

# Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory

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Edited by David B. Wake, University of California, Berkeley, CA, and approved March 27, 2009 (received for review October 26, 2008)

**Amphibians are in decline worldwide. However, their patterns of diversity, especially in the tropics, are not well understood, mainly because of incomplete information on taxonomy and distribution. We assess morphological, bioacoustic, and genetic variation of Madagascar's amphibians, one of the first near-complete taxon samplings from a biodiversity hotspot. Based on DNA sequences of 2,850 specimens sampled from over 170 localities, our analyses reveal an extreme proportion of amphibian diversity, projecting an almost 2-fold increase in species numbers from the currently described 244 species to a minimum of 373 and up to 465. This diversity is widespread geographically and across most major phylogenetic lineages except in a few previously well-studied genera, and is not restricted to morphologically cryptic clades. We classify the genealogical lineages in confirmed and unconfirmed candidate species or deeply divergent conspecific lineages based on concordance of genetic divergences with other characters. This integrative approach may be widely applicable to improve estimates of organismal diversity. Our results suggest that in Madagascar the spatial pattern of amphibian richness and endemism must be revisited, and current habitat destruction may be affecting more species than previously thought, in amphibians as well as in other animal groups. This case study suggests that worldwide tropical amphibian diversity is probably underestimated at an unprecedented level and stresses the need for integrated taxonomic surveys as a basis for prioritizing conservation efforts within biodiversity hotspots.**

biodiversity estimates | new candidate species | phylogeny | DNA barcoding

The current biodiversity crisis demands the study of broad-scale spatial variation in species richness and endemism to identify areas that merit special conservation attention. Global efforts to minimize biodiversity loss have led to prioritizing biodiversity hotspots (1) which are defined as areas with high concentrations of endemic species and that are undergoing exceptional habitat loss (2–4). A second step is the efficient implementation of conservation measures at a local scale which requires an understanding of spatial patterns of richness and endemism within these hotspots (5). Assessments of such regional priority areas are often hampered by incomplete distributional and taxonomic information. The operational units used to assess conservation priority areas are described species, whereas estimates of undiscovered and undescribed species are usually ignored. Recently, many potential new species have been identified by DNA barcoding, but a taxonomic validation of these species will outdate the short time span left for efficient biodiversity conservation prioritization, and so far it is not clear how this undescribed diversity can nonetheless be considered. This undescribed diversity also bears relevance for understanding the tree of life: The completeness of taxon sampling is one of the major prerequisites for reliable phylogenetic analysis, reconstruction of character evolution, and inference of macroevolutionary processes (6, 7).

Among terrestrial vertebrates, amphibians are characterized by a rapid rate of species discovery (8, 9), with an overall increase in the number of amphibian species globally of 19.4% during the last decade, reaching 6,449 currently recognized species (10). An important acceleration in the rate of new discoveries, mainly from tropical areas, is obvious from many recent studies (11–16). These discoveries are not the result of taxonomic inflation (9, 14, 17), but correspond to real divergent species (18, 19). Although high numbers of undescribed amphibians have been estimated to exist in poorly studied tropical regions (11, 15), these results remain unverified for complete, highly diverse amphibian faunas. In parallel, an increase of threatened amphibian species has been reported worldwide (8, 20, 21). Amphibians are of high conservation concern, with 43% of species being globally threatened (20), most of them in tropical regions with high amphibian diversity.

Madagascar is one of the top priority global hotspots for biodiversity conservation (1), affected by a high rate of habitat destruction (22). Its fauna and flora evolved largely in isolation (23), and many taxa are characterized by a high degree of microendemism within Madagascar (24–27). The native amphibian fauna is constituted by 5 endemic evolutionary lineages of frogs with 100% species-level endemism, 2 of which (the mantellids and the cophyline and scaphiophrynine microhylids) are very species-rich. Large-scale taxonomic inventories conducted since 1991 have led to an increase from 133 to 244 described species, largely due to the exploration of new areas and the application of more efficient techniques. About 46 species were identified during the 1990s mainly based on bioacoustics; the application of combined methods, including molecular genetics, was crucial in the discovery of 51 new species since 2000 as well as in the resurrection of species formerly considered to represent synonyms.

