Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama

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Amphibian populations around the world are experiencing unprecedented declines attributed to a chytrid fungal pathogen, *Batrachochytrium dendrobatidis*. Despite the severity of the crisis, quantitative analyses of the effects of the epidemic on amphibian abundance and diversity have been unavailable as a result of the lack of equivalent data collected before and following disease outbreak. We present a community-level assessment combining long-term field surveys and DNA barcode data describing changes in abundance and evolutionary diversity within the amphibian community of El Copé, Panama, following a disease epidemic and mass-mortality event. The epidemic reduced taxonomic, lineage, and phylogenetic diversity similarly. We discovered that 30 species were lost, including five undescribed species, representing 41% of total amphibian lineage diversity in El Copé. These extirpations represented 33% of the evolutionary history of amphibians within the community, and variation in the degree of population loss and decline among species was random with respect to the community phylogeny. Our approach provides a fast, economical, and informative analysis of loss in a community whether measured by species or phylogenetic diversity.

amphibian decline | biodiversity | chytridiomycosis | DNA barcoding | phylogenetic diversity

Catastrophic amphibian declines have been reported from multiple locations for at least 40 y, although many populations disappeared before the principal cause of the problem had been identified (1–3). It is now known that the chytrid fungal pathogen *Batrachochytrium dendrobatidis* (4, 5) causes the disease chytridiomycosis in amphibians (6, 7) which has led to the decline, extirpation, and extinction of amphibian populations and species around the world (8–11). Disease-driven declines and extinctions of amphibians have now been reported on four continents (12, 13), yet data on amphibian abundance and diversity for a given community before and following an epidemic disease outbreak remain rare. The lack of before-and-after data has limited accurate quantification of amphibian loss, and study of the ecological consequences of community disassembly that follows catastrophic loss of species (14).

Our knowledge of amphibian species diversity is far from complete (8, 15). Increased exploration in the tropics and new molecular approaches to the identification of cryptic amphibian lineages are leading to a rapid description of new species (8, 16). Countervailing trends of species decline and discovery suggest that many amphibians could be locally extirpated or driven to global extinction before scientists become aware of their existence (8, 17). Thus, scientists need to be able to quickly identify and quantify diversity even without a complete taxonomic framework.

Alternative measures of diversity may be especially helpful to those who need to prioritize conservation actions with incomplete or limited data. DNA barcoding uses a rapidly expanding database to efficiently match species names to voucher specimens via DNA sequences, or to recognize DNA lineages that may represent species not yet described (16, 18). A second metric, phylogenetic diversity (PD), is free of taxonomy. PD was developed as a measure of the proportion of the phylogenetic history of a monophyletic group that could be protected by alternative habitat reserves (19). PD has been used more recently to explore spatial variation in local PD (20, 21). We adopt PD here to provide a measure of change in community diversity following epidemic disease, but it could be used for any temporal analysis in which species diversity of a community has been completely described at two or more points in time.

The correlation between the loss of species diversity and the loss of PD from a community is difficult to predict on theoretical grounds because the pattern and magnitude of evolutionary loss depend on tree shape (22) and on the phylogenetic relationships among species (23–25). Many consider PD to be the more profound measure of loss (21, 26), although the relationship between species loss and PD loss within a single community has not been studied (27). The difference between PD and species loss has important implications for the conservation of biodiversity, as these measures may be decoupled (28).

Many phylogenetic, ecological, or environmental covariates of amphibian decline and extinction have been identified (29), and many species respond similarly across geographic locations, although environmental conditions can influence local responses. Over evolutionary time, extinction rates themselves may have a significant phylogenetic component, as shown in the fossil record (30). Over ecological time, decline in abundance among species may be phylogenetically correlated, as shown recently for a tree community (31). Not surprisingly, ecology and phylogeny likely influence the degree of decline among amphibians (29, 32). International Union for Conservation of Nature Red List threat categories are correlated with amphibian species phylogeny (23, 29), and infection by *B. dendrobatidis* has a phylogenetic component when analyzed on a global phylogeny of amphibian families (33). One limitation of these analyses, however, is that geography may be a confounding variable. Some lineages of amphibians are endemic to specific regions where *Batrachochytrium* is not yet present (e.g., the family Mantellidae in Madagascar) (34), and regional assemblages of amphibians have received unequal sampling effort.

