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Deciphering amphibian diversity through DNA barcoding: chances and challenges

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Amphibians globally are in decline, yet there is still a tremendous amount of unrecognized diversity, calling for an acceleration of taxonomic exploration. This process will be greatly facilitated by a DNA barcoding system; however, the mitochondrial population structure of many amphibian species presents numerous challenges to such a standardized, single locus, approach. Here we analyse intra- and interspecific patterns of mitochondrial variation in two distantly related groups of amphibians, mantellid frogs and salamanders, to determine the promise of DNA barcoding with cytochrome oxidase subunit I (*cox1*) sequences in this taxon. High intraspecific *cox1* divergences of 7–14% were observed (18% in one case) within the whole set of amphibian sequences analysed. These high values are not caused by particularly high substitution rates of this gene but by generally deep mitochondrial divergences within and among amphibian species. Despite these high divergences, *cox1* sequences were able to correctly identify species including disparate geographic variants. The main problems with *cox1* barcoding of amphibians are (i) the high variability of priming sites that hinder the application of universal primers to all species and (ii) the observed distinct overlap of intraspecific and interspecific divergence values, which implies difficulties in the definition of threshold values to identify candidate species. Common discordances between geographical signatures of mitochondrial and nuclear markers in amphibians indicate that a single-locus approach can be problematic when high accuracy of DNA barcoding is required. We suggest that a number of mitochondrial and nuclear genes may be used as DNA barcoding markers to complement *cox1*.

Keywords: DNA barcoding; amphibia; *cox1*; 16S rRNA; Mantellidae; *Aneides*

1. INTRODUCTION

Amphibians are a vertebrate class that recently has been in the centre of research and public attention. This partly results from the phenomenon of multi-causal global amphibian declines, which in the most alarming cases occur through the spread of novel pathogens into undisturbed and even protected areas (Berger *et al.* 1998; Daszak *et al.* 2003), but in many cases the causes of decline are unknown (Stuart *et al.* 2004). While on one hand amphibian species are disappearing, on the other hand a large number of new species are being described every year (Hanken 1999). The rise of new species is remarkable (Glaw & Köhler 1998) and does not reflect taxonomic inflation in which known subspecies or variants are elevated to species status but is due to true first-hand discoveries (Köhler *et al.* 2005). Due to their dependence, in most cases, on both aquatic and terrestrial habitats, amphibians may be especially sensitive to environmental change (Stuart *et al.* 2004), and have been used as indicator species for habitat degradation (e.g. Welsh & Ollivier 1998; Alford & Richards 1999; Welsh & Droege 2001; Davic & Welsh 2004).

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One contribution of 18 to a Theme Issue 'DNA barcoding of life'.

The morphology of amphibians is plagued with homoplasy (e.g. Emerson 1986; Wake 1991), and molecular phylogenetic analyses have uncovered several remarkable radiations, demonstrating that similar ecological and morphological adaptations have occurred in parallel, often in different regions of the world (e.g. Bossuyt & Milinkovitch 2000; Parra-Olea & Wake 2001; Mueller *et al.* 2004; Van der Meijden *et al.* in press).

A particular challenge to amphibian taxonomists is when patterns of convergence and parallelism occur among relatively closely related taxa (Wiens *et al.* 2003), which can completely mask species diversity. Many frog species are morphologically similar to other, partly sympatric taxa, but are strongly differentiated by advertisement calls and genetic divergences. Similarly, many species of salamanders are only reliably distinguishable by molecular methods (Larson & Chippindale 1993). Also, most amphibians have complex life cycles (Wilbur 1980), with a larval phase radically different in morphology (i.e. tadpoles of anurans). This complicates the identification and description of larvae, as they cannot be easily assigned to an adult phase based on their morphology (Thomas *et al.* 2005).

The plethodontid salamanders of North America have been in the centre of debate of species concepts

and the value of genetic distances for species delimitation (e.g. Highton 2000 versus Wake & Jokusch 2000). As summarized below, analysis of diverse data sets in amphibians indicates common discordances between nuclear and mitochondrial signatures, and distinct phylogeographic structuring of species disrupted by at least occasional hybridization and mitochondrial introgression. Mitochondrial divergences among unanimously recognized species are usually large, but in some cases intraspecific divergence values approach or overlap with interspecific values (Vences *et al.* 2005), potentially confounding species identifications based on a single sequence.

Altogether, these patterns define amphibians as a challenging group for DNA barcoding purposes. On one hand, the spread of amphibian declines and the indicator value of amphibians claim for a rapid assessment of their species diversity, and for fast and reliable species identification tools. On the other hand, the genetic structure of amphibian species may not in all cases allow reliable species identification through DNA barcodes based on cytochrome oxidase subunit I (*cox1*) sequences. The usefulness of molecular identification of amphibians has already been explored in the allozyme age, with electrophoretic methods developed to identify embryonic stages in ecological studies (Arntzen 1989) and frog leg meat in the international trade (Veith *et al.* 2000). Here we (i) briefly review patterns of mitochondrial variation in amphibians, (ii) define two major goals of DNA barcoding in amphibians, the identification of larval stages and of candidate species and (iii) provide novel data on *cox1* variation in two intensively studied amphibian groups, mantellid frogs and plethodontid salamanders.

