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BIODIVERSITY RESEARCH

Long-term isolation and endemicity of Neotropical aquatic insects limit the community responses to recent amphibian decline

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ABSTRACT

Aim Neotropical highland streams have shown diminished ecosystem functioning after amphibian extirpation infected by the chytrid fungus *Batrachochytrium dendrobatidis*. The loss of amphibians could affect communities of aquatic insects co-occurring in these streams in various ways. We examined patterns of species and genetic diversity of these communities and their evolutionary history along the chytrid expansion gradient to elucidate potential community responses.

Location Six streams over a 320-km transect in Panama affected by chytrid expansion from west to east for up to 14 years, and two apparently chytrid-free streams in the east.

Methods Patterns of α- and β-diversity were investigated at three hierarchical levels: genus, species and haplotypes. Genus identification was based on morphology, and putative species were inferred by grouping the DNA barcodes (749 *cox1* sequences) with the GMYC method on all collected individuals of Ephemeroptera, Trichoptera, Coleoptera and Plecoptera.

Results A total of 96 genera in 43 families (9 orders) of insects were encountered. Genus-level α-diversity was higher in the easternmost streams, possibly due to a separate biogeographical history, whereas β-diversity was constant along the chytrid expansion gradient. Community DNA barcoding resulted in 426 *cox1* haplotypes and 154 putative species, most of them limited to single sites. High β-diversity along the gradient at both species and haplotype levels argues against community homogenization by migration in the wake of amphibian declines. In contrast, phylo-β-diversity was low, indicating community similarity at deep levels.

Main conclusions Aquatic insect communities in this region are influenced by long-term limited dispersion that generated high endemicity. The pattern persists mostly unperturbed after disease-driven amphibian declines; hence, if indeed insects fill the niches vacated by tadpoles, they would originate from local communities rather than immigration. Given the unique evolutionary history and physical isolation of local assemblages, the ecosystem deterioration carries the risk of losing unique diversity.

Keywords

***Batrachochytrium dendrobatidis*, community DNA barcoding, genetic diversity, SGDC, species diversity, stream ecology.**

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INTRODUCTION

The accelerated rate of species extinction resulting from human activities is closely associated with the loss of ecological function (Hooper *et al.*, 2005; Cardinale *et al.*, 2006). However, the interrelationships of species extinctions, altered community composition and abiotic environmental changes are complex and hamper our ability to predict the effects of biodiversity loss (Vaughn, 2010). Current empirical approaches have focused mainly on microcosms to examine effects of diversity changes on system-scale functional processes such as nutrient recycling, primary production and organic matter dynamics (but see Atkinson *et al.*, 2013; Whiles *et al.*, 2013). However, experimental studies conducted over short periods on local communities may not reflect broader evolutionary and ecological processes such as the possible local loss of intraspecific genetic diversity and the compensation by gene flow across metapopulations. Moreover, the time delays between species losses and their impacts on altered ecosystems may exceed predictions based on small-scale experiments (Duffy, 2009). Similarly, experimental manipulations generally do not account for potential new colonizers with similar functional roles that may compensate for the species loss.

Amphibian populations world-wide are experiencing massive declines, many of which are linked to *Batrachochytrium dendrobatidis* (*Bd*), a pathogenic fungus that causes chytridiomycosis (Lips *et al.*, 2006). *Bd* thrives in cool, humid conditions, making pristine high-elevation sites in the tropics, where endemism is high, most vulnerable. Pathogen prevalence is lower, and amphibian populations often remain stable at warmer lowlands (Bustamante *et al.*, 2010; Becker & Zamudio, 2011). Studies in Central America have documented a steady advance of *Bd* from west to east in Panama, with massive die-offs of highly diverse, abundant amphibian communities in high-altitude streams (Lips *et al.*, 2005, 2006). For instance, the arrival of *Bd* coincided with the extirpation of 40% of taxonomic diversity and 33% of the phylogenetic diversity of frogs at one site in Panama (Crawford *et al.*, 2010). The ongoing *Bd* wave in Panama provides a chronosequence of sites affected since 1996 to chytrid-free streams (Fig. 1a). This situation represents a unique natural