In Central America, the decimation of highland amphibian populations was first noted in Monteverde, Costa Rica (35, 36), in the late 1980s, and since then the spread of *B. dendrobatidis*

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has been moving in an epidemic wave from the northwest toward the southeast through the cordillera of Isthmian Central America (9, 37–40). Anticipating the arrival of *B. dendrobatidis*, an intensive field survey and monitoring program was established in 1998 in the G. D. Omar Torrijos H. National Park (latitude 08.667, longitude −080.592) at 800 m elevation, near El Copé, Panama (9).

The arrival of *Batrachochytrium* in El Copé in 2004 provided a unique opportunity to collect standardized data on amphibian abundance and diversity before and following the epidemic outbreak of chytridiomycosis. Given the frequency of cryptic lineages among Panamanian amphibians (41, 42), we estimated amphibian diversity using DNA Barcode of Life protocols (18, 43) to establish the number of lineages and quantify PD. We combined molecular genetic data with long-term field surveys to describe the change in abundance of each amphibian species, the change in community composition at El Copé including known and cryptic lineages, and the resulting loss of evolutionary history as measured by PD. Our approach provided a fast, economical, and highly informative analysis of loss in a community whether measured by species or PD.

Results

Seven years of predecline surveys in El Copé, Panama, identified 63 species of amphibians within a 4-km^2 area. Standardized surveys during 2000 through 2003 provided estimates of predecline abundance for most species in the El Copé study area, whereas surveys during 2006 through 2008 provided postdecline abundance data (Table S1). Abundance was defined as the average species-specific density along standardized transects, and percent change in abundance for each species was obtained by dividing the difference between pre- and postdecline abundance by the predecline abundance. Repeated-measures ANOVA revealed a significant community-wide decline in abundance between pre- and postdecline periods ($F_{1,317} = 7.926$; $P = 0.0052$). A Wilcoxon rank-sum test with continuity correction revealed that the median annual abundance declined significantly in 17 species (Table S2), although given the limited number of years of sampling, this test has little power. The distribution among species of changes in abundance permitted us to categorize each species by its level of decline (Fig. S1).

DNA Barcode of Life protocols (18, 43) were used to generate mitochondrial cytochrome oxidase subunit 1 (COI) and 16S ribosomal RNA gene genotypes with published primers and protocols (44, 45) for 300 individuals representing between one and 12 individuals for each of the 63 named species known from the study site before the arrival of *B. dendrobatidis* in 2004. These DNA sequences were used to establish a phylogenetic tree providing an evolutionary context for analyzing the magnitude and pattern of community loss of amphibian diversity following epidemic disease. We counted the number of candidate species (16) as the total number of independent lineages inferred from the phylogenetic and barcode gap analyses (Fig. 1) minus the number of named species in the dataset.

Twenty-five named species of amphibians observed before 2004 were not observed in the 2006–2008 transects surveyed. We categorize these species as extirpated: a loss of 40% of the nominal species diversity of the El Copé amphibian community (Table 1). Moreover, our genetic analyses identified an additional 11 candidate species, of which at least five are extirpated, increasing the total loss of amphibian lineages to 30 (41%). In addition to the loss of 30 named and candidate amphibian species, another nine named species have declined by 85% to 99% in abundance. These taxa are at significant risk (46) and we categorize them as critical (Fig. S1).

Our DNA barcode results suggest that amphibian lineage diversity in the El Copé community had previously been underestimated by 17% despite 7 y of intensive study and collection (9). If we count four morphospecies (Table S2) as previously identified diversity, the increase is 11%. All candidate species differed from their sister lineage by a genetic distance at the COI gene of 8.6% to 24.6% or 1.9% to 11.2% at the 16S gene (Fig. 1). Three of the candidate species did not form monophyletic lineages presumed to be conspecific (*Diasporus* aff. *diastema*, *D.* aff. *quidditus*, and *Pristimantis* aff. *museosus*; Fig. 2). Samples we continue to consider to be conspecific showed as much as 6% divergence at COI (in *Hyalinobatrachium colymbiphyllum*) or 1.2% divergence at 16S (in *Cochranella euknemos*). We never observed two individuals identified as heterospecific based on morphology showing genetic divergence less than 1.3% at 16S or less than 12% at COI (Fig. 1). Application of a general mixed Yule coalescent (GMYC) model–based approach to identifying discrete evolutionary clusters (47, 48) yielded an additional 40 "entities" beyond the 74 identified by the bivariate barcode gap analysis. The restricted geographic sampling in our study, however, could be hampering the GMYC analysis (49, 50), and we therefore prefer the more conservative estimate of 74 total species named and candidate.