2. METHODS

A fragment of *cox1* was sequenced in an array of frogs of the family Mantellidae using a pair of primers proposed for arthropods (Hebert *et al.* 2003): LCO1490, 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′, and HCO2198, 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ that amplify a region near the 5′-terminus of this gene. As expected (Vences *et al.* 2005) this pair of primers worked in a large proportion of specimens but not in all. In salamanders, amplification success was poor, and new primers were required; two primers that amplify a region of 1259 bp starting from the 5′-terminus of this gene were designed by DRV, based on available complete mitochondrial sequences: MVZ_201, 5′-TCA ACA AAY CAT AAA GAT ATT GGC ACC-3′ and MVZ_202, 5′-GCG TCW GGG TAR TCT GAA TAT CGT CG-3′. For comparative purposes, the fragment of the large mitochondrial ribosomal subunit (16S) proposed by Vences *et al.* (2005) for amphibian DNA barcoding was sequenced from the same individuals, using the primers 16Sa-L and 16Sb-H (Palumbi *et al.* 1991). Sequences obtained in this study were deposited in Genbank (accession numbers DQ105329-DQ105345, DQ116461-DQ116497). Our dataset was complemented by 16S and *cox1* sequences obtained in a previous study (Vences *et al.* 2005): AY883978–AY883995, AY847959–AY848683, and by complete mitochondrial genome sequences available from Genbank.

To assess patterns of *cox1* evolution, and intra- and interspecific variation in amphibians, we used three separate data sets. Selection of taxa was as follows.

(a) *Mantellid frogs (family Mantellidae)*

These are an endemic and species-rich clade from Madagascar that has been intensively studied using different data sets (morphology, bioacoustics and genetics). The occurrence of sibling species (species that are sister to each other, or very close relatives, but morphologically very similar) is very common in this clade and adds to the value of molecular approaches to understand their diversity. We, here, compared mantellids of three genera (*Aglyptodactylus, Boophis, Mantidactylus*) at three levels, extending the data set of Vences *et al.* (2005): (i) intrapopulational variation was assessed for six species (*Aglyptodactylus madagascariensis*, *Boophis goudoti*, *Mantidactylus blommersae*, *M. brevipalmatus*, *M. enki*, *M. tschenki*) by sequencing two to five specimens per population. (ii) interpopulational variation was assessed by comparing individuals from mainly the two eastern rainforest regions Andasibe and Ranomafana; only species were selected where morphological and bioacoustic uniformity, and probably non-fragmented distribution areas, strongly suggest that populations from the study sites are conspecific (*B. goudoti*, *B. luteus*, *B. septentrionalis*, *Mantidactylus asper*, *M. blommersae*, *M. liber*, *M. melanopleura*, *M. redimitus*, *M. tornieri*); (iii) interspecific divergence was assessed by comparing several pairs of sibling species (*B. erythrodactylus* versus *B. tasymena*; *B. goudoti* versus *B.* cf. *periegetes*; *B. luteus* versus *B. septentrionalis*; *B. sibilans* versus an undescribed species *B.* sp.; *M. blommersae* versus *M. domerguei*; *M. depressiceps* versus *M. tornieri*).

(b) *Climbing salamanders (genus* Aneides*)*

We selected four species of *Aneides*, which present several characteristics that make them suitable for comparative analysis of *cox1* variation. All belong to the same clade on the basis of their morphology and genetic relationships (Wake 1963, 1966; Larson *et al.* 1981; Jackman 1993; Mahoney 2001). They are morphologically distinguishable and their distribution ranges are well known, occurring in the same biogeographic region (western North America), with several recognized contact zones between the species. We selected one specimen per species from the type locality or the closest locality available in the Museum of Vertebrate Zoology (University of California at Berkeley, USA) tissue collection. One or several specimens were selected from the contact zones between species (*A. vagrans* versus *A. flavipunctatus*, *A. vagrans* versus *A. lugubris* in Mendocino County; *A. flavipunctatus* versus *A. lugubris* in Mendocino and Santa Clara counties; *A. ferreus* versus *A. vagrans* in Del Norte County (California, USA)). When possible, one specimen per species from a population distant to the distribution range of the other species was also sequenced. *Aneides shardii* was also included for comparison as it is currently recognized as the sister species to all other *Aneides* (Wake 1966; Mahoney 2001).

(c) *Salamanders of which complete mitochondrial genomes have been sequenced*

Four mitochondrial genes, 16S, *cox1*, NADH dehydrogenase subunit 4 (*nd4*) and cytochrome *b* (*cob*) were aligned from 33 mitochondrial genomes available from Genbank. These include mostly plethodontid salamanders, but also five closely related ambystomatid species, and altogether representatives of six salamander families: Cryptobranchidae,

Ambystomatidae, Salamandridae, Plethodontidae, Hynobiidae and Rhyacotritonidae. These genomes allowed us to compare the variability of the selected genes within salamanders.