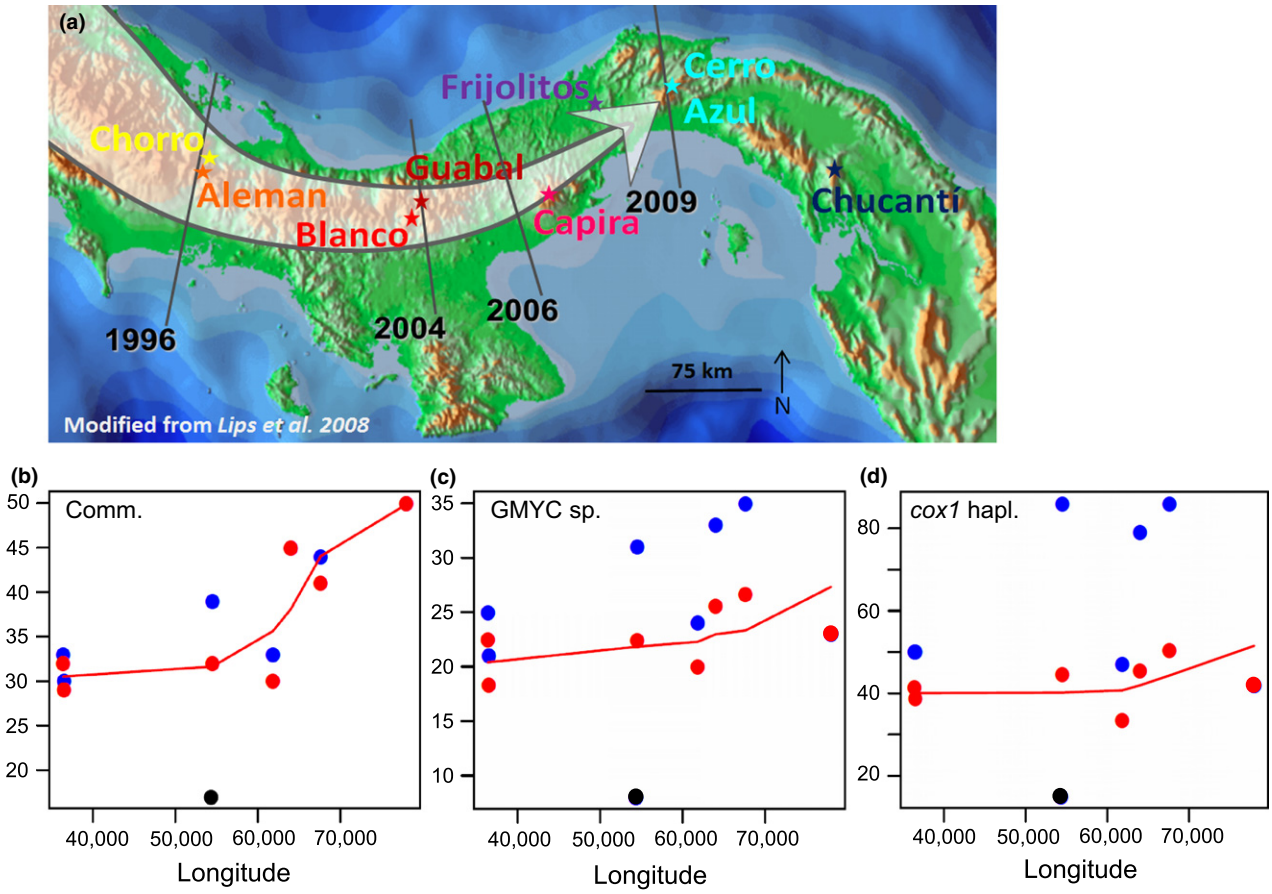

Figure 1 (a) Distribution of sampling sites across Panama and diversity patterns after amphibian declines across the longitudinal gradient for (b) richness of genera within communities, (c) GMYC species richness and (d) *cox1* haplotypes richness. Blue circles are total richness and red circles are richness after rarefaction based on Chuncanti, except río Blanco (black circle). Lines indicated the best fit for a local polynomial regression for the rarefied richness.

experiment for testing fundamental questions about the extirpation of a major trophic group and associated changes in ecosystem function and their effects on unrelated lineages.

Larval amphibians feed on algae (grazers) and detritus and may act as 'ecosystem engineers' driving nutrient recycling because of their high abundance and consumption rates (Flecker *et al.*, 1999; Whiles *et al.*, 2006). Their massive declines have altered ecosystem function through changes in nutrient uptake and cycling efficiency, resource availability and overall biological activity (Whiles *et al.*, 2006, 2013; Colón-Gaud *et al.*, 2009; Rugenski *et al.*, 2012). These streams are also occupied by diverse communities of aquatic insects, including some that could replace tadpoles ecologically because they have similar functional roles, such as various Ephemeroptera, many of which are grazers, while other species may also be affected in various direct and indirect ways by the loss of amphibians (Colón-Gaud *et al.*, 2010a). Previous local-scale studies examining the ecological effects of amphibian extirpation on the macroinvertebrate communities comparing pre- and post-decline conditions found subtle shifts in species composition, biomass and secondary production (Colón-Gaud *et al.*, 2010a,b; Whiles *et al.*, 2013), whereas another study conducted over longer periods showed a reduction in macroinvertebrate richness by ~40% as time progressed (Rantala *et al.*, 2015). However, all previous studies assessed changes in macroinvertebrate communities at local sites and used coarse identification to genus because of the difficulties with species-level taxonomy. This limits assessment of the precise species composition and its variation among affected communities and therefore does not permit the evaluation of local communities in the context of larger-scale patterns and the evolutionary history of the encountered lineages.