We estimated the magnitude of the loss of evolutionary history using change in PD (19, 26, 51), as obtained from the total branch length connecting the El Copé subset of lineages to a phylogenetically constrained amphibian phylogeny based on 10 published analyses (*SI Materials and Methods*), including the 17-gene dataset of Heinicke et al. (52). Although PD is independent of taxonomy, we relied on taxonomy to connect field survey data with molecular genetic data. The El Copé amphibian community lost 33% of its PD (Fig. 2), or 32% of its PD using a temporally calibrated phylogeny (not shown). The loss of PD increases to 41% when we included species categorized as critical (Fig. S1, Fig. 2, and Table 1). If all declining species (Fig. S1) are eventually lost, only 39% of the original PD of the El Copé amphibian

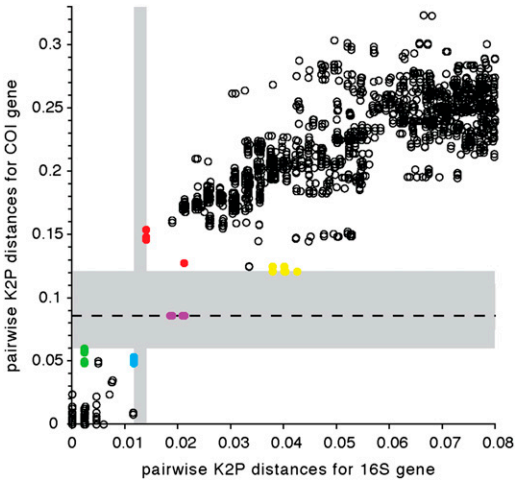

Fig. 1. Bivariate graph of average pair-wise distances of the COI versus 16S gene fragments showing the barcode gap for sympatric samples representing the amphibian fauna of G. D. Omar Torrijos H. National Park, Panama. Gray zones indicate the gap in genetic distances between the most divergent intraspecific samples and the least divergent interspecific samples, as measured by K2P distances: 0.01163 versus 0.01397 for 16S, and 0.06026 versus 0.12107 for COI. The width of the 16S gap is defined by the intraspecific divergence within *Cochranella euknemos* (blue dots) versus the interspecific distance between *C. euknemos* and *C. granulosa* (red dots). The width of the COI gap is defined by the intraspecific divergence within *Hyalinobatrachium colymbiphyllum* (green dots) versus the interspecific distance between *Pristimantis cruentus* and *P.* aff. *museosus* (yellow dots). *Silverstoneia nubicola* lineage A versus lineage B (purple dots) is an intermediate case showing a divergence of 0.08611 at COI (dashed line). We count lineage B as a candidate species. To aid in visualization of the gap, the *x* axis was arbitrarily cut off at 0.08 divergence.

Table 1. Loss of amphibian diversity

Species removed (by decline category)	Named species lost (n = 63)	Candidate species lost (n = 11)	Lineages lost (n = 74)	PD lost (%)
Extirpated and DD-extirpated	25 (40%)	5 (45%)	30 (41%)	33
Extirpated, DD-extirpated, and critical	34 (54%)	5 (45%)	39 (53%)	41
Extirpated, DD-extirpated, critical, and declined	42 (67%)	6 (55%)	48 (65%)	61

Decline and loss of amphibian diversity from El Copé study site with increasingly inclusive categories of decline (Fig. S1). Loss of diversity was measured by four metrics: named species, candidate species, lineages (named + candidate species), and PD (sum of branch lengths obtained by MPL analysis of topologically constrained phylogenetic tree; Fig. 2).

community would remain (Table 1). Thus, our data establish empirically that the loss of amphibian biodiversity is severe by any measure.