BLAST searches (Altschul *et al.* 1990) were carried out after setting up local sequence databases using BIOEDIT (http://www.mbio.ncsn.edu/BioEdit/bioedit.html). Uncorrected pairwise distances (*p*-distances) for *cox1* sequences of mantellid frogs and *Aneides* salamanders were obtained using PAUP* (Swofford 2002). Pairwise Kimura-Two-Parameter (K2P) distances for mantellid frogs and a more extensive taxon sampling of salamanders for the *cox1* and 16S genes were calculated with the program TAXI (Steinke *et al.* 2005). All *cox1* distances reported herein refer to the 5′-terminal portion (550–650 bp in length), whereas *cob* and *nd4* distances refer to the complete genes.

3. PATTERNS OF MITOCHONDRIAL VARIATION AND PHYLOGEOGRAPHY

Although amphibians are known to be able to disperse across oceanic barriers (e.g. Vences *et al.* 2003), they are usually poor dispersers and highly philopatric (Blaustein *et al.* 1994), with a limited osmotic and desiccation tolerance (Balinsky 1981). This influences the often strong phylogeographic structuring in this group (summary in Avise 2000, plus many subsequent studies). Amphibian species are not only well structured phylogeographically, with distinct mitochondrial haplotypes characterizing most geographic subpopulations, but these different haplotypes can also be strongly divergent. Vences *et al.* (2005) report on conspecific 16S rRNA haplotypes of up to 6% pairwise divergence in mantellid frogs. Some of these deeply divergent haplotypes, especially in salamanders, are in lineages where nuclear DNA data do not support full reproductive isolation, thus corroborating that they do not simply characterize cryptic unrecognized species.

The existence of species that are non-monophyletic in their mitochondrial structure has been extensively observed in many animal groups (Funk & Omland 2003). Although in some cases reflecting incomplete taxonomic knowledge, there certainly are numerous examples of non-monophyletic species due to introgression or incomplete lineage sorting. One example that is conspicuous because it concerns an economically relevant species is that of the pelagic Atlantic bluefin tuna, which included rare sequences sister to the Pacific bluefin tuna and introgressed haplotypes of other species in the study of Alvarado Bremer *et al.* 2005. The frequency of these divergent haplotypes was about 5% (10 and 20 out of 334 specimens), thus nearing a level where this commercially relevant species could not be identified by mitochondrial barcoding with statistical support of $p<0.05$. In amphibians, introgressed or incompletely sorted haplotypes have been found in plethodontid salamanders, *Batrachoseps* (Wake & Jockusch 2000) and *Plethodon* (Weisrock *et al.* 2005), European newts, *Triturus* (Babik *et al.* 2005), and Malagasy poison frogs, *Mantella* (Chiari *et al.* 2004). Also, indiscriminant amplexus and subsequent hybridization of some toads (especially the genus *Bufo*) results in genetic introgression of distantly related, otherwise broadly sympatric species (Masta *et al.* 2002). Discordance between the geographical signature of mitochondrial and nuclear markers has also been extensively observed in amphibians, in the phylogeographical structure of one species (García-Paris *et al.* 2003; Monsen & Blouin 2003; Kuchta & Tan 2005), contact zones of phylogeographical subgroups of one species (Sequeira *et al.* 2005) and hybrid zones between two species (Babik *et al.* 2003).

A special situation is that observed in hybridogenetic and gynogenetic amphibians. Such phenomena are known in Palearctic water frogs (genus *Rana*, subgenus *Pelophylax*: hybridogenesis) and North American salamanders (genus *Ambystoma*: gynogenesis). These highly complex genetic systems may also lead to situations where mitochondrial phylogeny does not correspond to species phylogeny, and DNA barcoding would thus lead to wrong identifications, but these patterns are still insufficiently studied.

However, the available data indicate that problems of real non-monophyly of mitochondrial haplotypes in a species, through introgression, incomplete lineage sorting or other phenomena, are exceptions rather than the rule in amphibians, and—as in other animal groups—may be unusual enough to only rarely confound species identification. For example, in a large mitochondrial screening of mantellid frogs (Vences *et al.* 2005), introgression was observed in three out of 200–300 species (1–1.5%), which might be considered as an acceptable error margin, and the affected taxa were always closely related and largely allopatric species pairs. The lesson to be learnt from discordances of geographical signatures of nuclear and mitochondrial markers is that DNA barcoding in closely related, allopatric, and hybridizing taxa (be they considered as species or subspecies) should never rely on mitochondrial markers alone but should always include nuclear markers. Exploring the usefulness of nuclear ribosomal genes for this purpose appears to be promising (Tautz *et al.* 2003; Markmann & Tautz 2005; Monaghan *et al.* 2005).

Hence, the one major query to DNA barcoding in amphibians refers to their large mitochondrial variability within and especially among populations. Three potential problems could affect mitochondrial markers under these conditions: (i) the priming sites may be too variable to allow the use of universal primers in all species, or even in all populations of one species, (ii) the gene fragment may be too saturated with mutations to allow reliable assignation of genetically divergent populations to the correct species, (iii) the gene fragment may be too saturated to allow a distinction of conspecific sequences from sequences of a potentially new species. In a previous paper we have shown that the 16S rRNA gene appears to be a suitable barcoding marker for amphibians, and predicted some difficulties in the universal use of *cox1* in this animal group (Vences *et al.* 2005). In the following we will present patterns of *cox1* variation in amphibians and discuss major applications of DNA barcoding in amphibians.