Here, we sampled previously studied sites together with additional streams for a broader assessment of the variation of aquatic insect communities affected by the chytrid disease wave. The lack of an established taxonomy for insects forming these local assemblages may be overcome using mitochondrial DNA (mtDNA) variation in the 'barcode' marker cytochrome C oxidase I (*cox1*). Clusters of sequence variation in *cox1* approximate the entities traditionally recognized as Linnaean species in many invertebrates (Hebert *et al.*, 2003). This permits rapid assessment of taxonomically complex species assemblages from multiple sites and various life stages by grouping individuals using quantitative procedures (Tänzler *et al.*, 2012). Moreover, intraspecific variation of *cox1* is commonly used to determine genetic diversity and assess gene flow among populations, while interspecific variation is used to infer their evolutionary histories (Avise, 2009). Thus, analysing the nucleotide variation of the *cox1* gene for entire communities does not only serve to identify species, but also integrates this information with their evolutionary differentiation and current gene flow, linking processes at different temporal scales (Marske *et al.*, 2013; Joly *et al.*, 2014). Community DNA barcoding therefore has great potential for inferring population dynamics, phylogeography, macroecological patterns and evolutionary history of diverse groups (Hajibabaei *et al.*, 2007; Tänzler *et al.*, 2012) and for predicting large-scale biodiversity patterns using local genetic inventories (Papadopoulou *et al.*, 2011).

The simultaneous analysis of taxonomy, population genetics and evolution of species assemblages may lead to a more complete understanding of processes affecting the persistence and turnover at various time-scales and the potential effects of tadpole loss on aquatic insects. Moreover, understanding the evolutionary mechanisms and current population dynamics should be valuable for assessing the fragility of tropical macroinvertebrate communities to perturbation and their response to a rapidly changing environment. Species composition and their evolutionary and biogeographical history are likely influenced by barriers to dispersal among streams and by the vagility of species, along with the particular functional roles of each species. The biogeography of aquatic insects in Central America remains relatively unexplored, but high diversity, endemicity and turnover among mountain ranges are expected based on the stability hypothesis of habitats, which posits that weak climatic fluctuations during the Pleistocene in the tropics promoted the persistence of isolated populations and allopatric speciation (Fjeldså *et al.*, 1999; Cadena *et al.*, 2011; García-López *et al.*, 2013). Similarly, in temperate regions, the population persistence in permanent streams has led to higher endemism and smaller geographical species ranges for running (long-term stable habitat) than standing (instable habitat) water species (Ribera *et al.*, 2003; Hof *et al.*, 2006). Given the complexity of factors affecting the distributions of aquatic insects and their potential responses to amphibian declines, we assessed the diversity and turnover using a similar analytical framework at three hierarchical levels from the genus level to intraspecific genetic differentiation, which provides a wider view of the importance of the historical and contemporary factors determining current diversity patterns (Bonada *et al.*, 2009; Dexter *et al.*, 2012; Marske *et al.*, 2013).

Considering the rapidity and severity of amphibian declines and consequent changes in ecosystem function (Colón-Gaud *et al.*, 2010a,b; Whiles *et al.*, 2013), two scenarios may be considered for the effects on insect assemblages. First, if long-term habitat stability and limited dispersal drive the evolutionary history of aquatic insects in the region, we expect substantial isolation among local communities even at small spatial scales. This scenario would predict that the post-decline communities are composed of species already present locally, and even if the ecological roles of tadpoles could be assumed by functionally similar aquatic insect species, the arrival of new immigrants is unlikely. Therefore, the local species would change in abundance after amphibian declines, but not in composition (or some ecological functions remain unfilled), and β-diversity should be high and stay high. In that case, we predict a positive correlation between species and genetic diversity because limited migration among discrete, undisturbed sites over extended time-scale would have parallel effects on local diversity at these

two levels (Vellend & Geber, 2005; Vellend *et al.*, 2014). An alternative scenario is expected if there is historical and recent long-distance dispersal. In this case, novel widespread aquatic insect immigrant species may occupy available niche space in the altered ecosystem, and we expect increasing homogenization at both interspecific and intraspecific levels at sites with the longest time since amphibian decline, possibly at the expense of the original residents, which may reduce β-diversity when studied across multiple sites. This could also lead to a distortion of diversity at species and genetic levels because rapid range expansions, possibly driven by selection only at species level, would undermine the processes (such as stochastic dispersal) needed for the multihierarchical patterns to form (Vellend & Geber, 2005).

METHODS

Study area and sampling methods

A total of 8 streams were sampled (Fig. 1a). These included six pristine post-decline streams located across four mountain ranges, spanning from sites at Fortuna in the west to Cerro Azul in the east and representing a chronosequence of decline dates from 1996 to 2009. We also sampled one chytrid-free site in the Chucanti Nature Preserve and one lowland pristine stream (río Frijolitos) in the Soberanía National Park (Table 1). All streams, except río Frijolitos located in the lowlands, were sampled at similar altitudes between 700 and 900 m, or at 1200 m in the westernmost sites (Chorro, Aleman), and were located in five unconnected rain forests and surrounded by agricultural areas that are expanded at the expense of rain forest. Only Aleman and Chorro were connected by water (3.48 km apart in different subcatchments), while the remaining sites were unconnected and separated by mountains. Geographical distance between sites ranged from 2.11 km (Guabal-Blanco) to 415.85 km (Aleman-Chucanti), with distances among most sites between 50 and 180 km (Appendix S1). Except río Blanco that was smaller, all streams had similar habitats and substrate which ranged from large boulders and cobbles in riffles, to silt and sand in pools. For example, between 2008 and 2011 for Chorro, Guabal and Chucanti, respectively, the average width was 4.5 m, 3.7 m and 3 m; the temperature was 19 ± 2°C, 21 ± 1.5°C and 21 ± 2°C; and discharge was 72 ± 8 l s^{-1}, 60 ± 8 l s^{-1} and 55 ± 12 l s^{-1} (Rugenski, 2013).