Undescribed diversity may have an important impact on understanding the spatial patterns of endemic radiations on the island, but objective estimates of species numbers are not available so far. We report a comprehensive assessment of morphological, bioacoustic, and genetic variation of the anuran fauna of Madagascar. Our goals are (i) to provide a reliable estimate of the proportion of yet-undescribed amphibian species from Madagascar and their phylogenetic and geographic distribution, (ii) to discuss the impact of our findings for global estimates of amphibian diversity, and (iii) to propose a novel terminology to be better able to assess the increasing number of identified but taxonomically undescribed candidate species of animals.

Author contributions: D.R.V. and M.V. designed research; D.R.V., K.C.W., F.A., J.K., F.G., and M.V. performed research; D.R.V., K.C.W., J.K., and M.V. analyzed data; and D.R.V. and M.V. wrote the paper.

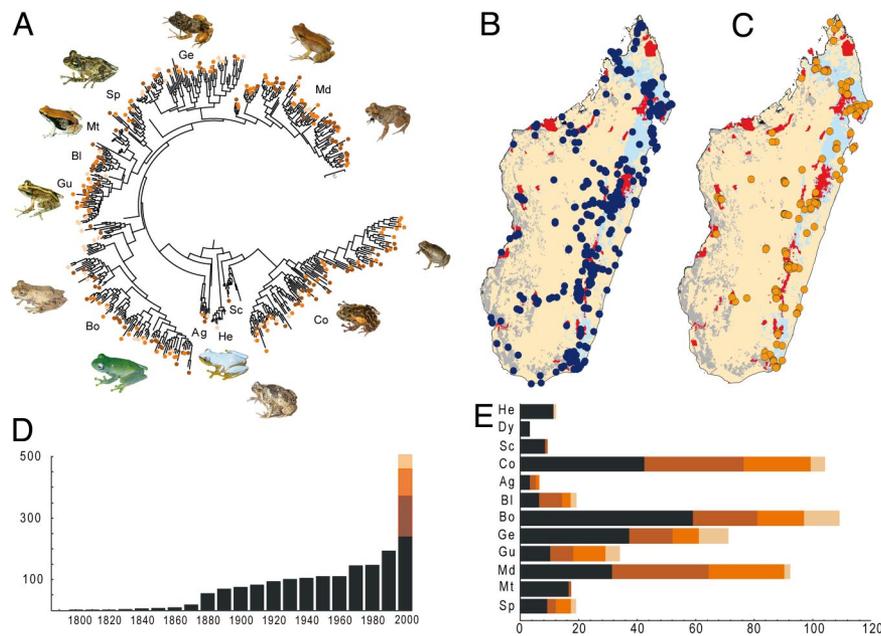
The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the Genbank database (accession nos. FJ559069–FJ559372 and FJ217329–FJ217345)

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This article contains supporting information online at [www.pnas.org/cgi/content/full/0810821106/DCSupplemental](http://www.pnas.org/cgi/content/full/0810821106/DCSupplemental).



**Fig. 1.** Phylogenetic, geographic, and historical patterns of undescribed amphibian diversity in Madagascar. (A) Bayesian phylogenetic tree of 236 (out of 244) described species and 258 deeply divergent genealogical lineages of Malagasy frogs (among them 129 CCS and 92 UCS and 37 DCL with  $>3\%$  genetic divergence to nearest described neighbor) based on a fragment of the mitochondrial 16S rRNA gene. Circles represent CCS (brown), UCS (orange), and DCL (light orange). Inset photos show some of these forms (see *SI Appendix*). He, *Heterixalus* (Hyperoliidae); Dy, *Dyscophus*; Sc, Scaphiophryninae; Co, Cophylinae (Microhylidae); Sp, *Spinomantis*; Md, *Mantidactylus*; Mt, *Mantella*; Gu, *Guibemantis*; Ge, *Gephyromantis*; Bo, *Boophis*; Bl, *Blommersia*; Ag, *Aglyptodactylus* (Mantellidae). (B and C) Maps of collecting localities of described species (B) and of CCS and UCS (C) of Malagasy frogs, and remaining primary vegetation (evergreen forests, blue; non-evergreen forests, gray). Current protected area network is shown in red. (D) Cumulative number of species currently considered as valid per 10-year intervals (2001–2008 for the current decade), and the CCS, UCS, and DCL identified in the present paper. (E) Total numbers of described species and CCS, UCS, and DCL in major clades of Malagasy frogs.