4. THE *COX1* GENE IN AMPHIBIANS

When compared with other mitochondrial genes, *cox1* has not been used in a particularly large number of

studies in amphibian phylogeny or phylogeography (e.g. in James & Moritz 2000; Rissler & Taylor 2001; Symula *et al.* 2001, 2003; Goldberg *et al.* 2004), and rarely as the only marker. Without doubt, the most commonly used genes include 16S, the small mitochondrial ribosomal subunit (12S) and *cob*. In April 2005 there were 537 hits in Genbank when searching for *cox1* in amphibians, whereas there were 3641 hits for *cob*, 3301 for 16S and 2316 for 12S.

An important question addressed by the present study is how variable is *cox1* in amphibians. Several lines of evidence indicate that this gene is highly variable. Vences *et al.* (2005) analysed standard priming sites for the 3′-terminal segment based on 10 complete mitochondrial sequences of frogs, salamanders and caecilians, and found that the variability (restricted to 3rd codon positions) among these amphibians was higher compared to 59 sequences from an array of taxa spanning across all vertebrates. James & Moritz (2000) regularly observed, in the 3′-terminal *cox1* fragment of the Australian sedge frog *Litoria fallax*, pairwise divergences higher than 5% among haplotypes from neighbouring populations, and 11–12% between two major haplotype clades within the species. The high variability of this gene was also obvious from the fact that, in the study of James & Moritz (2000), 87 individuals showed 84 unique haplotypes. Data presented here (figure 1) indicate that in mantellid frogs, 10–14% divergence is regularly found within species and up to 18% might be possible. Our data also indicate up to 7.8% divergence within species of climbing salamanders (figure 1). Compared to the 16S rRNA gene, and below 20% uncorrected divergence, the *cox1* substitution rates in both salamanders and frogs appear to be about two times higher than the 16S divergences (figure 2).

A striking example of the species definition issue in amphibians is *Ensatina eschscholtzii*, a salamander that in many respects can be understood as a ring species (e.g. Moritz *et al.* 1992; Wake 1997). Genetic admixture is observed throughout the ring, except for some areas of secondary contact where strongly differentiated subspecies behave as distinct species in sympatry. We compared the 3′ terminal portion of the available *cox1* sequence from the complete mitochondrion of *Ensatina e. eschscholtzii* with that of newly obtained sequences for *E. eschscholtzii platensis*, and found an uncorrected pairwise divergence of 12%. This provides a further example of very high *cox1* divergences within amphibian units that are considered as species, although this is not unanimous in this case (e.g. Highton 1998).

Compared to the situation at least in birds (Hebert *et al.* 2004*b*), and probably also in some insects (e.g. Hebert *et al.* 2004*a*), these high intraspecific mitochondrial divergences are certainly a striking character of amphibians. However, several other animal groups may exhibit equivalent rates of variation as amphibians. Very high intraspecific haplotype divergences have been observed in pulmonate snails (Thomaz *et al.* 1996), and among lizard populations assumed to be conspecific, e.g. in *Tarentola* (Harris *et al.* 2004), or in insular populations of the gecko *Cyrtodactylus kotschyi* (up to 20% *cox1* divergence; Kasapidis *et al.* 2005). Although such lineages may turn out to be separate species in the future, there is little doubt that they are closely related to each other and morphologically largely conserved. This latter study also found intrapopulational divergences of up to 7% in these geckos, which is higher than any value observed in amphibians so far.