Sampling was conducted over a 4-week period in February–March of 2011 during the dry season. Stream flow had been stable during the dry season, except río Blanco that experienced a flood event 3 weeks prior to sampling. Macroinvertebrates were sampled at all available habitats from riffles and pools along a 150-m reach using a 250-μm mesh kick net. Standard sampling took place for at least 150 min until no new taxa were captured. Samples were preserved in absolute ethanol, and macroinvertebrates were identified to genus in the laboratory (Roldán, 1988; Merritt *et al.*, 2008) prior to molecular analysis. For each genus, functional feeding group assignments were based on Merritt *et al.* (2008).

Molecular analysis and estimates of molecular species identities

The three most abundant orders (58 of 96 genera) encompassing all functional feeding groups were selected for community DNA barcoding (see functional feeding groups in Appendix S2), including Ephemeroptera classified as grazers and collectors (17 genera); Trichoptera, of which the majority are classified as filterers and shredders, but also including a few grazers and collectors (Glossossomatidae, Hydroptilidae) and predators (Polycentropodidae) (18 genera); and Coleoptera, with the majority classified as collectors, but also including predators (Dytiscidae, Gyrinidae) and grazers (Psephenidae) (23 genera). Additional individuals belonging to Plecoptera (predators) were sequenced because they included the only genus (*Anacroneuria*) distributed at all sites. The *cox1* gene was sequenced for all available specimens up to 20 individuals per genus and site (see Appendix S3 for details of DNA sequencing).

Putative species were delimited separately for each order using the Generalized Mixed Yule-Coalescent (GMYC) model

Table 1 Site description and diversity at genus level for all orders of aquatic insects. Year decl., year of decline; Ind., number of individuals collected for the sampling period; Gen. Rich., number of genera per site, Chironomidae and Tipulidae were not included; Gen.Rich.R., rarefied Gen.Rich

Site	Code	Area	Altitude	X_UTM	Y_UTM	Year decl.	Ind.	Gen. Rich.	Gen. Rich.R
Chorro	CO	R.F. Fortuna, Chiriquí	1218	364,119	963,913	1996	306	33	32
Aleman	AL	R.F. Fortuna, Chiriquí	1206	365,157	967,239	1996	299	39	29
Blanco	BL	P.N. Omar Torrijos, Coclé	771	543,390	957,542	2004	147	19	–
Guabal	GU	P.N. Omar Torrijos, Coclé	684	545,100	958,785	2004	395	39	32
Capira	CP	P. N. Altos de Campana, Panamá	808	618,314	960,341	2007	321	33	30
Frijolitos	FR	Pipeline Road, Panamá	104	640,012	1,011,706	–	236	45	45
Cerro Azul	CA	P. N. Chagres, Panamá	860	676,275	1,021,286	2009	291	44	41
Chucantí	CU	R. Chucanti, Panamá	937	779,864	973,300	–	248	50	50
Total							2243	96	–

(Fujisawa & Barraclough, 2013) from the *cox1* haplotype data after all identical sequences were collapsed. To increase the robustness and the accuracy of the GMYC model, DNA sequences of outgroups and all available *cox1* data for the genera encountered were downloaded from GenBank (120 sequences, see Appendix S4 for GenBank accession numbers used) and added to the final molecular data (Fig. 2). The GMYC analysis was conducted on a maximum-likelihood phylogenetic tree obtained with RAxML 7.0.4 (Stamatakis, 2006). The tree was made ultrametric using penalized likelihood as implemented in r8s v. 1.7 (Sanderson, 2003). The GMYC analysis was conducted using the R package *Splits* (http://r-forge.r-project.org/projects/splits//) with the 'single threshold' option.