We analyzed the phylogenetic distribution of percent decline of each species by mapping the direct impact of disease as a continuous variable rather than a conservation threat category (29). Because our data come from a single site, we eliminated geography as a potentially confounding variable affecting the correlation between population decline and phylogeny (33, 51). To test for a correlation between phylogeny and percent decline in abundance, we evaluated Pagel's generalized least squares (GLS) model of character evolution as a constant-variance random walk (53) on species-level phylogenies with one sample chosen arbitrarily to represent each of the 74 named and candidate species. The hypothesis of phylogenetic independence ($\lambda = 0$) was not rejected by maximum likelihood (ML) analysis conducted on a phylogeny constrained to match known higher-level amphibian relationship, as in Fig. 2 (likelihood ratio test, $\chi^2 = 0.5866$, one degree of freedom, $P = 0.44$). Similar results were obtained using a Bayesian method of analysis that accounted for phylogenetic uncertainty on unconstrained trees (highest Bayes factor among analyses was 1.09). Alternative coding of trait values did not change these results, and credibility intervals around λ always included zero, indicating a lack of significant correlation between percent decline and phylogenetic relationships in the El Copé amphibian community.

Discussion

The irony of increased species discovery coupled with enigmatic population declines in amphibians was recognized at least 10 y ago (17), yet until now we have lacked quantitative data demonstrating the direct impact of epidemic disease on amphibian diversity and community phylogeny. Combining changes in species-specific abundance with DNA barcode identities permitted us to quantify the loss of individuals, lineages, and evolutionary history resulting from chytridiomycosis. This disease has caused the extirpation of 25 species, 11 genera, and four families from El Copé. DNA barcode data establish that 11 of 74 species (15%) went unrecognized, and at least five of these 11 are already extirpated from the site (Table 1). Molecular data increased the number of extirpations recorded at El Copé by 20% (from 25 species to 30 species lost). Given that El Copé is one of the better-studied amphibian faunas in the Neotropics, the loss of undescribed species is likely far greater in sites that have not been the object of such intensive investigation as El Copé.

Our count of undescribed species may be regarded as a conservative estimate, and recent morphological systematic work suggests we still have not accounted for all the cryptic diversity at El Copé (54). The ability of two forms to coexist in sympatry provides a litmus test of species status (55). Because all our samples were sympatric, identifying candidate species from El Copé via the DNA barcode gap (56) was straightforward. Our DNA barcode analysis showed no overlap in the intraspecific versus interspecific genetic distances at the COI or 16S gene (Fig. 1). Our inferred candidate species at El Copé were separated from their named sister species by 8.6% to 24.6% as measured by COI divergence. By way of comparison, sister species of frogs in the family Mantellidae are differentiated by 8% to 17% at the COI barcode locus, whereas sympatric conspecifics of Mantellidae differ by only 1% to 5% (57).

Given the substantial level of cryptic lineage diversity uncovered by DNA barcodes, our estimate of the loss of amphibian diversity is greater than would have been obtained from morphological-based identifications alone. Although the percent diversity lost is equivalent between named species and total number of lineages, the DNA barcode data revealed an additional five candidate species that have already disappeared from the study site (Table 1), at least one of which is likely already globally extinct (58). These candidate species remained unrecognized despite 7 y of field surveys. The wave of epidemic declines continues spreading unchecked into eastern Panama (40), which is further threatened by invasion of *B. dendrobatidis* from the southeast, as this pathogen is widespread in Andean South America (37, 59, 60), including Colombia (61, 62).

The amphibian phylogeny based on the 17-gene dataset of Heinicke et al. (52), along with nine other recent molecular phylogenetic analyses (*SI Materials and Methods*), provided a superb context within which to place our DNA barcode to measure the loss of PD following catastrophic disease. The relationship of PD to species diversity has been characterized for complete clades, but not for phylogenies based on local communities (22, 24, 51). At El Copé we showed that the loss of PD lagged behind loss of lineage diversity: 33% versus 41% (Table 1). PD loss might be overestimated if tip branches in a local community phylogeny are longer than in a complete phylogeny (e.g., sister lineages are not found at El Copé), and if extirpation of lineages is biased toward tip branches (as suggested by phylogenetic independence). However, the consistency in percent PD loss estimated from unconstrained, ultrametric, and time-calibrated trees (Table 1 and *SI Materials and Methods*) suggests that our results are robust, and that the relationship between species loss and PD loss observed here could be informative of the loss of diversity in other ecological communities.