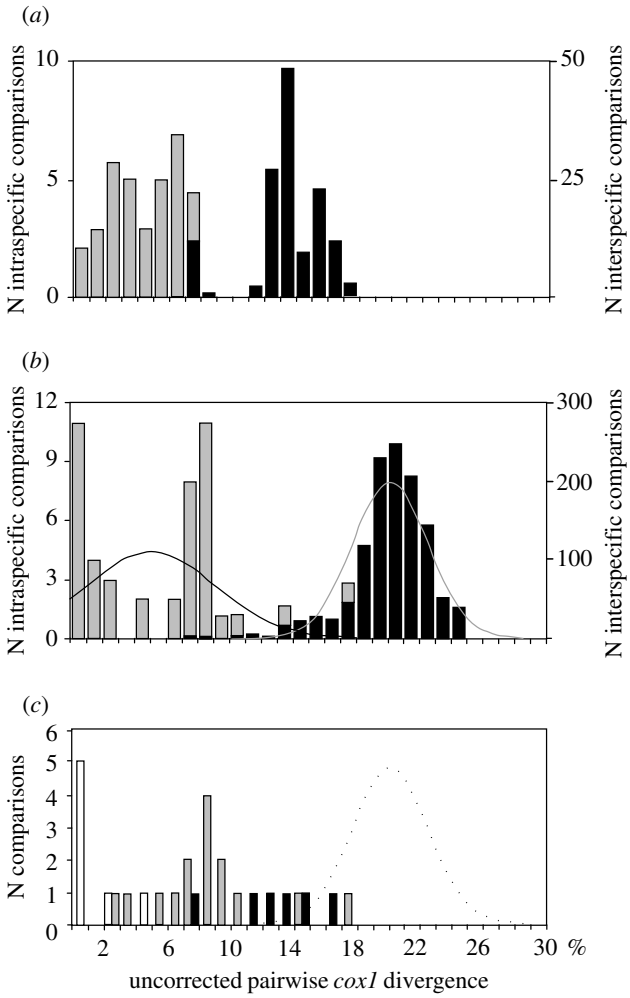

Figure 1. Uncorrected pairwise distances in the *cox1* gene in (*a*) climbing salamanders (*Aneides*) and (*b*) and (*c*) mantellid frogs (*Aglyptodactylus*, *Boophis*, *Mantidactylus*). Grey bars are comparisons among conspecific sequences (left axis); black bars represent comparisons among different species (right axis). In (*c*), white bars refer to comparisons of conspecific specimens from the same site whereas grey bars are comparisons of conspecific specimens from different localities, black bars are comparisons among closely related sibling species only, and the dotted line is the envelop of all interspecific comparisons as shown in (*b*).

To test whether the large *cox1* divergences encountered in amphibians were due to an especially fast molecular evolution of this gene in this taxon, we compared substitution rates in this gene with those in two other mitochondrial genes commonly used in amphibian phylogenetics. Analysis of the data set of complete mitochondrial genomes of salamanders (figures 2 and 3) indicates that mutations at first and second positions are rare as compared to other mitochondrial protein-coding genes. Indeed, *cox1* reaches a plateau of saturation faster and, therefore, is less variable than the two other mitochondrial protein-

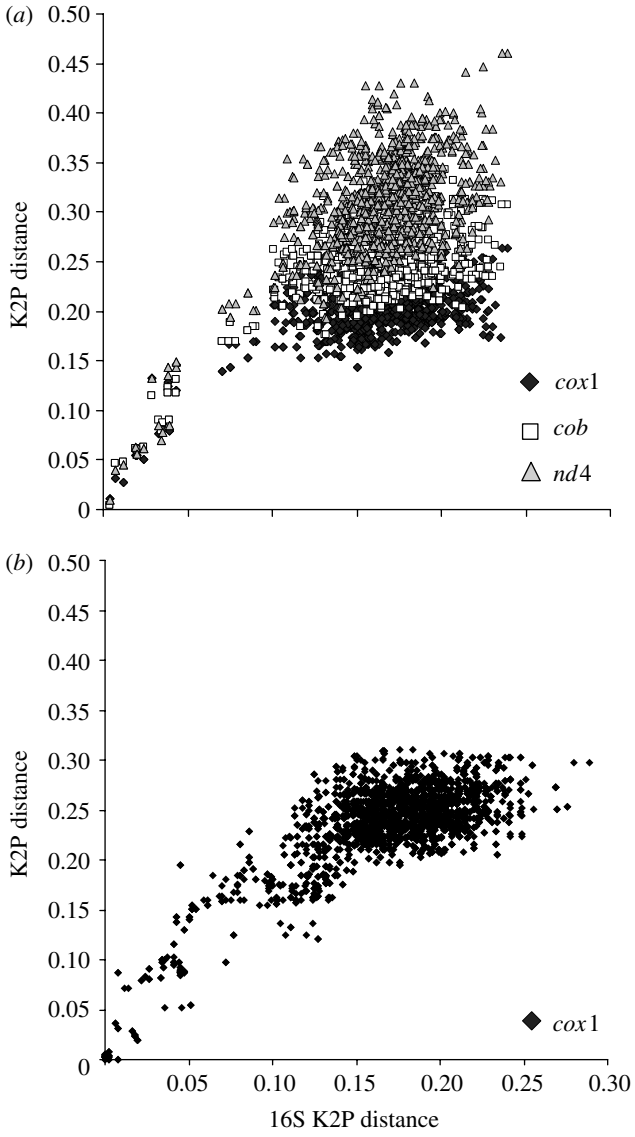

Figure 2. Kimura-2-parameter distances (Kimura 1980) among (*a*) 33 salamanders for which complete mitochondrial genomes have been published, for *cox1*, *cob* and *nd4*, and (*b*) mantellid frogs for which *cox1* and 16S sequences are available, for *cox1*. The protein-coding distances are plotted against the 16S distances.

coding genes analysed, *cob* and *nd4*. In the data set from complete mitochondrial salamander genomes, substitution rates at the third codon position of *cox1* (synonymous substitutions) are similar to those in *nd4* and *cob*, whereas these rates are distinctly lower at first and, especially, second codon positions (non-synonymous substitutions), and amino acid substitution rates are distinctly lower in *cox1* (figure 4). These molecular evolutionary patterns of the *cox1* gene in salamanders are in accordance with observations from other animals (Saccone *et al.* 1999). Hence, the high divergence values found in amphibians are not due to a putative comparatively faster evolution, in this taxon, of *cox1*, but a general feature that we expect to be reflected also in divergences of other mitochondrial protein-coding genes.