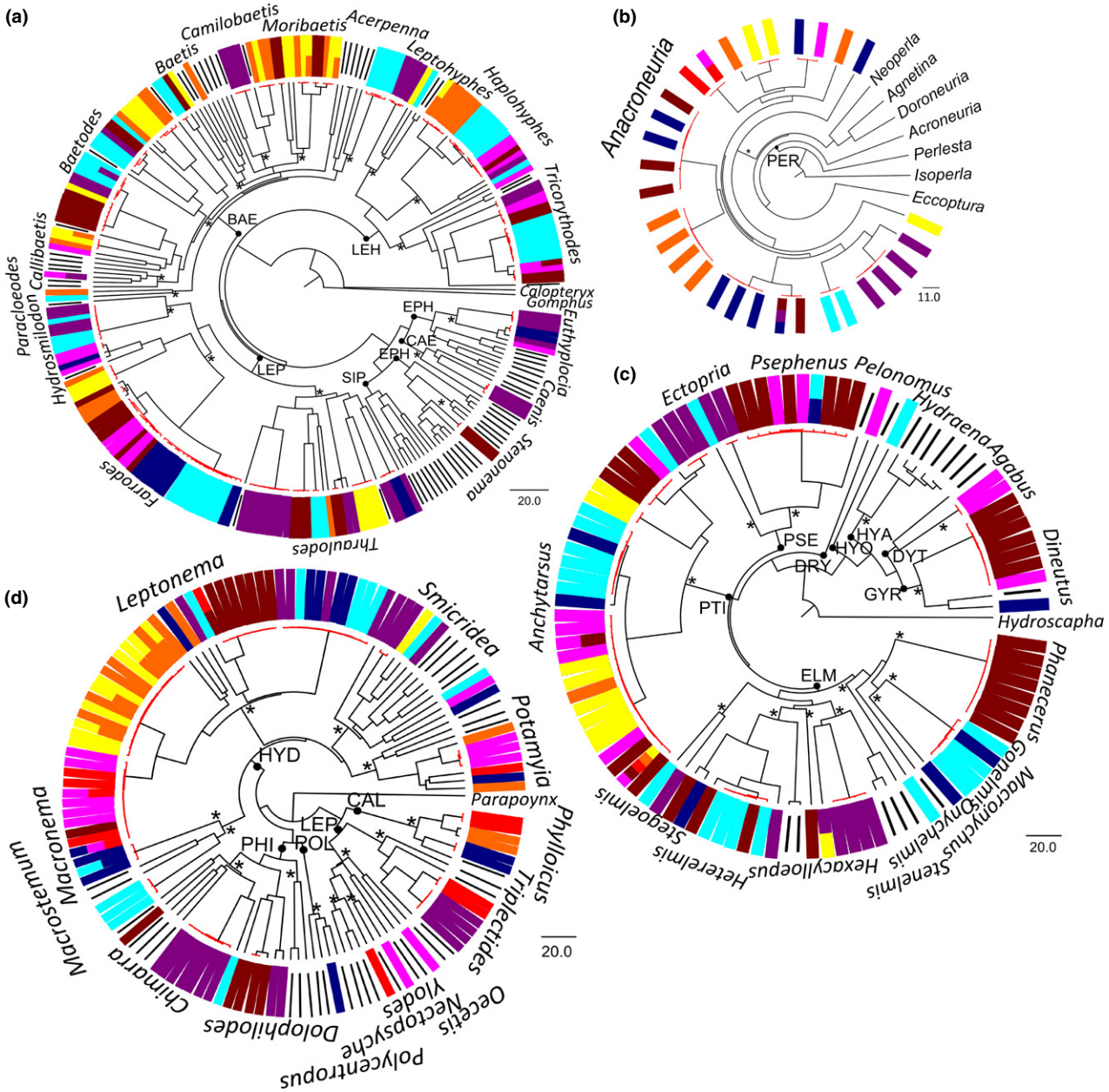

Figure 2 Maximum-likelihood phylogenetic trees of unique *cox1* haplotypes for all four lineages: (a) Ephemeroptera, (b) Plecoptera, (c) Coleoptera, (d) Trichoptera. Branches coloured in red correspond to variation within the inferred GMYC groups. Each haplotype (terminal on trees) is coloured in accordance with its locality in Fig. 1. Note that haplotypes located in more than one site are coloured with different colours. Black bars represent sequences downloaded from GenBank (see details in Appendix S4). Family names were abbreviated to the first three letters: (a) LEH, Leptohyphidae; BAE, Baetidae; LEP, Leptophlebiidae; EUT, Euthyplociidae; CAE, Caenidae; HEP, Heptageniidae; SIP, Siphlonurus. (b) PER, Perlidae. (c) HYO, Hydrophiloidea; HYA, Hydraenidae; GYR, Gyrinidae; DYT, Dytiscidae; DRY, Dryopidae; PSE, Psephenidae; PTI, Ptilodactylidae; ELM, Elmidae. (d) CAL, Calamoceratidae; LEP, Leptoceridae; HYD, Hydropsychidae; POL, Polycentropodidae; PHI, Philopotamidae.

Analysis of community diversity at genus, inter- and intraspecific levels

To compare differences in α-diversity among sites, richness was calculated at three levels: (1) individuals identified to genus for the entire community, (2) individuals sequenced and clustered into GMYC groups and (3) genetic variation within GMYC groups (number of haplotypes and nucleotide diversity). Measures of α-diversity were rarefied for the site that had the lowest sample size. Río Blanco exhibited significantly lower abundance and diversity than other sites (see below), possibly as a result of its smaller size and the recent flood, and hence was removed from comparisons among sites. The trend of variation in rarefied α-diversity along the chytrid expansion was explored using a local polynomial regression (LOESS) fitting simple models to variation of richness site-by-site to build up a function that described the deterministic part of the variation.

To assess turnover in communities along the chytrid disease path, β-diversity at the three levels and phylogenetic distance were measured for each pair of sites. Taxonomic β-diversity was measured using the rarefied matrix of genus composition and the Bray–Curtis dissimilarity index, which takes into account abundance (Anderson *et al.*, 2011). β-diversities of GMYC groups and haplotypes were calculated using the Sørensen index because of its analogy with the PhyloSor index (Bryant *et al.*, 2008), which was used for measuring the phylo-β-diversity. The PhyloSor index measures the overlapping fraction of phylogenetic branch lengths connecting haplotypes between communities (Bryant *et al.*, 2008). Values of all these similarity indexes range from 0 (complete dissimilarity) to 1 (complete similarity) for a pair of communities. Indices of β-diversity were also used to examine the community-similarity distance decay by fitting exponential decay curves using nonlinear regression (Soininen *et al.*, 2007).