## Results

From our integrative analyses of morphological, bioacoustic, and genetic data, we identified many more species of Malagasy frogs than there are names available. The number of described species of Malagasy frogs has slowly increased during the 20th century, reaching 100 described species around 1940, and 133 at the time of the last monographic account in 1991 (28) (Fig. 1). The current number of 244 valid native species indicates an increase of 83.5% since 1991.

We classify frog lineages from Madagascar in 3 categories (Table 1): (i) confirmed candidate species (CCS) are those differing clearly by morphological and bioacoustic characters and usually showing high genetic differentiation that we hypothesize are distinct, undescribed species; (ii) unconfirmed candidate species (UCS) are deep genealogical lineages (15)—bioacoustically and morphologically unstudied and usually derived from geographically distant populations—for which general indications exist that they are distinct, undescribed species; and (iii) deep conspecific lineages (DCL) are deeply divergent genealogical lineages, studied but not having clear morphological or bioacoustic differences with described species. Our data provided an additional 129 CCS, 92 UCS, and 37 DCL. If CCS plus UCS are assumed to represent distinct undescribed species, a 90.6% increase from the current 244 to 465 frog species is projected. This projection would constitute an overall increase of 250% since 1991.

Undescribed diversity is phylogenetically widespread in Malagasy frogs (Fig. 1A and *SI Appendix*). We found candidate species in most clades except in a few monospecific or species-poor genera (e.g., *Wakea*, *Dyscophus*). Genera such as the colorful *Mantella* or *Heterixalus* are rather well studied and consequently contain only a few candidate species, whereas in the 3 most speciose and diverse clades (*Mantidactylus*, *Boophis*, and Cophylinae), the number of candidate species is close to or even exceeds the number of described species (Fig. 1E). In general, in most genera the average differentiation in the 16S rRNA gene of CCS and UCS is  $>4\%$ . Most described species of Malagasy frogs show genetic distances of 6–8% to their closest relatives depending on the period of discovery (9), but these values refer to distances corrected by the Kimura-2-parameter-model (K2P) which are higher than the uncorrected distances we report. The molecular differentiation of CCS and UCS is thus at similar levels as between described species of Malagasy

frogs, indicating that they are not the result of taxonomic inflation (e.g., elevating subspecies to specific status).

New discoveries are also geographically ubiquitous. They occur throughout Madagascar, both in poorly explored and in better-studied areas. Described species are known from 451 sites and 87.3% are found in protected areas, whereas CCS and UCS are known from 168 sites and only 66.4% are found in protected areas (Fig. 1B and C). Even in 2 of the best-studied sites for amphibians in Madagascar, Mantadia/Analamazaotra and Ranomafana National Parks, harboring a total of 94 and 112 species of frogs, we found 10 and 31 CCS and UCS (see *SI Appendix*). Most described species are known from one or a few localities and have not been found elsewhere. This pattern is consistent among the undescribed species; only 6 of the 219 CCS and UCS are widespread, whereas 63 are currently known from small ranges and 154 from single localities.

## Discussion

**The Concept of Candidate Species and Their Delimitation.** By integrating molecular, morphological, and bioacoustic data, we have identified a large number of undescribed species of amphibians in Madagascar. The remarkable increase in estimated species numbers is independent of the species concept applied. Almost all described species are well defined as reciprocally monophyletic and strongly differentiated units by molecular datasets and by morphological and/or bioacoustic evidence. The only exceptions are *Mantella milotympanum*, *Mantella nigricans*, *Dyscophus antongilii*, *Heterixalus andrakata*, *Heterixalus variabilis*, and *Heterixalus tricolor*, which are not clearly diagnosable by molecular data and require further study (29). The same combination of character sets defines all CCS (e.g., Fig. 24), and, based on our ongoing studies, we expect the same for most UCS. Many of these taxa occur in syntopy with their nearest relatives without any signal of admixture, corroborating their species status under biological, evolutionary, and phylogenetic species concepts. In contrast, many DCL correspond to genetically differentiated allopatric populations of widespread species for which the application of a biological species concept is inherently difficult. If criteria such as those for Malagasy primates (30) were applied to these DCL, many of them would be considered evolutionary species as well. Because the process of species formation in amphibians is an active and controversial area of research (31), we currently discourage such conclusions.