Figure 3 further indicates that also among clades of amphibians there are distinct differences in substitution rates of *cox1* and other mitochondrial genes (see also Hoegg *et al.* 2004): the mean and maximum

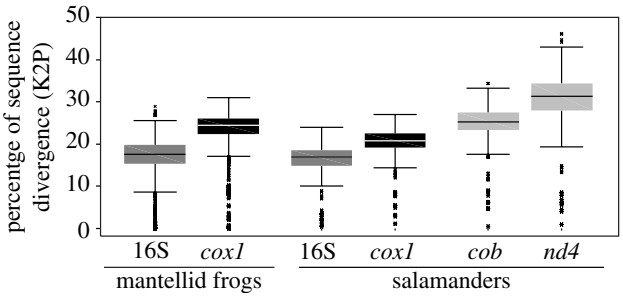

Figure 3. Boxplots of uncorrected pairwise distances for *cox1* and 16S in mantellid frogs, and for *cox1*, 16S and two other genes commonly used in amphibian studies (*cob* and *nd4*), based on analysis of 33 complete salamander mitochondrial genomes. Boxes represent mean (black or white line) plus/minus standard deviation; error bars represent 5 and 95% percentiles; stars represent outliers.

divergences observed in mantellid frogs are higher than those observed in salamanders, although the former refer to species of a single family, and the latter to comparisons among several families that represent much deeper phylogenetic lineages.

5. IDENTIFYING LARVAL AMPHIBIANS

One potential major application of DNA barcoding in amphibians is certainly the species identification of larvae. Especially the tadpoles of frogs are morphologically highly divergent from their adult stages (Altig & McDiarmid 1999), a situation comparable with that in holometabolous insects. These larvae can be significant components of aquatic ecosystems (e.g. Ranvestel *et al.* 2004), and at certain periods of the year are the only available evidence for the occurrence of certain secretive species at a site. Amphibian larvae may also contain relevant taxonomic and, especially, phylogenetic information (e.g. Haas 2003). For such studies, it is of course necessary to collect some basic information: which tadpole belongs to which species, how can it be recognized, and which morphological adaptations does it have? Such fundamental knowledge is scarce in species-rich tropical amphibian communities, because identifying tadpoles to species, or even genus, is an extremely time-consuming task. It involves either (i) laborious rearing of eggs laid by a well-identified pair of adults, or (ii) even more laborious rearing of tadpoles collected in the wild, to obtain metamorphosed juveniles, which then are tentatively assigned to one of the species known to occur at the site. Parmelee *et al.* (2002) discuss these problems of morphological tadpole identification, and anticipate that rapid molecular DNA techniques suitable for field identification will be available within the next few decades. DNA barcoding clearly offers these tools and has been applied in several studies to successfully identify tadpoles (Malkmus & Kosuch 2000; Ziegler 2002; Ziegler & Vences 2002; Thomas *et al.* 2005).

The high mitochondrial variability of amphibians may be the source of wrong identifications of tadpoles, if reference and test specimens have different geographical origins. If the within-species differentiation approaches saturation, phenetic and phylogenetic comparisons may not be able to accurately assign