Nucleotide diversity π (i.e. the average number of nucleotide differences per site between two sequences; Nei, 1987) was measured for GMYC groups that had at least 5 individuals sequenced per site. To test for differences in mean π among sites, a nonparametric Kruskal–Wallis test was performed. Parallel evolutionary processes at species and genetic levels (i.e. species and genetic diversity correlation (SGDC); Vellend, 2003) were tested by correlating separately the number of genera and GMYC groups per site with the mean π per site using Pearson's correlation. All statistical analyses were carried out using the R packages *stats* (R Development Core Team, 2011), *Vegan* (Oksanen *et al.*, 2011) and *Ape* (Paradis *et al.*, 2004).

RESULTS

At the genus level, we identified 2243 individuals belonging to 96 genera in 43 families and 9 orders (Appendix S2). The highest α-diversity was at Chucanti with 50 genera followed by Frijolitos (45) and Cerro Azul (41). These three streams were located at the easternmost side of the gradient, and among these sites, only Cerro Azul was infected with chytrid in 2009. The remaining four sites had similar α-diversity ranging between 29 and 32 (Fig. 1b, Table 1). Many of the identified genera were located across all sites; however, several genera within the families Gomphidae (Odonata), Dytiscidae, Elmidae and Hydraenidae (Coleoptera) were located only at the easternmost sites from Capira, which led to higher richness. None of the genera exclusive to the east had the same functional role as tadpoles (Gomphidae and Dytiscidae: predators; Elmidae and Hydraenidae: collectors). β-diversity at the genus level was similar along the geographical gradient and ranged from 0.53 to 0.3 and did not show a geographical distance decay of similarity ($R^2 = 0.068$; $P = 0.25$, Fig. 3a).

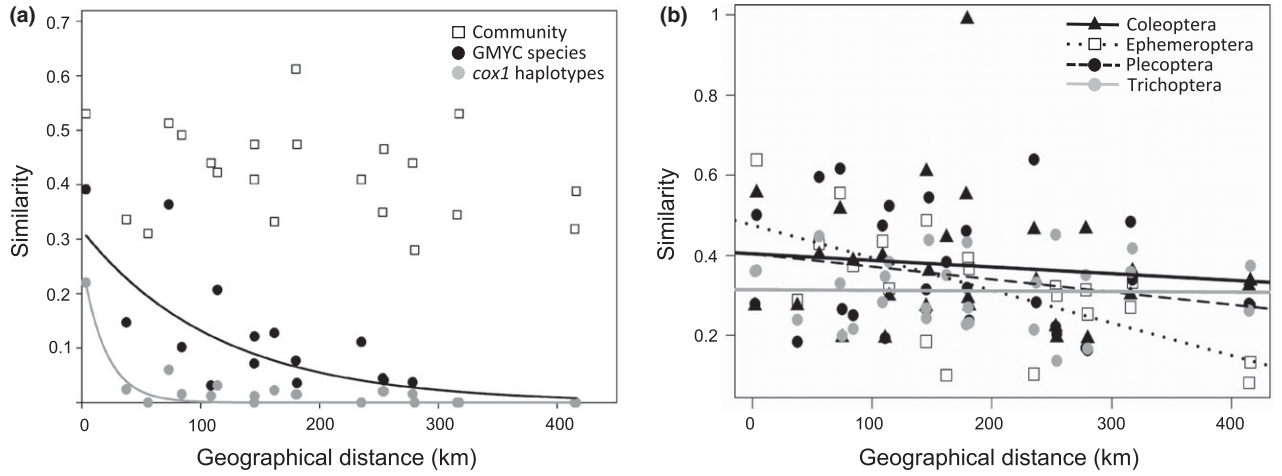

Figure 3 (a) Exponential distance decay of similarity fitted at the genus level (white squares; Bray–Curtis index; $r^2 = 0.08$, $P = 0.21$), species level (black circles; Sørensen index; $r^2 = 0.51$, $P < 0.001$) and haplotype level (grey circles; Sørensen index; $r^2 = 0.85$, $P < 0.001$). (b) Lineal geographical distance decay of similarity (PhyloSor index) fitted for Ephemeroptera (white squares; $r^2 = 0.423$, $P = 0.001$), Trichoptera (grey circles; $r^2 = 0.0003$, $P = 0.45$), Coleoptera (black triangles; $r^2 = 0.012$, $P < 0.28$) and Plecoptera (black circles and black dash line; $r^2 = 0.057$, $P = 0.09$).

DNA sequences were obtained for 749 individuals representing 426 unique *cox1* haplotypes (GenBank accession numbers KR134410-KR134835) (Table 2, Appendix S5–S6). The maximum-likelihood tree including external DNA sequences was consistent with our genus-level identifications in all cases (Fig. 2). GMYC analysis grouped these haplotypes into 154 putative species (Appendix S6), Ephemeroptera had the highest richness, followed by Trichoptera, Coleoptera and Plecoptera (Table 2). Rarefied α-diversity at species (around 20 species per site) and genetic (around 40 haplotypes per site) levels were similar among sites except for río Frijolitos and Cerro Azul, which had higher diversity. Accordingly, LOESS analysis of rarefied α-diversity at species and genetic levels did not show a trend along the chytrid disease expansion (Fig. 1C–D).

