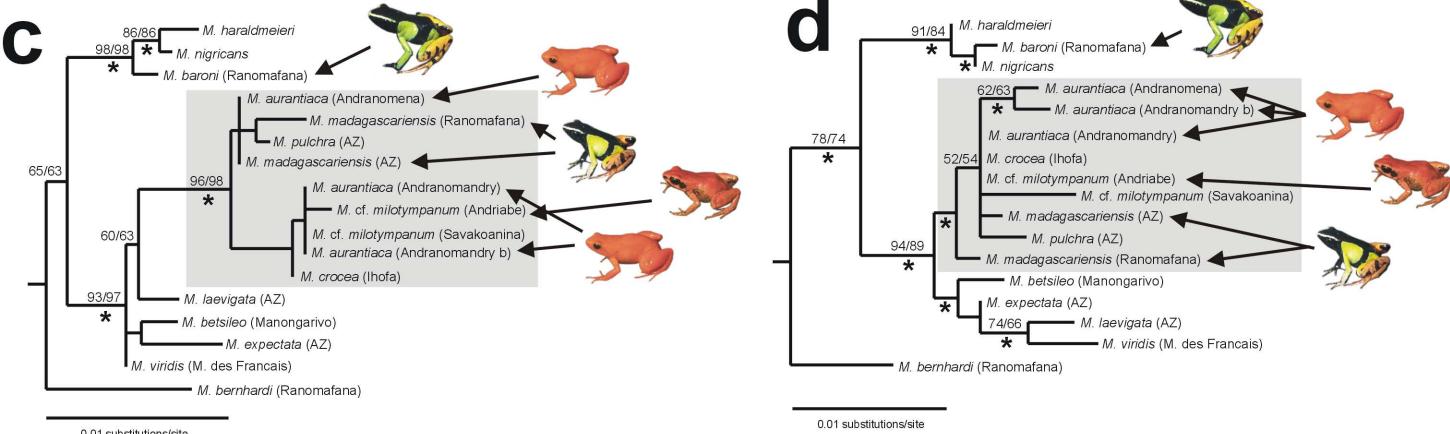
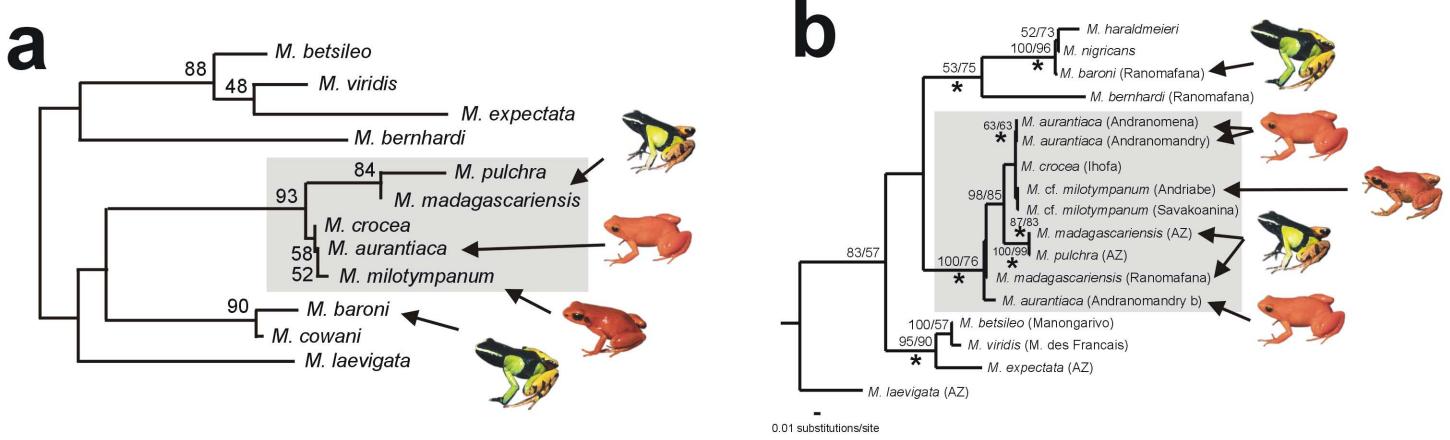
In North American *Rana cascadae*, mtDNA and nuclear (microsatellite) analyses were concordant in suggesting a substantial genetic differentiation of the California populations versus the Oregon/Washington populations (Monsen and Blouin 2003). Regarding mtDNA alone, a similar degree of differentiation was observed between a geographically isolated population on the Olympic Peninsula, Washington, and other Oregon/Washington populations. The Olympic population was fixed for one specific, highly divergent haplotype. In microsatellite analyses, however, it clustered with other Washington populations and showed no conspicuous differences from these.

Iberian painted frogs, genus *Discoglossus*, are taxonomically a difficult (and highly debated) group, where much of the disagreement stems from conflicts between datasets. Two species have been described from the area, *Discoglossus galganoi* and *D. jeanneae*, the latter initially thought to occur only in southernmost Spain, south of the Guadalquivir river. These are largely seen to correspond to two main haplotypic lineages that roughly occupy a southern and eastern portion of the Iberian peninsula (*jeanneae*) and a northern and western portion (*galganoi*) (García-Paris and Jockusch 1999). The two lineages are sister to each other but do not have relevant differentiation in morphology or calls. Zangari *et al.* (2006) compared mitochondrial data with allozyme differentiation, and found that the two taxa have only limited allozymic differentiation, and that furthermore the geographic distribution of nuclear *jeanneae* alleles does not extend as far east as that of *jeanneae* haplotypes. Consequently, these authors see the pattern as a result of admixture among these forms and reject their status as separate species.

Within *Batrachoseps*, several species contain haplotype clades that are non-sister. Northern and southern haplotype clades of *B. major* meet along a narrow border. Despite the large differentiation (more than 9% pairwise divergence in the cytochrome *b* gene), allozyme evidence suggests that gene flow is taking place between the groups; they are considered conspecific (Wake and Jockusch 2000). The species known in earlier literature as *B. nigriventris* was separated into two species, *B. nigriventris* in southern and coastal California, and *B. gregarius*, in the Sierra Nevada and its foothills. These morphologically similar forms were placed in different species because their haplotypes were not sister and were divergent by 9–10%.  $D_N$  from allozymes ranged from 0.16–0.40, with at least one fixed difference. Within *B. gregarius* non-sister haplotype clades (diverged by 7–8% in cytochrome *b*), however, are found what would be considered species, yet a minimum  $D_N$  between the clades of 0.04 was taken as evidence of conspecificity (Jockusch *et al.* 1998). In *B. nigriventris*, even with *B. gregarius* removed, problems remain. MtDNA haplotypes of northern and southern populations are from 5.2–11.5% divergent and are non-sister. Furthermore, allozymic differences between the groups are large ( $D_N$  from 0.16–0.29) (Wake and Jockusch 2000). These units appear to be approaching species-level divergence, but