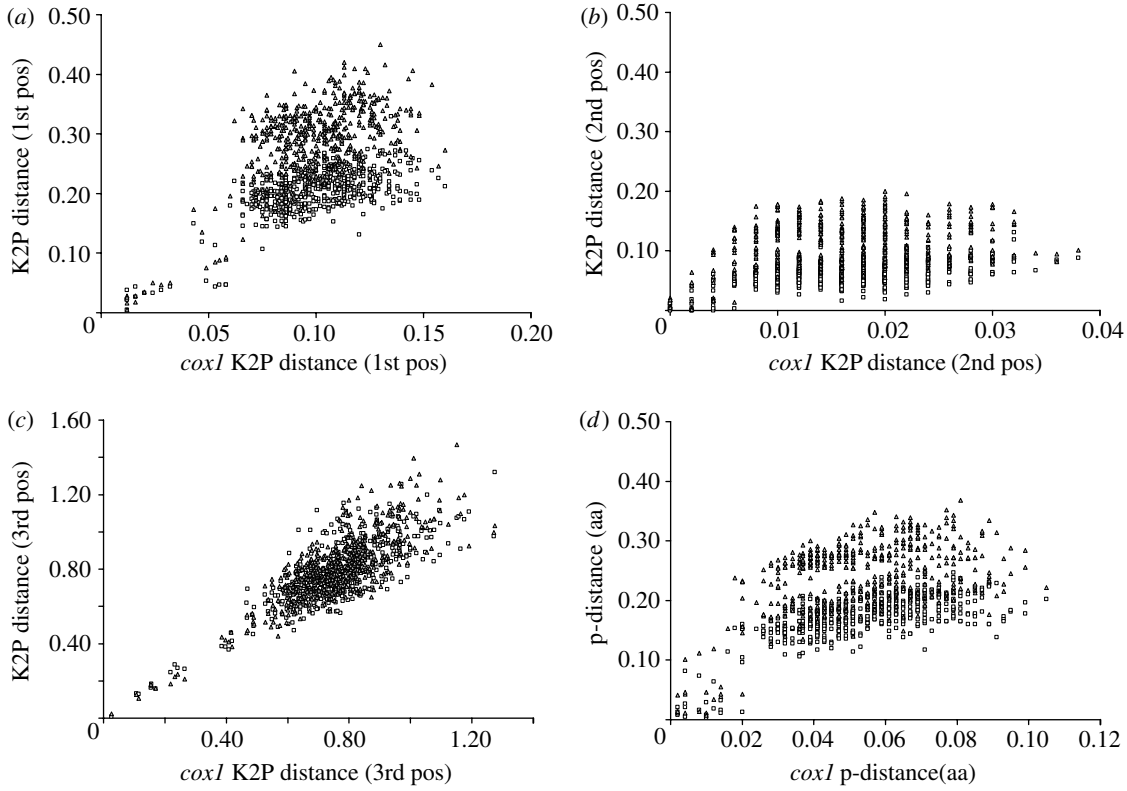

Figure 4. Scatterplot of Kimura-2-parameter distances (Kimura 1980) of the first (*a*), second (*b*) and third (*c*) codon positions and amino acid substitutions of *nd4* (squares) and *cob* (triangles) versus the distance of the first, second, third codon positions and amino acid substitutions of *cox1*, respectively. In (*d*) the amino acid p-distances from *nd4* and *cob* are plotted against the amino acid p-distance of *cox1*. Based on data from 33 complete mitochondrial sequences of salamanders.

test sequence to reference sequence any more. When comparing various vertebrate taxa to each other, the *cox1* gene was not able to recover most major clades (Vences *et al.* 2005), and would not have been able, for example, to reliably identify an unknown amphibian sequence as belonging to this class.

To test the performance of *cox1* in phenetic identification of amphibians, we carried out two analyses using the BLAST algorithm (Altschul *et al.* 1990). We used a database that contained all vertebrate *cox1* sequences available from Genbank as of April 2005 ($N=1563$). This database contained a number of amphibian sequences, in particular those obtained by Vences *et al.* (2005). We removed all 13 sequences of mantellid frogs, and then 'BLASTed' our complete set of mantellid frog sequences against the database. In 23 out of 40 searches, the first hit did not refer to an amphibian sequence, results spanning from fishes over birds to mammals. In a second search we added our mantellid sequences from one locality in Madagascar to the vertebrate database, and 'BLASTed' the conspecific sequences from other localities against this expanded database. In 21 out of 22 searches, the searches were successful in identifying a conspecific sequence from another locality as the most similar to the query sequence. These results confirm that *cox1* does not perform well in assigning specimens to major vertebrate lineages when taxon sampling is poor, but corroborates that identification becomes reliable with dense taxon sampling in the reference database. As a conclusion, 16S (Vences *et al.* 2005) and *cox1* are suitable markers to identify unknown life-history stages of amphibians (eggs, larvae, juveniles, the opposite sex) to species. A crucial aspect for such applications is the development of new software tools to allow fast and reliable comparisons across large sequence sets. Besides pairwise alignments of indel-rich rRNA sequences (e.g. Steinke *et al.* 2005) this could encompass a better visualization of BLAST output, e.g. in the form of guiding trees or scatterplots, and simultaneous consideration of multiple markers.

6. SCREENING FOR CANDIDATE SPECIES

Considering the high rate of discovery of new species of amphibians in times of global amphibian declines, DNA barcoding can be a useful tool to speed up the initial recognition of new units that may represent undescribed species, here termed candidate species (see figure 5). For this purpose, it is necessary to define threshold values that ideally provide a sharp distinction between intraspecific and interspecific divergence values. If an unknown sequence differs from the closest reference sequence by a divergence above the threshold, the individual from whom the sequences were obtained belongs to a candidate species, which means that its taxonomic status merits further investigation. Bradley & Baker (2001), for mammals, set this threshold at 11% for the cytochrome *b* gene, whereas Hebert *et al.* (2004*b*) propose a *cox1* threshold of only 2.7% for birds. These authors propose to calculate a standard sequence threshold as 10 times the mean intraspecific variation observed. In our data sets, mean intraspecific *cox1* divergence was 5.4% in mantellids and 4.3% in *Aneides*, whereas the mean interspecific divergences were 20.7% and 13.5%, respectively.