Within El Copé, decline and extirpation were random with respect to the community phylogeny (Fig. 2), an unexpected result given that previous studies have documented a phylogenetic component to species loss (29, 33). One explanation for the contrast between our El Copé results and previous phylogenetic analyses of extinction risk may involve geographic scale; the risk of population extirpation of a given species could be distinct from its global risk of extinction. For example, given the positive relationship of chytridiomycosis and elevation (29, 63, 64), a species with a wide geographic distribution could be lost from montane sites such as El Copé but persist in the lowlands. On the other hand, analyses of amphibian declines across Central America suggest that for most species local extirpations lead to regional extinctions, lower beta diversity, and the homogenization of the regional amphibian fauna (65). Nonetheless, a deeper understanding of local versus regional extinction patterns and processes will require better sampling at the regional scale (27). Obtaining pre- and post-decline abundance data from more localities in a region would

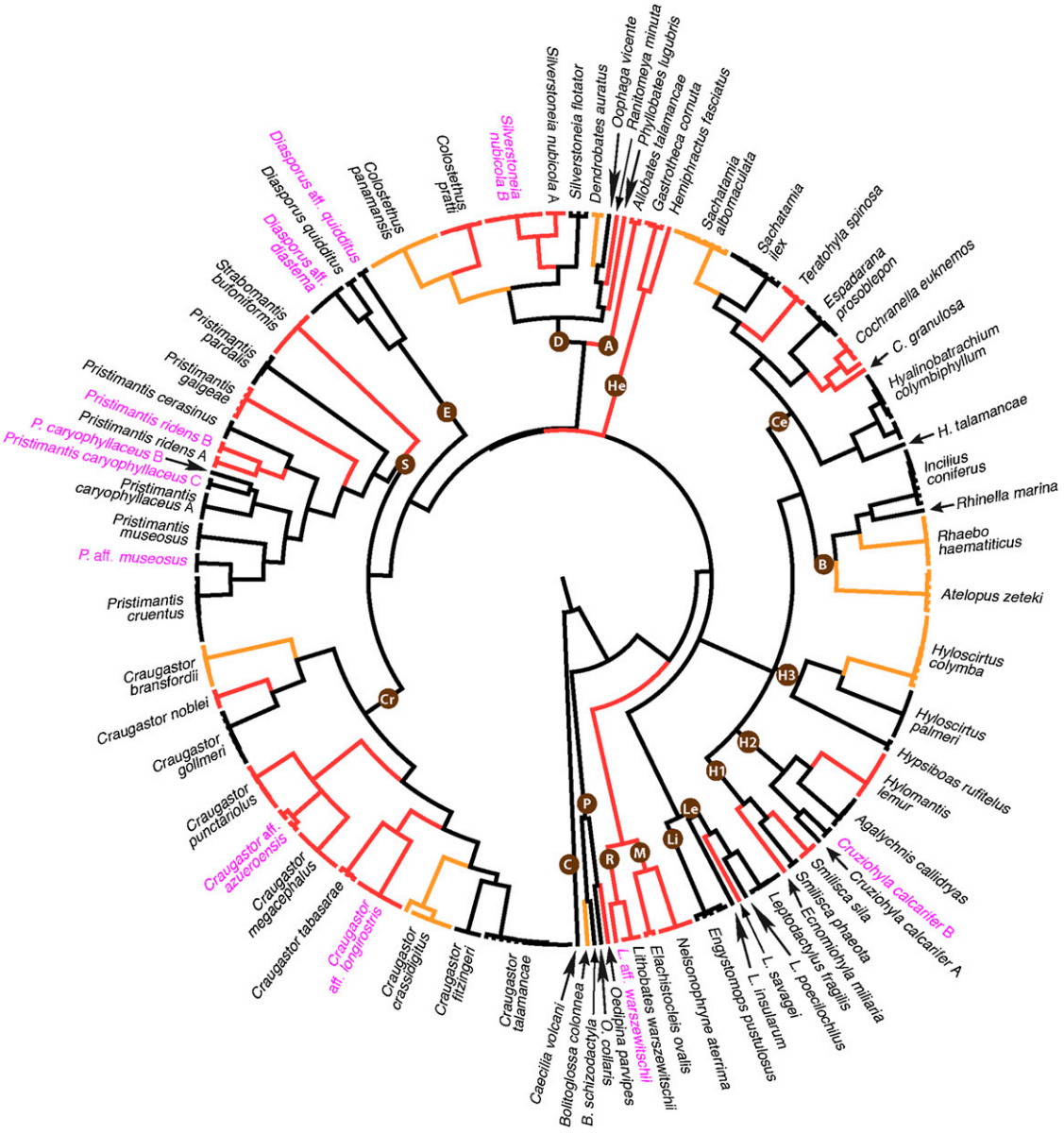

Fig. 2. Community phylogeny of the amphibians of El Copé, Panama, study site. Maximum likelihood phylogeny constrained to match known amphibian relationships (*Materials and Methods*) and inferred from concatenated DNA sequences of the COI and 16S gene fragments from 300 samples representing all 63 named amphibian species plus 11 candidate species (labeled in purple) known from the study transects of approximately 4 km^2 established in Omar Torrijos National Park. Branch lengths made ultrametric using the MPL algorithm. Branches are color-coded by percent decline in relative abundance following the catastrophic decline caused by the arrival of *B. dendrobatidis* (5). Red branches indicate 100% decline in relative abundance (extirpated category). Orange branches indicate a decline of 85% to 99% (critical category). Black indicates less decline or an increase in relative abundance. Taxonomic families of amphibians are indicated by brown circles: A, Aromobatidae; B, Bufonidae; C, Caecilidae (a caecilian); Ce, Centrolenidae; Cr, Craugastoridae; D, Dendrobatidae; E, Eleutherodactylidae; H1–H3, Hylidae (H2, Phyllomedusinae); He, Hemiphractidae; Le, Leptodactylidae; Li, Leiuperidae; M, Microhylidae; *P*, Plethodontidae (salamanders); R, Ranidae; and S, Strabomantidae.