**Fig. 5.** Complex patterns of genetic and chromatic variation in Malagasy poison frogs of the *Mantella madagascariensis* group. The trees in (a), (b), (c) and (d) show the phylogeny of species in this group based (in this order) on allozyme distances, on one mitochondrial gene sequence (cytochrome *b*), and two nuclear gene sequences (Rag-1 and Rag-2). The *M. madagascariensis* group is shaded grey. The convergent evolution of the same aposematic pattern in the largely sympatric *Mantella madagascariensis* and *M. baroni* (the latter belonging to a different species group; Nei's genetic distance based on allozymes = 0.56) is supported by all three datasets, and may have been triggered by Müllerian mimicry. The markers in (b-c), however, fail to group individuals from the same population and species (*M. aurantiaca* and *M. madagascariensis*), probably due to haplotypic introgression in the mitochondrial gene and incomplete lineage-sorting in the nuclear genes. The species *M. aurantiaca* and *M. crocea/M. milotympanum* are not grouped as monophyletic by any of the DNA sequences, but by allozymes (a). Sequences marked with "AZ" in (b-c) refer to the same individuals used in the allozyme study (a). These species occupy a limited area in central eastern Madagascar as shown by the map (d) and are swamp-forest specialists. Haplotype sharing is observed among all three taxa (e), and *M. milotympanum* is most probably a local colour variant of *M. crocea*. After Vences *et al.* (1998) and Chiari *et al.* (2004).



new data from nuclear gene sequences (ITS, Jockusch, pers. comm., 2004) finds no difference between the two geographic units of *B. nigriventris*.

Beyond amphibians, a striking case of discordance is found in greenish warblers of the genus *Phylloscopus* (Irwin *et al.* 2002), a supposed ring-species complex assigned to *P. trochiloides*. Two major haplotype clades meet south of the Himalaya Mountains, and the two groups merge genetically. The expected explanation for such a pattern would be that it represents secondary contact of previously isolated and genetically differentiated population segments, but Irwin *et al.* (2002) argued that the discordance (the levels of divergence are low) might have arisen *in situ*, without any geographic barriers to gene flow.

Monsen and Blouin (2003) discussed the disagreement of mitochondrial and nuclear markers in *Rana cascadae* in terms of the expected fourfold reduction of effective population size ( $N_e$ ) of mitochondrial DNA, caused by its matrilineal inheritance. Such a reduction of the effective population size should lead mtDNA to reach reciprocal monophyly faster than nuclear DNA (Moore 1995). Because of this reduction of  $N_e$ , even low levels of introgression may be sufficient to establish a neutral mitochondrial genotype in a foreign population (Takahata and Slatkin 1984). This could explain the presence of haplotypes typical for *Mantella crocea* in a population of *M. aurantica* which chromatically shows no signs of hybridization (Vences *et al.* 2004a). Wake and Jockusch (2000) attributed the discordance of mitochondrial and nuclear markers in *Batrachoseps* to the fact that male salamanders move more than females, which could cause the persistence of divergent, maternally inherited mtDNA haplotypes in peripheral populations, even if gene flow is maintained or re-established through the more mobile males. A male-biased dispersal pattern has also been inferred by Lampert *et al.* (2003) in túngara frogs, and observed by Joly and Grolet (1996) for juvenile Alpine newts, *Triturus alpestris*. If female anurans were more sedentary than males, this could explain the pattern observed in *Rana cascadae* (Monsen and Blouin 2003; see above), but evidence for female-biased dispersal was found in bullfrogs, *Rana catesbeiana*, by Austin *et al.* (2000), and in common frogs, *Rana temporaria*, by Palo *et al.* (2004a). Funk and Omland (2003) emphasized that in female heterogametic taxa, hybrid females can show a reduced viability that might restrict the introgression of maternally inherited mtDNA between species. Sex determination and sex chromosomes in amphibians are poorly understood, but female heterogametic systems are known (King 1990) and could influence interspecific and even intraspecific mitochondrial gene flow. Possible associations between heterozygosity and survival probability, as found by Lesbarres *et al.* (2005) in tadpoles of *Rana temporaria*, could further influence differences between mitochondrial and nuclear genetic structure of amphibian populations as they probably would influence more strongly the diploid nuclear genome than the haploid mitochondrial genome. Related to the problem of understanding the impact of all these population genetic factors is the contradictory information available on effective population sizes (numbers of reproducing individuals) in amphibians. The values summarized by Funk *et al.* (1999) for various salamander and frog species are all below 400 individuals, and often below 100 individuals (see also Zeisset and Beebee 2003; Scribner *et al.* 1997). In contrast, Crawford (2003a) estimated extremely high effective population sizes of  $10^5$  for species of *Eleutherodactylus*, and Hoffman *et al.* (2004) estimated values of hundreds to at most a few thousand in leopard frogs (*Rana pipiens*).

How organellar genomes versus nuclear genomes are influenced by range shifts might be a very relevant source of discordance among mtDNA and nuclear datasets, given that the smaller  $N_e$  of organellar genomes increases the probability of loss of allelic diversity at the leading edge of a range expansion (due to stronger genetic drift). Kuchta and Tan (2005) studied phylogeography in the rough-skinned newt, *Taricha granulosa*, using allozymic and mitochondrial (cytochrome *b*) sequences. They found evidence for a post-glacial range expansion northwards, reflected by isolation by distance in southern populations but no such patterns in the northern populations. The mtDNA range expansion signature, however, had its southern limit in Oregon whereas the allozyme signature had its limits further northwards in Washington. Kuchta and Tan (2005) stressed that due to the lower

effective population size of mtDNA (and the resulting low number of haplotypes per population), any colonization event starting from a single population will result in low levels of haplotype diversity in the newly colonized area. Short-range colonizations are not different from long-range (leptokurtic) colonizations, although the latter will result in even lower diversity. By contrast, the authors hypothesized that allozymes may not necessarily suffer a reduction in intrapopulational diversity if the expansion is not leptokurtic. In the example of *Taricha granulosa*, the newts may first have expanded their range slowly northwards out of California, the signature of this process only recorded in mtDNA, and then in a second step performed a rapid expansion into Alaska, with a signature also recorded by allozymes (Kuchta and Tan 2005). In order to better understand processes of range expansion from historical refugia, statistics based on coalescence theory were used by Carstens *et al.* (2005) for the salamander *Dicamptodon aterrimus*; testing explicit hypotheses by such methods may become crucial for understanding the dynamics of mitochondrial and nuclear allele distributions in time and space.