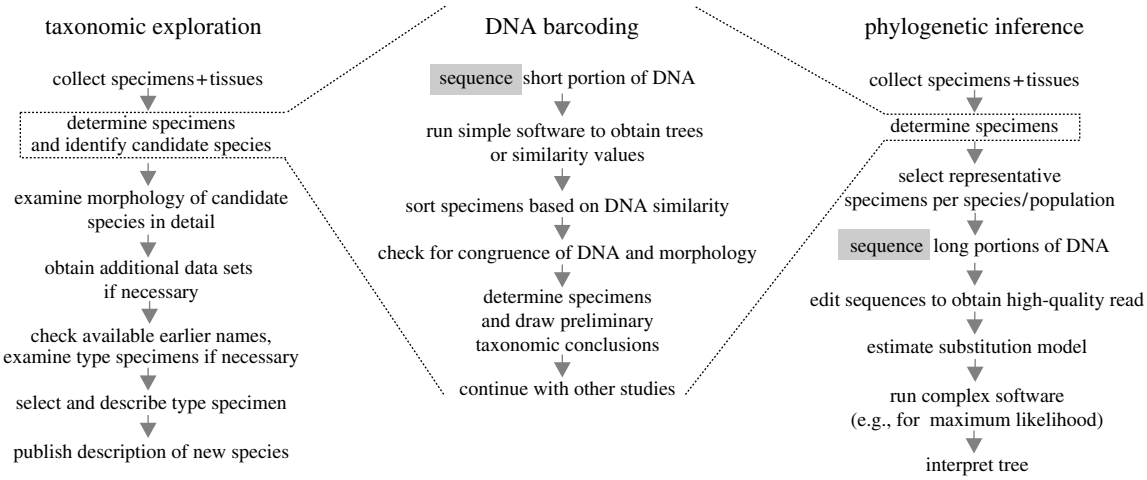

Figure 5. Flowchart illustrating the utility of DNA barcoding in amphibian systematics, with emphasis in its fundamental difference from phylogenetic inference. DNA barcoding serves as a fast and often preliminary identification tool that accelerates both taxon selection in phylogenetic studies and progress in taxonomic exploration, but cannot supplement either of these fields. DNA sequences are obtained both in DNA barcoding and phylogenetic studies, but phylogenetic studies are more demanding for accurateness and sequence length.

A calculation of threshold values as proposed by Hebert *et al.* (2004*b*) would yield unrealistic values of 43–54% pairwise divergence, which is much above the saturation plateau of *cox1* and exceeds the highest divergence values observed among any pair of amphibian species (e.g. figure 3). Threshold values for amphibians can therefore only be tentative, and may be placed around a value of 5% 16S divergence (Vences *et al.* 2005) or 10% *cox1* divergence (figure 1).

The major problem with defining this threshold value is the wide range of overlap between intra- and interspecific divergence values, which seems to be a generalized problem in amphibians (figure 1). In climbing salamanders, the two species with the smallest interspecific divergences are *Aneides ferreus* vs. *A. vagrans* (6.5–7.9%), whereas the largest intraspecific divergences (above 7%) were found in comparisons of the Santa Clara sequence with those from other populations of *A. flavipunctatus*. In mantellid frogs, many species had divergence values among populations that were largely overlapping with those of closely related allopatric species pairs.

The selection of taxa and specimens to calculate averages is a further issue. Figure 1*b* is based on all pairwise comparisons in the set of available mantellid sequences. The amount of overlap between intraspecific and interspecific divergences appears relatively limited and far from the respective average values. In contrast, figure 1*c* shows only the mean values for intraspecific comparisons (one value per species), and only the interspecific comparisons of closely related sibling species where identification problems are most acute. As easily visible from the graph, the overlap of values is much more pronounced in figure1*c*, which suggests that thorough statistical tests are needed to estimate the probabilities of correct identification of species and candidate species, using different threshold levels of genetic divergences.

7. CONCLUSIONS

Based on data presented and reviewed herein there seems to be no convincing evidence for mitochondrial introgression and incomplete lineage sorting being much more common in amphibians than in other animals (see also Funk & Omland 2003). The *cox1* gene shows high divergences within and among amphibian species, but this is due to general high mitochondrial variability rather than to a particularly fast evolutionary rate of this gene. Despite this high variability, *cox1* seems to be able to correctly identify sequences from different localities to the species level.

The major problems with DNA barcoding of amphibians are related to this high mitochondrial variability. First, there is a distinct overlap of intraspecific and interspecific divergence values, which complicates the establishment of threshold values to identify candidate species. Second, because high variation is also observed in the *cox1* priming sites, a mix of several primers will be needed to reliably amplify this gene from all amphibian species, and the use of alternative markers with more conserved priming sites, such as 16S rRNA, should be considered, at least for some applications (Vences *et al.* 2005).

These conclusions may hold not only for amphibians, but also for other animal groups. In addition, mitochondrial introgression may return as a major problem in cases when identifications through DNA barcodes must have very high reliability. Depending on the required level of accuracy, nuclear barcoding markers need to be established to be able to corroborate any disputed mitochondrial species identification.

The *cox1* gene has been proposed as a standard marker, and we support attempts to build up a global and complete *cox1* database of eukaryotes, except plants (see Chase *et al.* 2005). However, there also seems to be consensus that additional markers will be helpful, and needed for certain applications. Their number will probably be limited. Besides *cox1*, and leaving aside the situation in plants, genes used for molecular taxonomy approaches (although usually not under the term DNA barcoding) include the mitochondrial 16S rRNA (which is being sequenced by the AmphibiaTree consortium for a large set of amphibian species at present, see http://www.amphibiatree.org),

12S rRNA, and cytochrome *b* genes, and the nuclear 28S rRNA and ITS genes.

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