allow investigation of potential environmental correlates of intraspecific variation in disease susceptibility, while simultaneously improving phylogeny-based forecasts of extinction risk.

Our results provide a phylogenetic model of the extirpation process that results from chytridiomycosis. Regional analyses show that some of the populations extirpated from El Copé represent global extinctions (65), whereas global analyses show that current rates of extinction are far greater than historical rates (66). Together, these results demonstrate that disease-induced amphibian declines are not temporary, should not be regarded as part of naturally recurrent demographic processes, and have already brought about the loss of an unknown quantity of species heretofore unrecognized by scientists.

Materials and Methods

Field Data. We generated a list of 63 named amphibian species (Table S2) identified from surveys of permanent transects combined with casual observations of species off the transects for the years 1998 through 2004 (no new species were added during 2005–2008). Forty-nine species were regularly encountered on transects and we were able to estimate pre- and postdecline abundances. Four of these 49 species were morphospecies that were confirmed by genetic data post hoc as candidate species (defined below). We were unable to obtain quantitative abundance data for 18 species. Nine of

these 18 species occurred primarily in ponds, habitats that we did not formally survey. The other nine species were encountered very rarely (Table S2). Predecline abundances were calculated on the basis of fieldwork during 2000 to 2003, after species identification and survey methods were standardized. Field sampling effort is shown in Table S1. We did not use survey data from 2004 to 2005 to calculate changes, as this was the peak of the die-off and many populations were in flux (9). We used survey data from 2006 to 2008 to calculate postdecline abundances. To test for a significant change in abundance in pre- versus postdecline periods for (*i*) community-wide data, we ran a linear mixed-effects repeated-measures ANOVA; and for (*ii*) individual species abundance data, we used Wilcoxon rank-sum tests with continuity correction (*SI Materials and Methods*).