**Table 1. Definitions of proposed categories of candidate species**

Category	General definition	Definition used in Malagasy frogs
Unconfirmed Candidate Species (UCS)	Default category for deep genealogical lineages of unknown status. The genetic differentiation must be above a threshold value typical for comparisons among closely related species in the group of animals under study. Data deficient for morphology, ecology, and distribution.	Uncorrected pairwise genetic divergences in 16S rRNA gene >3% to all other described species. No data on morphology and bioacoustics due to unavailability of voucher specimens or immature state of vouchers.
Confirmed Candidate Species (CCS)	Specimens or populations characterized by a detectable genetic differentiation to all described species, not necessarily above any threshold, but in concordance with at least one of the following criteria: (i) a distinct differentiation in a character that mediates a pre mating reproductive barrier, (ii) a diagnostic morphological difference in a character that in the respective group of animals is known to be of low intraspecific variability and of high value to discriminate species, (iii) sympatric occurrence without admixture, and with at least one phenotypic character state difference, even if subtle, strictly correlated to the genealogy inferred from a neutral molecular marker.	Uncorrected pairwise genetic divergences in 16S rRNA gene to all other described species in most cases >3%, sometimes only 1–2%. Concordance of this molecular differentiation with one of the following: (i) a qualitative difference in advertisement calls, (ii) diagnostic difference in at least one morphological character known to be generally species-specific in Malagasy frogs: e.g., presence and extent of dermal spines, ridges and tubercles, extension of terminal finger tips, morphology of femoral glands, relative tympanum size, iris color, or tadpole mouthparts.
Deep Conspecific Lineage (DCL)	Deep genealogical lineages above a threshold value typical for comparisons among closely related species in the group of animals under study. One or several of the following must apply: (i) morphological and chromatic data reveal no differences to topotypic populations of a described species, or only subtle differences in characters that are known to show intraspecific variability, or only quantitative differences (in characters such as body size), (ii) if characters relevant for pre mating isolation are known, then these show no or only quantitative differences to topotypic populations of described species, (iii) phylogeographic studies are available and show indications for genetic admixture with other genealogical lineages within a species.	Uncorrected pairwise genetic divergences in 16S rRNA gene > 3% to all other described species in combination with one or several of the following: (i) no morphological differences, or differences only in characters known to show intraspecific variability: e.g., size, relative hindlimb length, body color and pattern, (ii) no difference in advertisement calls, or only differences in variables known to show intraspecific variability, (iii) co-occurrence with other deeply divergent haplotypes within the same populations, not concordant with any distinct or subtle morphological difference among individuals.

Recent progress in molecular techniques leads to acquiring newly determined DNA sequences at a faster pace than taxonomists are able to follow up with results. In many cases, divergent DNA sequences are observed that probably correspond to distinct, undescribed species, but a taxonomic analysis of these data are impracticable in the short term, especially in morphologically cryptic and highly diverse groups (32, 33). The term “candidate species” is rarely used by zoologists, but has recently been proposed for newly discovered units that probably correspond to undescribed species (31), and we advocate using it in a less formal way than the *Candidatus* status in microbiology (34–36).

Delimiting species is a resurgence issue in biology for which various explicit procedures have been proposed (37, 38). Most of these procedures require a relatively good state of taxonomic knowledge. For tree-based methods, organisms usually need to be sampled from several populations and to have their phylogenetic relationships well resolved. Many nontree methods require extensive datasets as well. These datasets are usually not available for those genetically divergent individuals for which we propose the category of candidate species. Also, species are known to be often paraphyletic in their mitochondrial haplotypes (39). Even more importantly, phylogenies based on single DNA fragments, such as used in DNA barcoding approaches, are often not sufficiently resolved. We therefore do not recommend explicit tree-based delimitations of candidate species, although such methods are certainly a great improvement for the eventual taxonomic description of species.