Discordance between nuclear and mitochondrial clines are also observed in hybrid zones. Sequeira *et al.* (2005) analysed an area in Portugal where distinct phylogeographical lineages of the golden-striped salamander *Chioglossa lusitanica* meet. Based on a detailed population genetic analysis they concluded that hybridization proceeds according to a neutral diffusion process and that the observed pattern is panmixis in genetically admixed populations. In this area of secondary contact, however, the mitochondrial cline is (1) much narrower than that of four (largely concordant) allozyme markers, and (2) displaced 20 km to the north, possibly because increased effects of drift may cause mtDNA to cross contact zones slower and remain more stationary whereas nuclear markers would spread faster through populations in contact (Sequeira *et al.* 2005). A different pattern was observed by Babik *et al.* (2005) in a hybrid zone of closely related newts (*Triturus vulgaris* and *T. montandoni*) in the Carpathian mountains. While geographical clines of nuclear markers were highly concordant, mtDNA showed much wider bi-directional introgression. This pattern may be due to historical range shifts, the mitochondrial signature being representative of the original distribution of the two species, but other explanations may also apply (Babik *et al.* 2005).

Phylogeographic discontinuities in the distribution of mtDNA haplotypes are sometimes interpreted as species' borders in the absence of other information (e.g., Bradley and Baker 2001; Burbrink 2002). There are now many instances of phylogeographic discontinuities based on haplotypes that are not considered to mark species' borders among amphibians, including those mentioned above (*Ensatina*, *Batrachoseps*, *Rana*, and *Mantella*) and in *Salamandra salamandra* and *Rana temporaria* in the Cantabrian mountains of northern Spain (Fig. 6).

#### D. DNA Barcoding and the Concepts of MOTUs and Candidate Species

Considering the recent progress in DNA sequencing techniques, proposals have been made to screen the genetic diversity of large samples of organisms — even of pooled environmental samples — using standard molecular markers. Such procedures have gained attention under the terms DNA barcoding (Hebert *et al.* 2003) or DNA taxonomy (Tautz *et al.* 2003). These authors suggested either the mitochondrial COI gene (Hebert *et al.* 2003) or the nuclear small subunit ribosomal genes (Tautz *et al.* 2003) as standard markers. DNA barcoding has the potential to accelerate taxonomic progress (Wilson 2004), and a claim for portable molecular species-identification tools to increase "bioliteracy" has been made (Janzen 2004).

For such standardized high-throughput screenings of genetic diversity, pair-wise divergence values among DNA sequences are more universally applicable than are allozyme distances. On the one hand, the results of different workers can be directly compared. On the other hand, DNA sequences can be resolved from non-frozen tissues, avoiding the difficulty of preserving and transporting frozen samples which is especially acute in remote tropical areas.

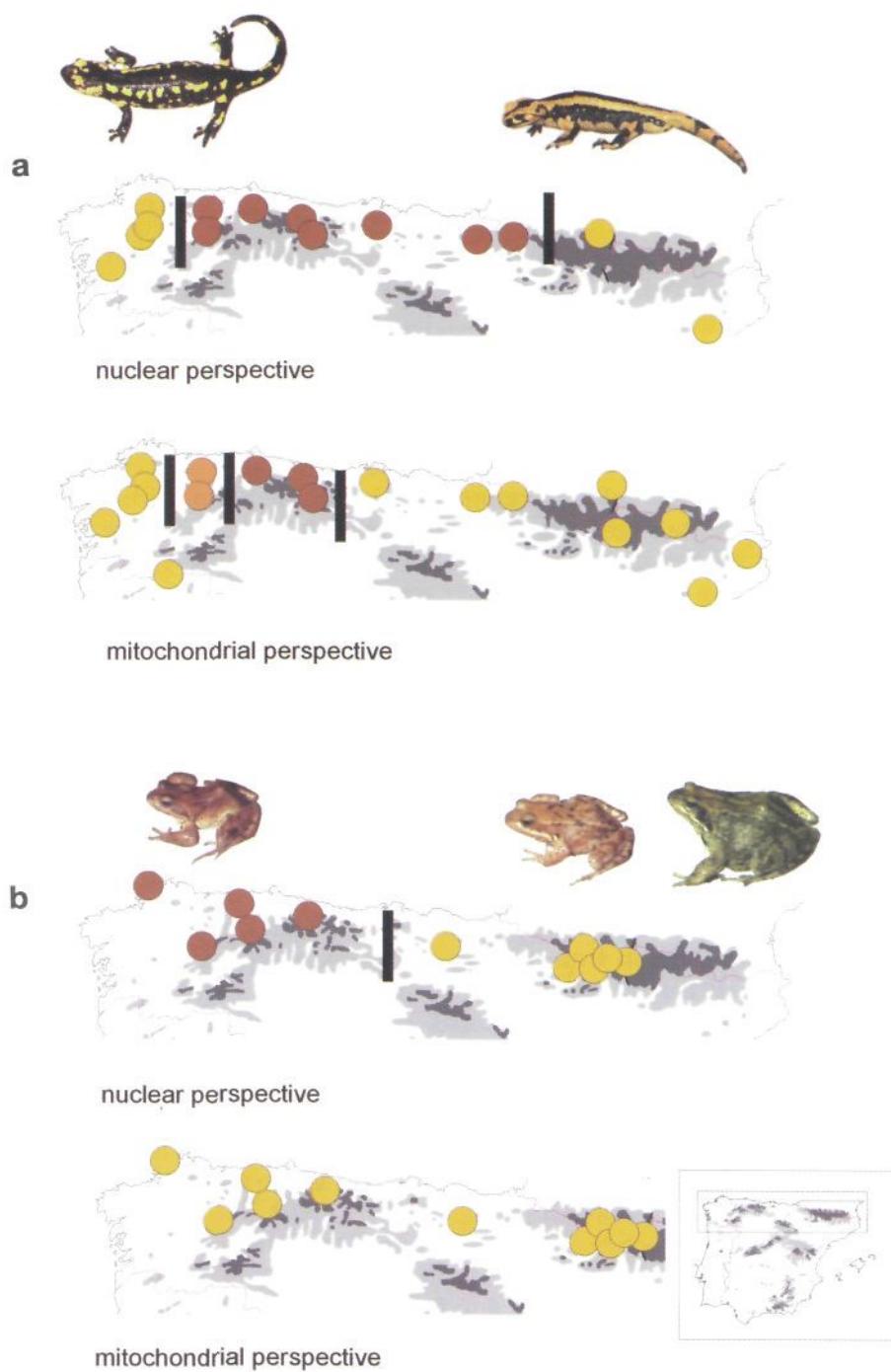
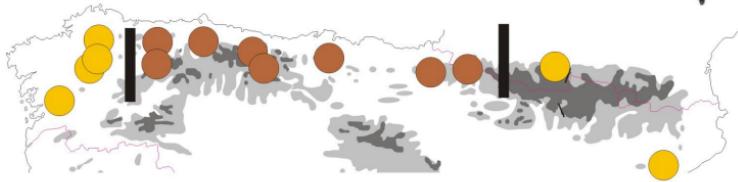