The mtDNA genealogies were characterized by high intraspecific and interspecific genetic structure at local sites (Appendix S5-S7). Almost all haplotypes were located only at one site (mono-coloured bar in Fig. 2), with the exception of 19 haplotypes that were located in two close sites, two haplotypes located in three sites, and only one haplotype (*Anchytarsus,* Coleoptera) found across the five westernmost sites. GMYC groups mirrored the structure of haplotypes, and generally, each GMYC group was located exclusively at one site. Rarely, GMYC groups were distributed in two nearby sites or, in the coleopteran genera *Psephenus*, *Anchytarsus* and *Heterelmis*, at four or five sites.

β-diversity at the species level ranged from 0.39 to 0 and, at the haplotype level, ranged from 0.22 to 0 (Fig. 3a). For both levels, the highest values of β-diversity were between the closely adjacent río Chorro and Alemán. At the species level, communities separated by more than 300 km showed complete turnover, and this distance was reduced to ~100 km at the intraspecific level. Nonlinear regressions of community-similarity distance decay fit an exponential decay for species ($r^2 = 0.51$, $P < 0.001$) and genetic ($r^2 = 0.85$, $P = 0.001$) levels. In contrast, phylo-β-diversity for Trichoptera, Coleoptera and Plecoptera ranged from 0.6 to 0.2 and did not show any geographical distance decay of similarity ($P > 0.05$), while Ephemeroptera showed a moderate linear decay ($r^2 = 0.423$, $P = 0.001$) (Fig. 3b). The largely constant values of phylo-β-diversity indicated that turnover of variability was at the terminal levels (species or haplotypes), but the same major lineages were represented at all sites and diversified locally.

Genetic diversity was measured for 60 GMYC groups that ranged from 5 to 21 individuals sequenced per site (mean = 8.3). Nucleotide diversity π ranged from 0 to 0.013, and these values were independent of location of the site along the gradient (Fig. 4a) and the number of individuals sequenced per site or order (Appendix S7). For instance, the highest values of π were found for *Farrodes* (Ephemeroptera) at Guabal ($\pi = 0.0135$) and Cerro Azul ($\pi = 0.0116$) that had 10 and 13 individuals sequenced, respectively, whereas the 9 individuals sequenced for *Farrodes* from Aleman showed $\pi = 0.00$. At Capira, the genus *Farrodes* had two GMYC groups that showed dissimilar values of $\pi = 0.0105$ and $\pi = 0.0021$ for 6 and 7 individuals sequenced, respectively. The only possible generalization concerns the genus *Anacroneuria* (Plecoptera) that showed consistently low π (0.00–0.0033). The highest values of π were found in Ephemeroptera, whereas Coleoptera and Trichoptera showed intermediate values. Cerro Azul showed the highest mean π per site; however, there were no significant differences among sites ($\chi^2 = 10.64$, $P = 0.1$). Species and genetic diversities at local sites were positively correlated ($r^2 = 0.72$, $P = 0.007$), whereas neither was significantly correlated at the genus level ($r^2 = 0.27$, $P = 0.18$) (Fig. 4b).

DISCUSSION

Our study reveals high turnover among streams simultaneously at the species and genetic levels, which together suggests that dispersal between the sampled areas has been limited over extended time-scales. We also find that α-diversity at species and genetic levels did not significantly change along the chytrid disease expansion; hence, the time since amphibian extirpation (from up to 14 years to chytrid-free sites) was not

Table 2 Number of species (GMYC) and *cox1* haplotypes (hap) for the four selected insect orders. Ind., number of individuals sequenced; NucDiv, mean nucleotide diversity π per site for the GMYC groups with at least 5 individuals sequenced

	Ephemeroptera			Trichoptera			Coleoptera			Plecoptera			Total			
Site	Ind.	GMYC	Hap	Ind.	GMYC	Hap	Ind.	GMYC	Hap	Ind.	GMYC	Hap	Ind.	GMYC	Hap	NucDiv*
CO	48	14	25	19	2	12	14	3	10	5	2	3	86	21	50	0.00254
AL	46	14	26	22	7	16	2	1	2	6	3	6	76	23	50	0.00403
BL	0	0	0	23	6	12	1	1	1	12	1	2	36	8	15	0.00077
GU	62	14	35	31	6	14	45	9	32	11	2	5	149	31	86	0.00392
CP	22	11	19	53	5	13	20	6	13	8	2	2	103	23	47	0.00318
FR	64	15	40	34	9	22	16	6	12	9	3	5	123	33	79	0.00391
CA	68	17	51	14	7	12	28	6	20	7	2	3	117	35	86	0.00755
CU	15	4	14	20	8	14	14	7	7	10	4	7	59	22	42	0.00266
Total	325	69	202	216	42	104	140	29	90	68	14	30	749	154	426	

*See Appendix S5–S6 for details.