Distance-based DNA barcoding methods based on divergence thresholds are prone to 2 kinds of errors. Because there is no

fixed time span needed for speciation, there should be a continuum of pairwise genetic divergences of sister species, down to 0% in cases of rapid adaptive speciation. Any threshold will therefore miss a proportion of very young species (false negatives). The second kind of error is to wrongly identify intraspecific genealogical lineages as species (false positives). The accuracy of the method depends on the so-called barcoding gap between intraspecific and interspecific divergences, but previous work on amphibians has shown a wide overlap of these values and absence of a distinct barcoding gap (40). Because DNA barcoding can only be a preliminary tool for a first and rough identification of candidate species, we favor a conservative approach that minimizes the error probability of false positives. This approach will miss species of recent origin, but it will more efficiently help taxonomists to focus on those genealogical lineages likely to be undescribed species. The categories CCS, UCS, and DCL constitute an advance over uncritical approaches to DNA barcoding because they incorporate this useful molecular tool but emphasize the need of complementary data to understand biological reality.

Following our definitions, assignment of a genealogical lineage to the CCS category has the highest reliability of the 3 categories proposed here, and the number of 129 CCS thus provides a minimum estimate of undescribed species in Madagascar. Many DCL are largely based on the lack of evidence for CCS status. Some of these genealogical lineages may thus be upgraded to CCS when more extensive phylogeographic data and integration of nuclear genetic markers are available (e.g., *Boophis luteus*, Fig. 2B), although in many cases the DCL status is well assessed by comprehensive datasets (e.g., in *Guibemantis*



will help to define their potential distribution in space and thereby help to identify target areas for further exploration.

Our findings confirm that spatial patterns of endemism, rarity, and species richness within the Madagascar biodiversity hotspot are poorly known. The protected area network of Madagascar has increased significantly in recent years, and proposals for new protected areas based on multitaxon analyses (52) will cover most of the key biodiversity areas in Madagascar. Almost a quarter (23.3%) of the newly discovered frog candidate species are not found within the currently existing protected areas; many of these are range-restricted and are more likely to disappear given the pace of habitat destruction observed in Madagascar (22). This pattern implies that even a large network of protected areas may not fully protect the current diversity. A conservation strategy should consider protecting additional small rainforest fragments, because they can contain a relatively large proportion of amphibian species (53, 54). The apparent absence from Madagascar of emerging diseases such as the amphibian chytrid fungus, in concert with a strong commitment of national institutions to conserve its biodiversity, characterize Madagascar as a unique opportunity to proactively protect an amphibian fauna so far untouched from catastrophic declines other than those caused by ongoing habitat destruction (55).

**Implications for Global Biodiversity Estimates.** This study analyzes the complete amphibian diversity of a tropical biodiversity hotspot by using comprehensive genetic, morphological, and bioacoustic datasets. Previous studies of other hotspots reported high numbers of undescribed amphibian species as well (11, 15), but were more limited in taxonomic and geographic coverage. The estimated 250% increase since 1991 up to possibly 465 species is unparalleled and would make Madagascar one of the top 5 most diverse countries on the planet for amphibians, together with Brazil, Colombia, Ecuador, and Peru. The global importance of Madagascar's amphibian fauna is paramount, especially because of its extreme degree of endemism (100% among the native species). However, it should be taken into account that very few other tropical countries with diverse amphibian faunas have been surveyed as intensively as Madagascar with comparable approaches. The unexpectedly great increase in species numbers that we estimate may therefore not characterize Madagascar as being much more species-rich than other tropical regions. Rather, it exemplifies the power of integrative taxonomic assessments and predicts that applying these to other regions or other groups of organisms may lead to comparably high proportions of novel discoveries.