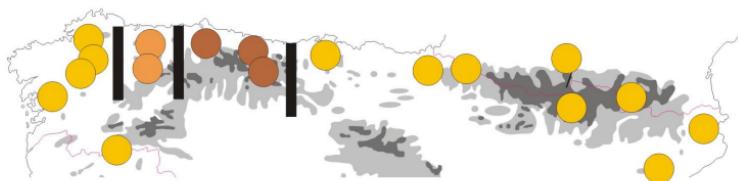
Fig. 6. Disagreement between nuclear markers (allozymes), mitochondrial DNA and morphology in identifying genetically divergent populations of salamanders (*Salamandra salamandra*) and common frogs (*Rana temporaria*) in northern Spain. Modified from García-París *et al.* (2003), Veith *et al.* (2000) and Veith, Kosuch, Dubois and Vences, in preparation. Common frogs in the Pyrenean mountain range show strong morphological variation among high-elevation and low-elevation populations that is not accompanied by relevant genetic divergence.



a



nuclear perspective



mitochondrial perspective



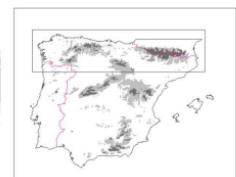
b



nuclear perspective



mitochondrial perspective



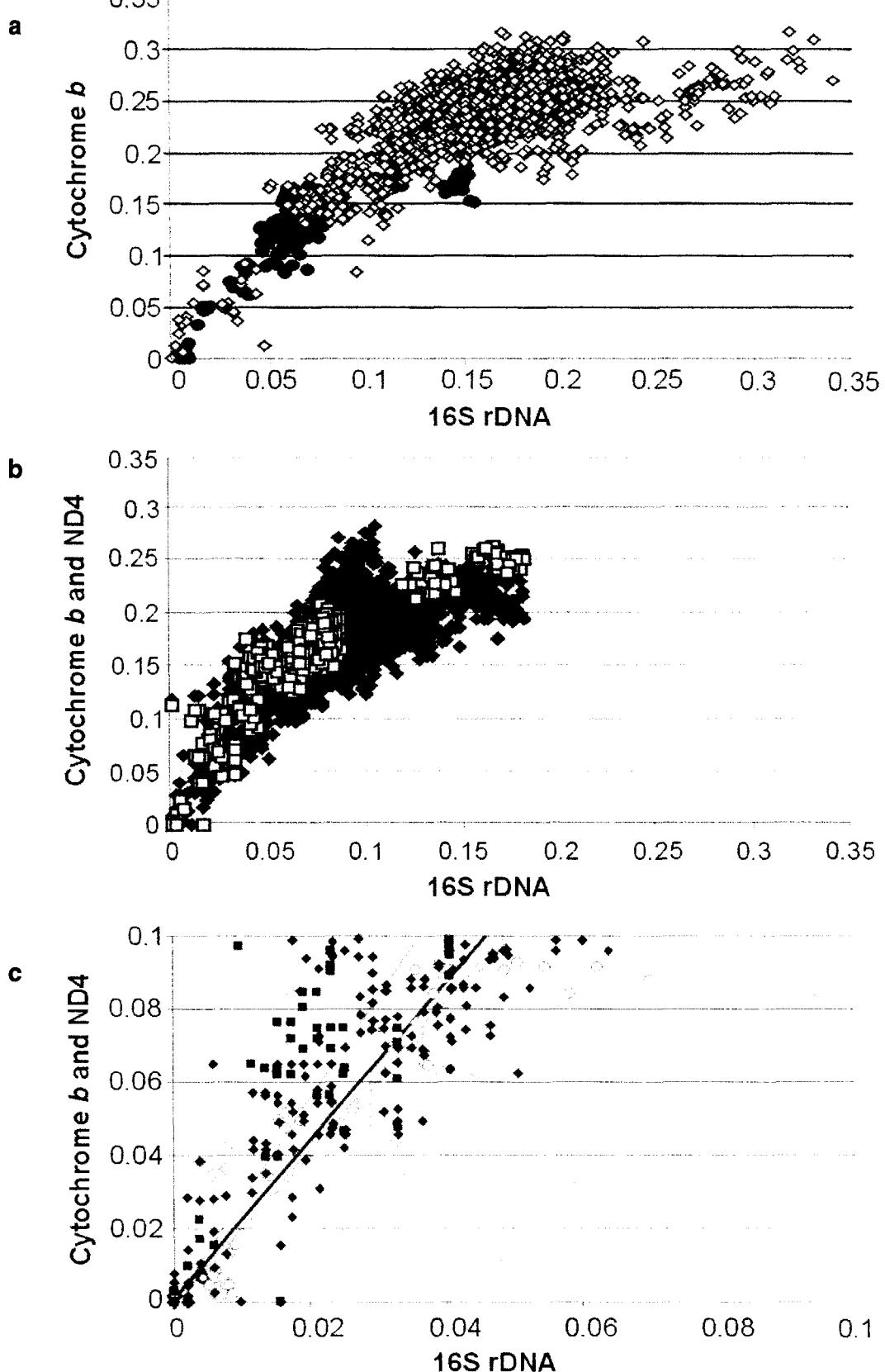
Unfortunately, amplification and sequencing of COI in amphibians is not straightforward from a technical point of view when standard primers and conditions are to be applied on a large scale in high-throughput approaches. Certainly it will be possible with optimized protocols to obtain high success rates in COI amplification in amphibians. But problems will remain in assays where false negatives are of concern, e.g., in analysis of pooled samples for assessment of tadpole diversity, because the priming sites of this gene are highly variable in amphibians (Vences *et al.* 2005a, 2005b).

Problems also apply to the use of nuclear ribosomal genes in amphibians. Early studies of 28S showed that the portions studied evolve too slowly even to resolve generic-level relationships among salamanders (Larson and Wilson 1989), but other, more variable parts of this gene exist and may hold promise. Direct PCR amplification of this gene, however, remains challenging and requires optimization of laboratory protocols.

Sequencing of another widely used marker, the mitochondrial cytochrome *b* gene, poses no problems in salamanders and archaeobatrachian frogs but is more difficult in neobatrachians in which a genomic rearrangement in the mitochondrion (Macey *et al.* 1997) makes the application of standard primers impossible and increases the risk of obtaining nuclear pseudocopies of the gene (Vences *et al.* 2004a). Because neobatrachians are the vast majority of amphibian species, it therefore seems reasonable to select the mitochondrial 12S and 16S rRNA genes as one standard marker for amphibian DNA barcoding in the short term, either combined (AmphibiaTree consortium 2004; Darst and Cannatella 2004) or as a fragment of the 16S rRNA gene that amplifies particularly well and has been most widely used in phylogenetic studies of amphibians (Vences *et al.* 2005a). As a general indication of the use of different genes in amphibian studies, the Genbank database (<http://www.ncbi.nlm.nih.gov/>), in April 2004, had 2 845 hits for 16S, 2 658 for cytochrome *b*, 466 for COI but only 90 for 28S. In May 2005, these values had increased to 4 130 for 16S, 3 705 for cytochrome *b*, and 539 for COI, reflecting the enormous increase of molecular studies on amphibians, and the wide use of the 16S gene.