We assigned species to categories of decline on the basis of the percent change in abundance as follows. Extirpated indicated a 100% decline in abundance (i.e., zero captures in postdecline surveys); critical indicated 85% to 99% reduction in abundance; declined indicated a reduction of 1% to 55%; and least concern indicated apparent increase in abundance. These categories were based on natural breaks in the distribution of declines (Fig. S1). Eighteen named species were classified as data deficient (DD) because they were found in the study area but not on the standardized transects used to quantify abundance (i.e., pond species and rare species). DD species were further classified as either DD–extirpated (nine named species) if they were not seen on postdecline transects and have largely disappeared from other declined sites, or as DD–least concern (nine named species and one candidate species) if they persisted (35, 38, 39). We conservatively assigned category of decline to candidate species (see below) by assuming that, if the morphologically most similar named species persisted, the candidate species persisted as well, and we assigned them the same category (Fig. S1).

Evolutionary Analyses. All 63 species in the study site were represented by between one and 12 genetic samples. ML and Bayesian criteria were used to infer unconstrained molecular phylogenies, and ML was used to infer trees constrained to match known higher-level relationships within Amphibia (*SI Materials and Methods*). The resulting phylogenies were transformed with a mean path length (MPL) algorithm implemented in the C program, PATHd8 version 1.0 (67) (Fig. 2). We used PAUP* 4b10 (68) to calculate patristic and pair-wise genetic distances. A candidate species (16) was identified as one member of a pair of lineages collected under the same species name that (*i*) did not form a monophyletic clade with its namesake or (*ii*) formed a monophyletic clade with its namesake that showed a Kimura two-parameter (K2P)–corrected divergence of more than 8% in COI and more than 2% at 16S (Fig. 1). The K2P model was chosen to facilitate comparison of our genetic distance data with previously published reports. We also estimated the number of lineages or evolutionary "entities" using a likelihood-based analysis of a GMYC model that assumes an ultrametric gene genealogy and identifies the points of transition between within-population rates of coalescence versus interspecific rates of lineage coalescence (47, 48). Using the code gmyc (47), written for R (69), a likelihood ratio test of a single-threshold model versus a multiple-threshold model rejected the former ($\chi^2_9 = 1956.236$, $P < 10^{-10}$), inferring four transition points and 112 entities including 60 clusters of more than one sample.

We evaluated the loss of PD (19) in the El Copé amphibian community, but we did not study community structure in terms of ecological niches or comparison with a regional species pool (27). Loss of PD was calculated by trimming species from the phylogeny according to their category of decline (Table S2) and recalculating total branch lengths among the remaining taxa without reoptimizing the tree. PD was quantified from the topologically constrained likelihood tree with three measures of branch length: unconstrained (Fig. S2), MPL (68) (Fig. 2), and time-calibrated MPL branch lengths (*SI Materials and Methods*). Loss of PD was roughly the same with or without topological constraints and approximately 1.0% lower using MPL relative to unconstrained branch lengths. Table 1 shows PD loss based on MPL branch lengths and topological constraints.

To test for a phylogenetic correlation of percent declines in abundance among species we used ML and Bayesian analyses of Pagel's GLS model (53). Likelihood evaluation of the GLS model assumed a ML phylogeny topologically constrained to match known amphibian relationships, obtained using *GARLI* (*SI Materials and Methods*). Bayesian analyses of the GLS model incorporated phylogenetic uncertainty by integrating across a set of 100 unconstrained trees sampled widely from the posterior probability distribution of trees. The GLS model assumes a Brownian model of evolution to obtain a matrix of expected covariation among species on the basis of the phylogeny or set of phylogenies. By using the software BayesTraits (available at www.evolution.rdg.ac.uk), we evaluated two competing ML models in which the trait of interest was the percentage decline of each species: an ML model with λ fixed at 0 versus λ as a free parameter. Models were evaluated by likelihood ratio tests and by Bayes factors (integrating over 100 trees) with marginal likelihoods approximated by the harmonic mean of likelihoods sampled from the Markov chain. All other options for BayesTraits followed suggestions in the user manual. As a visual aid, we present the phylogenetic distribution of amphibian declines coded as positive values, 0 to 100, on an unconstrained phylogeny that included all 300 samples (Fig. S2).

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