As a primary conclusion from our study, the number of tropical amphibian species is probably underestimated at an unprecedented level at a global scale. Extrapolating our data to other less-studied tropical regions predicts that the number of amphibian species worldwide could double or possibly even quadruple before saturation in new discoveries can be expected. Amphibians are the vertebrate group with the highest proportion of threatened species (20), and current declines may be affecting more diversity than previously thought. Paradoxically, we are living in an era of simultaneous mass extinction and mass discovery of amphibians (21). Integrative taxonomic inventories, including molecular assessments of diversity as a standard

technique, are urgently needed, especially for poorly explored, highly diverse regions.

## Materials and Methods

**DNA Sequencing and Analysis.** A fragment of the mitochondrial 16S rRNA gene was amplified and sequenced for ca. 2,850 specimens. Based on Neighbor-Joining trees, we identified sequences showing high divergences from reference specimens of described species. Selected sequences were used to compute a tree by using Bayesian inference after determining the appropriate substitution model (see *SI Appendix*). We used the software TaxI (56) to calculate pairwise distances between sequences, which avoids possible alignment artifacts in the distribution of indels from the global alignment that may affect this computation. Extended methods are available in *SI Appendix*.

**Assessment of Bioacoustic Differentiation.** The calls of many candidate species are documented in a recently compiled sound guide (57). Advertisement calls of anurans are excellent taxonomic indicators, but intraspecific call differences are known (58): Temporal variables and (to a far lesser extent) also frequency (spectral characters) depend on environmental temperature and state of sexual motivation, whereas frequency is mainly influenced by body size. However, these differences are in all cases quantitative and continuous, i.e., note or note interval duration, or fundamental and dominant frequency, become continuously larger or smaller with increasing or decreasing temperature or body size. Similar continuous differences are also known among populations of a species, often because of character displacement or adaptation to environmental factors (59). Most well-documented cases of geographic call variation in anurans refer to such variation in continuous characters (60–62). In contrast, qualitative differences such as the presence/absence of different call types or a melodious vs. unmelodious call structure are rare within species and the few documented cases show the signature of incipient speciation (63). We considered call variation in (i) dominant or fundamental frequency, (ii) note duration, (iii) note interval duration, and (iv) pulse rate as quantitative call differences, insufficient to define a CCS except in situations of sympatry. Differences in (i) number of note types, (ii) general arrangement of note types, and (iii) melodious vs. noisy or pulsed structure of notes were considered to be qualitative call differences, defining a CCS status.

**Geographic Analysis.** A georeferenced database was compiled containing amphibian records from literature and our own field inventories. Records were taken into account only if identification was considered reliable, i.e., type localities, records of morphologically distinct species, or records with bioacoustic or molecular data (50). The current protected area network was provided by Conservation International. Distribution maps of all described species and CCS (29) largely form the basis for the geographic analysis. Table S1 in the *SI Appendix* lists the working names applied herein and in the recently published field guide (29) for the candidate species. The 9 described species for which no genetic data are available (see *SI Appendix*) are morphologically distinct and have narrow distribution ranges (29), and their confusion with any CCS or UCS is unlikely.

**ACKNOWLEDGMENTS.** We thank Parfait Bora, Neil D'Cruze, Louis du Preez, Liliane Raharivoloniaina, Roger-Daniel Randrianiaina, Jasmin E. Randrianiaina, and Che Weldon for their help in the field. We also are grateful to Sandra Nieto Román, Louis Boumans, Angelica Crottini, Susanne Hauswaldt, Meike Kondermann, Gabriele Keunecke, Eva Saxinger, Axel Strauß, Meike Teschke, and Jens Weste who helped to obtain the DNA sequences. David Knox (Conservation International) provided the latest version of the maps of Madagascar's protected area network. The study was made possible by collaboration agreements of the authors' institutions with the Université d'Antananarivo, Département de Biologie Animale, the Association Nationale pour la Gestion des Aires Protégées, and the Parc Botanique et Zoologique de Tsimbazaza, Madagascar. We are grateful to the Malagasy authorities for research and export permits. This work was supported by National Science Foundation Assembling the Tree of Life Program Grant EF-0334939, Consejo Superior de Investigaciones Científicas Intramural Grant 2008301100, grants from the Volkswagen Foundation, Deutsche Forschungsgemeinschaft (VE247/1–1 and VE247/2–1), the European Association of Zoos and Aquaria, and the Wildcare Institute.

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