Protein-coding genes like ND1 or cytochrome *b* are known to evolve faster than 16S rDNA (Fig. 7a–b). A comparison from plethodontid salamanders and ranid and mantellid frogs (Fig. 7c) gives surprisingly concordant results, which suggests that, below an initial saturation plateau of 10% pairwise divergence, cytochrome *b* evolves, on average, almost exactly two times faster than does the typically used fragment of 16S rDNA. Mean divergence values can probably easily be compared by correcting for this factor. One study that extensively used COI sequences of an amphibian species to assess phylogeographic patterns is that by James and Moritz (2000). They found that the 3'-terminal COI fragment of the Australian sedge frog, *Litoria fallax*, regularly had more than 5% pair-wise divergences among haplotypes from neighbouring populations, and 11–12% between two major haplotype clades within the species. The large variability of this gene was also obvious from the fact that 87 individuals showed 84 unique haplotypes. This result agrees with the presumably high variability of this gene in amphibians generally (Vences *et al.* 2005a, 2005b).

As molecular analyses have become commonplace in amphibian systematics, much formerly cryptic biodiversity has been disclosed. The definition and diagnosis of species by mere genetic distances is controversial and problematic, as discussed here, and taxonomic conclusions based on genetic distances alone are strongly discouraged. On the other hand, molecular barcoding techniques can provide a very useful tool for preliminary screening of amphibian diversity, especially in poorly studied tropical regions, as long as the conclusions are not formalized by premature descriptions of new species. Floyd *et al.* (2002) and Blaxter (2004) proposed the term MOTU (molecular operational taxonomic unit) to refer to specimens grouped through a set of genetic markers, i.e., through DNA sequences that cluster within some defined cut-off level. Blaxter (2004) considered MOTU to be synonymous with the terms "phylogroup" and "genospecies", and to have a similar extent as traditional "species". A MOTU, however, does not inherently contain the biological information of an irrevocable evolutionary divergence as a species ideally does. Two



**Fig. 7.** Scatterplot of uncorrected pair-wise divergences from mitochondrial protein-coding genes (cytochrome *b* and ND4) and 16S rDNA in various amphibian datasets. (a) Cytochrome *b* vs. 16S rDNA in mantellid frogs (open symbols; M. Vences, unpubl. data) and Palearctic brown frogs (*Rana*; black symbols; Veith *et al.* [2003a]). (b) Cytochrome *b* (black symbols) and ND4 (open symbols) versus 16S rDNA in datasets of plethodontid salamanders (from Parra-Olea and Wake 2001; Parra-Olea 2002; D. B. Wake, unpubl. data). (c) Linear regression plots of the ND4 (thin black line; black squares) and cytochrome *b* (thick grey lines) in salamanders and frogs (data from plots a and b, only distances of 0–0.1; white circles, brownfrogs; white rhomboids, mantellids; black rhomboids, salamanders). The cytochrome *b* versus 16S regression lines of frogs (thick grey lines) and salamanders (thick black line) show almost identical slopes.

conspecific populations with divergent mitochondrial haplotypes could easily and correctly be defined as different MOTUs.

It does seem reasonable, however, to apply a rationale based on genetic distances to the exploratory identification of units that potentially could be differentiated at the species level. The term "provisional species" as proposed by Hebert *et al.* (2004) seems too definitive in that it already implies that the identified units are species, although not nomenclaturally described and therefore "provisional". Instead, it is proposed here that the term *candidate species* be used to refer to such units. Candidate species can be identified by any sort of rapid assessment, such as morphological or bioacoustic surveys, but in most cases will be diagnosed by DNA barcoding. When a DNA sequence shows divergences from other sequences above a certain threshold considered to be generally typical for differentiation of species, then the respective specimen can be assigned to a candidate species (e.g., *Plethodon elongatus/stormi* complex; Mahoney [2004]). Further detailed studies including a combination of various datasets (Wiens and Penkrot 2002) can then be directed towards this specimen to ascertain whether or not it actually represents a biological species.

#### E. Estimates of Amphibian Species Diversity

How many species of organisms currently live on Earth is a subject of ongoing controversy and is surprisingly poorly understood (May 1998, 1990). Insects have the highest estimated number of species of organisms globally; that estimate is also subject to the greatest uncertainty. Nevertheless, the trend of steadily increasing numbers of known species of amphibians (Köhler *et al.* 2005) indicates that even in vertebrates, which doubtless rank among the best studied organisms, the inventory is still far from complete. Blaxter's (2004) statement that only about 10% of vertebrate species still await discovery is certainly a strong underestimate.

The discovery and description of previously unknown species of amphibians is increasing at unprecedented rates (Table 2). Since the last comprehensive list by Köhler *et al.* (1998), the pace of species' descriptions again accelerated, and in the period 1998–2004 proceeded at a mean rate of over 100 species per year. At least in some geographic areas, the new descriptions do not seem to be biased towards small-sized species (Reed and Boback 2002) that presumably are more difficult to collect and less attractive to study. This indicates that taxonomic work is incomplete on entire batrachofaunas. Several plethodontid salamanders that are among the largest in the family have been discovered in Mexico: *Bolitoglossa alberchi* (García-París *et al.* 2003), *Pseudoeurycea nauhcampatepetl* (Parra-Olea *et al.* 2001), *P. papenfussi* (Parra-Olea *et al.* 2005a), *P. maxima* (Parra-Olea *et al.* 2005b), as well as in lower Central America: *Bolitoglossa magnifica* (Hanken *et al.* 2005) and even in such well explored areas as California: *Batrachoseps robustus* (Wake *et al.* 2002). Some spectacular discoveries have been made recently, notably of a new species, genus and family (Nasikabatrachidae) of frogs from southern India (Biju and Bossuyt 2003) and in Korea of a new genus and species (*Karsenia koreana*) of salamander from the Plethodontidae, a family not previously known from Asia (Min *et al.* 2005). It is correct, however, that in more thoroughly sampled areas new species are often subdivisions of taxa earlier thought to have

Table 2. Number of nominal amphibian species from three comprehensive accounts (Frost 1985; Duellman 1993; Köhler *et al.* 1998; the latter referring to counts until 1997), and recent numbers from Amphibiaweb (as of March 2004). The last column gives the cumulative increase in per cent, as compared to the initial number in 1985. The overall rate of increase is about 3% per year.

Year	Anura	Caudata	Gymnophiona	Total	Cumulative increase
1985	3 483	357	163	4 003	—
1993	3 967	392	163	4 522	12.9%
1997	4 371	436	163	4 970	24.2%
2004	4 917	520	168	5 605	40.0%

broad distributions (Hanken 1999). Examples include North American *Rana* (see Hillis *et al.* 1983), *Plethodon* (e.g., Highton and Peabody 2000), and *Batrachoseps* (Jockusch and Wake 2002), and European *Discoglossus* (Lanza *et al.* 1984; Capula *et al.* 1985; Busack 1986) and *Hydromantes* (Lanza *et al.* 1986). This reflects a general trend of finer taxonomic resolution of taxa from well-studied temperate areas also found in other animal groups (e.g., Chek *et al.* 2003a). Köhler *et al.* (2005), however, in assessing a now well-studied group of tropical amphibians, mantellid frogs from Madagascar, found that the many new species since 1992 were genetically as divergent from their closest previously described relatives as were species described between 1970 and 1991. In addition, most of these new species were based on newly collected specimens from poorly explored areas, and were also recognizable by morphology alone. They were not merely hidden among voucher specimens examined by previous researchers (Köhler *et al.* 2005). In salamanders of the genus *Aneides*, COI distances among conspecific populations amounted to as much as 7.8% (Vences *et al.* 2005b). Thus, these new species were true first-hand discoveries and not the result of a finer taxonomic resolution of already known species. The general claim of Isaac *et al.* (2004) that the increase in recognized species is due mostly to taxonomic inflation rather than to new discoveries is certainly unjustified for amphibians, and might rather reflect a widespread bias towards endothermic animals (Bonnet *et al.* 2002). The wealth of new amphibian species is in part due to refined techniques, i.e., molecular and bioacoustic methods that allow the recognition of sibling species, but, more importantly, it is strongly influenced by better and more intensive exploration of remote regions, especially in the tropics.

In conclusion, there is little doubt that by far the largest proportion of described taxa merit recognition as species independent of which species-concept or criteria are applied. Disputes about species' boundaries concern a few well-studied species complexes, mostly from temperate areas in North America and Europe. Taxonomic resolution is at a lower level in tropical regions, and it is unlikely that many recently described amphibian species from hyperdiverse tropical countries will have their status questioned by future, more detailed genetic studies. In the examples of conspecific populations of salamanders discussed previously, a cytochrome *b* divergence of 7–9% was found, which corresponds to a 16S rDNA divergence of 3.5–4.5%. In several widespread frog species from Madagascar (family Mantellidae), populations from two localities about 250 km apart, Ranomafana and Andasibe, show high haplotypic divergences. These frog species have a similar ecology, morphology and advertisement calls at both localities, and as far as is known they are continuously distributed in the intervening area. Hence, there is no reason to assume that they are differentiated at the specific level. The encountered divergences are of 2–5% pairwise 16S rDNA divergences, whereas the modal differentiation among sibling species of mantellids is at 8% (Vences *et al.* 2005a; Fig. 8). In these same frogs, intraspecific COI divergences of 10–14% appear to occur regularly, and up to 18% may be possible in single cases (Vences *et al.* 2005b). On the other hand, in a vast sample of mantellid frogs of which 5–10 individuals were sequenced per population, the maximum 16S rDNA haplotypic divergence within populations was on the order of 1%. Based on these data, it seems reasonable to apply a criterion for identification of candidate species of 6–7% 16S sequence divergence (12–14% cytochrome *b* divergence) in allopatry, and 2% 16S rDNA divergence (4% cytochrome *b* divergence) in sympatry. Preliminary estimates of the number of candidate species in the frog fauna of Madagascar, using these criteria, indicate a conservative proportion of at least 30%, but the number of undescribed species may indeed be as high as the number already described. Similar results have been reported from Sri Lanka, where the majority of frogs may be unnamed and a great many may already have become extinct because of conversion of forests for agricultural and other purposes (Manamendra-Arachchi and Pethiyagoda 2005; Meegaskumbura *et al.* 2002), and from South-East Asia, where molecular surveys indicate that several widespread species of frogs are indeed complexes of partly sympatric sibling species (Stuart *et al.* 2006).

A major challenge for the future will be to achieve a comparable state of knowledge on each major taxonomic and regional amphibian group by applying congruent criteria

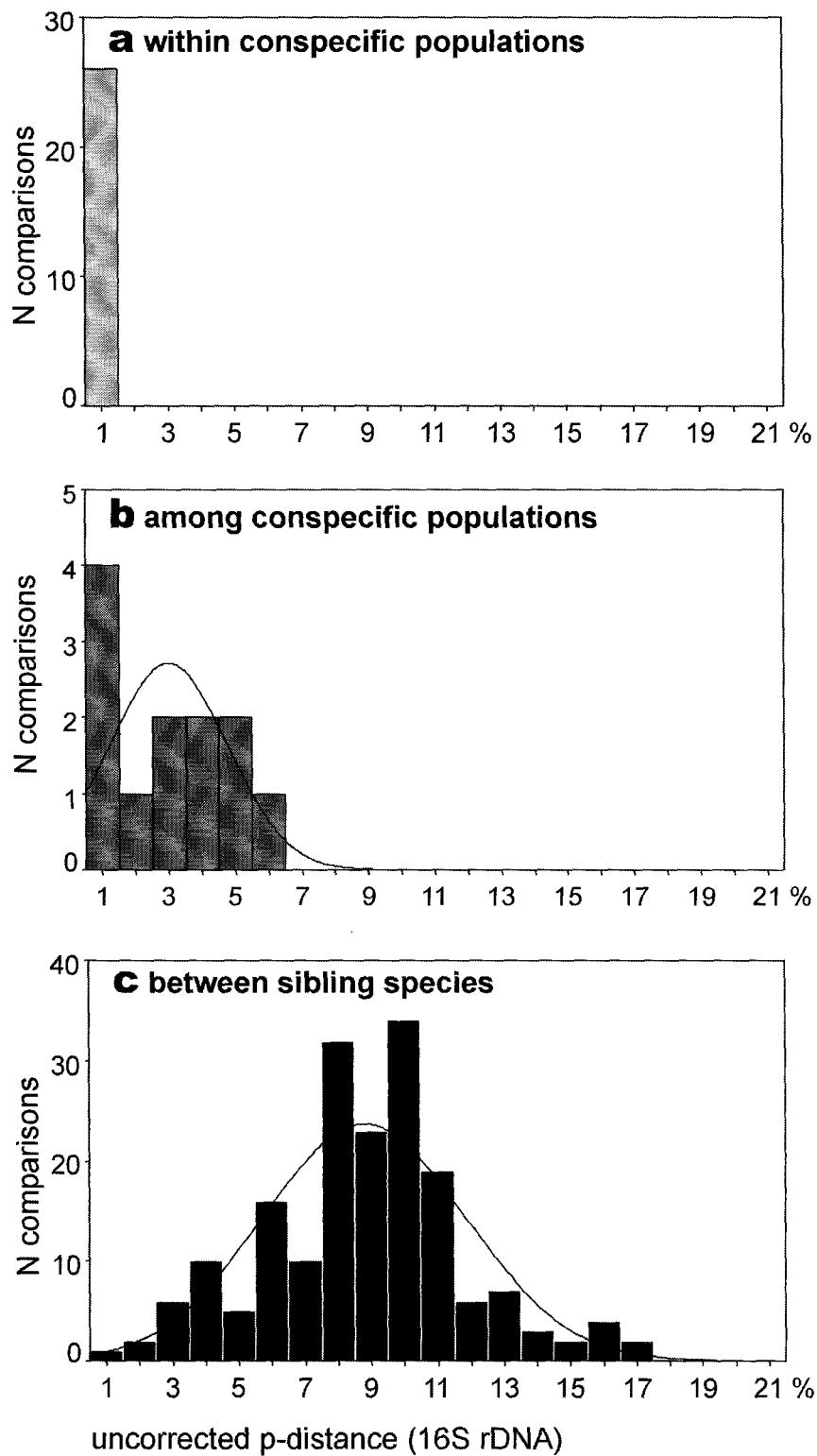


Fig. 8. Pair-wise DNA divergences between individuals of Malagasy frogs (family Mantellidae) that belong to the same population (a), to conspecific populations from the two hyperdiverse rainforest sites, Ranomafana and Andasibe (b) or to different sibling species (morphologically similar species that are either sister to each other or that cluster in an unresolved terminal polytomy) (c). The values for conspecific populations are, for each species, the average of all comparisons (2–10 individuals per site) and therefore do not include the maximum differences; a graph with the maximum inter-populational distances per species (not shown) does not fundamentally differ, however (Vences *et al.* 2005). Note that in this group of frogs, so far there are no examples of conspecific specimens with pair-wise divergences >6%, but there are an important number of examples (40 out of 182 pair-wise comparisons) of almost certainly distinct species with divergences of 1–6%. These would be missed by any DNA barcoding survey of Malagasy amphibian diversity that employs a cutoff point of 5% or 6% to define candidate species.

for species recognition, and in so doing elucidate global patterns of amphibian diversity. Only such a harmonized global approach, together with regional expertise on distributions and threats as assembled in the Global Amphibian Assessment, will provide a reliable basis for identifying hotspots of amphibian diversity and to outline priority areas for conservation. Future conservation prioritization is likely to make increasing use of molecular phylogenetic data to identify hotspots of genetic diversity or evolutionary history (e.g., Crozier 1997; Purvis *et al.* 2000; Mace *et al.* 2003), rather than relying solely on richness or endemism of nominal species.

## V. CORRELATES, RADIATION TRAJECTORIES AND HYPOTHESIS TESTING

### A. Correlates of Species Diversity

Species diversity is not evenly distributed among amphibian lineages. For example, one family (Plethodontidae) makes up almost 70% of all salamanders (373 out of 544 species), and a single frog genus (*Eleutherodactylus sensu lato*) contains more than 700 species while many other genera are monotypic (AmphibiaWeb as of May, 2005). This certainly, in part, reflects different taxonomic concepts, with some supraspecific groups being defined more inclusively than others, but there is little doubt that certain clades of amphibians are more species-rich than other, fully comparable ones. Identifying possible correlates of this unequal species diversity of amphibian clades is crucial for understanding factors and possible key innovations that led to rapid, explosive radiations. Bogart (1991) observed that in frogs, karyotypic diversity is higher in those lineages in which species produce a small number of eggs or have territorial behaviour, e.g., *Eleutherodactylus* and dendrobatid frogs. Such species are likely to occur in small isolated demes in which inbreeding serves to fix mutational events in a homozygous condition. However, the equally speciose plethodontid salamanders, also with direct development, do not show a conspicuous acceleration of their chromosomal evolution (Sessions and Kezer 1991).

Dubois (2005) recently proposed an hypothesis to account for the presumably higher rates of speciation in direct-developing amphibians. His idea is based on the fact that direct-developers usually deposit a single clutch in a terrestrial environment where it remains for a prolonged period until the juveniles hatch. If the clutch is discovered by a predator, it is likely that all eggs — and thus the complete reproductive effort of one female — will be destroyed. In contrast, aquatic clutches usually hatch after a few days, and the tadpoles then disperse in a pond or stream. Predation is then unlikely to affect all offspring of one female. There is no doubt that familial mortality can contribute to a faster fixation of alleles in a population, and thereby possibly to a more rapid formation of species. Similar arguments, however, could also be used to advocate a higher rate of speciation in frogs from arid environments where rapid drying of temporary ponds often destroys many clutches completely. Familial mortality is certainly one factor that could explain higher rates of speciation in direct-developing amphibians, but other factors, such as effective population size, dispersal capacity, or elevational and habitat specialization need to be taken into account as well.

Based on his experience with Caucasian amphibians, Tarkhnishvili (2004) proposed that a higher incidence of vicariant speciation (called speciation-by-isolation in his work) may be found in ecological specialists with low reproductive output, while adaptive speciation through mechanisms he called “landscape-dependent differential selection” may be more frequent in rapidly reproducing generalists. Vences *et al.* (2002) found higher speciation rates in brook-breeding clades of amphibians of Madagascar and discussed the ecological shift from ancestral pond-breeding to supposedly derived brook-breeding as a possible key innovation promoting diversification. Based on Lewis’ (e.g., 1981) work on the structure of the inner ear of frogs, Ryan (1986) postulated that auditory characters may be associated with higher speciation rates in major clades of frogs by increasing the frequency range in which frogs can communicate, thereby allowing a larger bioacoustic diversification.

## B. Correlates of Genetic Diversity

Life history and natural history may also influence genetic variation. Vences *et al.* (2002a, 2002b) found evidence that some lineages of explosive pond-breeders in Madagascar are characterized by slow rates of molecular (mitochondrial) evolution as compared to brook-breeders or lineages with direct development. Nevo (1985) sought possible correlates of allele polymorphism and heterozygosity in mammals from published allozyme studies. Lower heterozygosities characterized ecological specialists and species from arid environments or with narrow geographic ranges. In a subsequent paper, Nevo and Beiles (1989) postulated that heterozygosity generally is negatively associated with rainfall and increases with aridity and with homogeneity of the environment, as also previously assumed for populations of partly subterranean frogs of the genus *Pelobates* (Nevo 1976). Inger *et al.* (1974) compared allozyme data for seven species of frogs from southeastern Asia, and found evidence for lower genetic variation in habitat (forest) specialists that have linear ranges (along streams) and do not form breeding aggregations, conforming to their prediction that such species would tend to form local demes with restricted gene flow. Carnaval (2002) found a less-developed phylogeographic structure in generalist species of tropical treefrogs, genus *Hyla*, than in specialists from montane closed-canopy forest that presumably were unable to maintain gene flow among forest fragments. In European brown frogs, the widespread *Rana temporaria* shows a high degree of allele polymorphism whereas more geographically restricted species may be genetically more uniform (e.g., Veith *et al.* 2002). On the other hand the almost equally widespread *Rana dalmatina* has a very low genetic differentiation, both within and among populations from throughout its range (M. Veith and M. Vences, unpubl.). In Finnish populations of common frogs, *Rana temporaria*, Johansson *et al.* (2006) found decreasing genetic diversity within populations with increasing latitude, and with decreasing population size and regional site occupancy. Differentiation among populations also tended to increase with increasing latitude. These results indicate, as expected, that smaller populations and those at range borders are less genetic variable, while differentiation among populations can increase at the range borders where populations are more isolated. In a survey of allozyme data published for freshwater invertebrates, Marten *et al.* (2006) found that across groups, species occurring in lotic habitats had stronger genetic differences between populations than those in lentic habitats. They interpreted this difference in terms of a possible higher dispersal propensity of lentic species which would be an adaptation to the lower stability and predictability of lentic habitats over time. Genetic population differentiation was negatively correlated with body size, and size was seen as a possible surrogate for local abundance (Martens *et al.* 2006). A critical test of this hypothesis in amphibians is needed.

Fully terrestrial, direct-developing species of plethodontid salamanders and neobatrachian frogs, especially such large genera as *Eleutherodactylus*, might be expected to form locally isolated demes because they have no communal breeding sites, have small home ranges, and do not disperse very far (Dubois 2005). The observation of rapid karyological evolution in *Eleutherodactylus* (Bogart and Hedges 1995) seems to conform to this prediction, and their high mitochondrial substitution rates (Crawford 2003b) may be related to this pattern as well. Larson (1984; see also Larson *et al.* [1984]) summarized all published data and many data still unpublished at that time for plethodontid salamanders and showed that genetic variance (summarized as  $F_{ST}$ ) was extraordinarily high (mean  $F_{ST} = 0.53$ , s.d. 0.19). Since that time systematic revisions subdivided some of these taxa (e.g., *Plethodon dorsalis*) into two or more species, but even eliminating these, the values are high. In part, this is because the populations can be locally dense and hence sustain low frequency alleles, as indicated also by high heterozygosities. In other instances, such as in the extraordinary desert salamander, *Batrachoseps campi*, populations are small and widely scattered, so local heterozygosity is relatively low while regional genetic variance is high ( $F_{ST} = 0.59$  for 11 populations). The highest heterozygosities known are for direct-developing species that have dense populations, such as *Bolitoglossa subpalmata* (now

*B. pesrubra*) (Hanken and Wake 1982; García-París *et al.* 2000) and *Ensatina escholtzii* from northwestern California (Wake and Yanev 1986), both with heterozygosities in excess of 25%.

There are very few species of direct-developing salamanders that show low genetic variance over a large geographic region. Even *Plethodon cinereus*, a species that occupies a territory that was glaciated in the Pleistocene, has an *Fst* of 0.13. Larson (1984), in a study including 25 samples, each with 5 to 36 populations, on species with ecologies ranging from semiaquatic to terrestrial and arboreal, and including some species with larval development, found that all taxonomic subunits and adaptive types had *Fst* in excess of 0.5. He concluded that the explanation for this high fragmentation among conspecific populations must transcend those variables. He raised the major unresolved question as to whether these levels of differentiation represented reductions in gene flow or long histories of separations; both factors may be in play.

In sharp contrast to these situations is the Hellbender, *Cryptobranchus alleganiensis*, a very large and relatively widespread stream salamander in eastern North America. It shows very little regional variation and exceptionally low heterozygosity (0.02 or less) (Merkle *et al.* 1977; Shaffer and Breden 1989). There is almost no regional differentiation. This species is the sole American representative of an ancient lineage, so it is unlikely to be young, but it may experience relatively high levels of gene flow. Another aquatic species that shows almost no genetic variation across a relatively large geographic range is the mudpuppy, *Necturus maculosus* (Ashton *et al.* 1980). On the other hand, the Chinese giant salamander *Andrias davidianus*, phylogenetically related to and ecologically comparable with *Cryptobranchus*, does show distinct differentiation among populations, although the correlation with geography is poor (Murphy *et al.* 2000).

One study that contains an extraordinarily large amount of new data on mitochondrial and nuclear DNA and analysed from a population genetic perspective is that of Crawford (2003a) for some *Eleutherodactylus* from lower Central America. These frogs had been classified as from two to seven species by previous authors, who considered them to be close relatives. Extremely local *Fst* values were close to zero for pairs of populations, but these values rapidly increased to values between 0.5 to 1.0. Populations were estimated to be very large (on the order of  $10^5$  individuals), and the depth of differentiation very great, with morphologically indistinguishable species having been estimated to have diverged on the order of  $10^7$  million years ago. Crawford concluded that most of the incredible diversification of *Eleutherodactylus* was due to its great age rather than to its higher rate of speciation, and suggested that this explanation may apply to other tropical taxa as well. The possibility remains, however, that rates of molecular evolution may be elevated in some taxa relative to others for largely unknown reasons.

All these examples and counter examples illustrate the speculative nature of single-case correlations and show the need for thorough testing of general patterns using a comprehensive phylogenetic framework, including modern methods of phylogenetic contrast and appropriate null models (e.g., Barraclough *et al.* 1998). Vastly more data have become available since Larson's (1984) review, but there has been no recent comprehensive analysis. The extreme fragmentation characteristic of many amphibian populations is doubtless an important factor in the differentiation of plethodontid salamanders and mantellid, rhacophorid, ranid and leptodactylid frogs. An understanding of such general trends has the potential to provide insights into the factors that promote speciation in amphibians, but only very detailed analysis of incipiently speciating model taxa will foster an understanding of the underlying microevolutionary processes.

### C. Trajectories of Amphibian Radiations

The basic assumption of Lynch's (1989) approach was that the geography of speciation can be inferred from the distributional areas of species at the time of speciation, and that

these can be inferred from present distributions: if two species are currently sympatric, then speciation is inferred to have been sympatric, and if they are allopatric, speciation is inferred to have been allopatric (Losos and Glor 2003). Whether these assumptions are reasonable or not, it is remarkable that other studies using similar methods indicate that sympatric speciation is more common than expected from prior work (Losos and Glor 2003).

Streelman and Danley (2003) proposed a general model in which vertebrate radiations tend to follow similar evolutionary trajectories that consist of successive stages of divergence in (1) habitat, (2) trophic morphology and (3) communication, often in this order. In some groups, such as several radiations of cichlid fishes, there might be departures from the model in which the divergence in signalling occurred before habitat and trophic divergence, and therefore can be thought of as the initial trigger of speciation. The available evidence for frogs, in which distinct call differences seem to be associated with deep genetic divergences, support the hypothesis that in anurans the diversification of communicatory traits is the last stage to occur. Because trophic morphology in amphibians is not a particularly divergent trait among closely related species, the available evidence thus points to ecological (habitat) diversification as the initial step in the typical trajectory toward speciation and radiation, and in most cases this process appears to take place under conditions of geographic isolation.

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