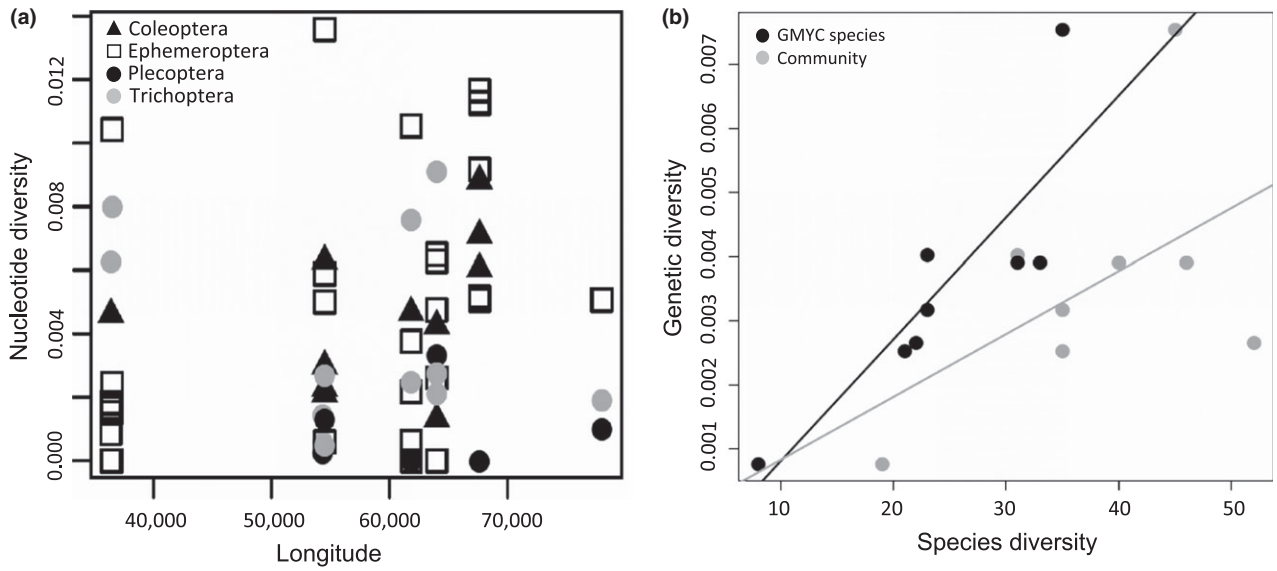

Figure 4 (a) Distribution of nucleotide diversity (π) across the longitudinal gradient for all GMYC groups that had at least 5 individuals sequenced: Ephemeroptera (white squares), Trichoptera (grey circles), Coleoptera (black triangles) and Plecoptera (black circles). (b) Species and genetic diversity correlation (SGDC). Species diversity refers the number of genera per site (grey dots, $r^2 = 0.27$, $P = 0.18$) and GMYC groups per site (black dots, $r^2 = 0.72$, $P = 0.007$). For the calculations of the local genetic diversity, the mean nucleotide diversity was established for each GMYC group at a site for those represented by at least 5 sequenced individuals (Appendix S6).

correlated with the local richness of aquatic insects. The high β-diversity and rapid exponential distance decay of similarity detected at both organizational levels are evidence against any large-scale processes sweeping through these insect communities and the homogenization through widespread species that might mirror the chytrid wave. This suggests that the effect of amphibian decline on the pattern of species and intraspecific diversity at large spatial scale is minimal.

Existing ecological studies have established changes in ecosystem function (Whiles *et al.*, 2006, 2013; Colón-Gaud *et al.*, 2009; Rugenski *et al.*, 2012) and declines of insect diversity several years after the loss of amphibians (Rantala *et al.*, 2015). These assessments were generally limited to particular local sites, hence lacking the regional perspective, and they were based on the unrefined identification to genus level only. Our phylogenetic analysis from DNA data extended across the entire region provides a novel, evolutionarily explicit framework to discriminate between different possible scenarios for the response of aquatic insect communities. The significant changes in ecosystem function following the tadpole loss at local sites (e.g. increase in community respiration and N flux to the epilithon, or decrease in N uptake length and N flux to fine particulate organic matter; Whiles *et al.*, 2013) are apparently not compensated for by other components of the ecosystem such as aquatic macroinvertebrates (Rantala *et al.*, 2015). Our results are consistent with this scenario, as the post-decline macroinvertebrate communities show very localized distributions that suggest they are not composed of recent immigrants that would occupy the vacant niche space after tadpole extirpations. With our data, we cannot exclude that species in post-decline sites might have been drawn from a local pool of *in situ* evolving lineages, which may have been restricted to pockets of the local sites, or existed at very low abundance when tadpoles were present and now increased in abundance (which we did not measure). While insects do not profit from the disappearance of tadpoles, it remains to be seen whether and how these profound ecological changes linked to shifts in basal resources associated with the loss of tadpoles will have direct (e.g. competition for food resources) or indirect (e.g. bioturbation, altering algal community structure) effects on macroinvertebrate communities (Rantala *et al.*, 2015), which may ultimately result in the disappearance of isolated aquatic macroinvertebrate communities.

The only feature unique to the chytrid-free sites was the composition of communities in the genus-level study, which detected several taxa limited to the easternmost side of the geographical gradient and α-diversity increased abruptly from Capira eastwards. The absence of these specific taxa at the westernmost sites was confirmed by species composition datasets from 2008 to 2011 from Chorro and Guabal (Colón-Gaud *et al.*, 2010a,b; Rugenski, 2013). Given that these taxa use resources different from tadpoles and are in different functional feeding groups, and furthermore, Cerro Azul experienced declines two years before our study but still harboured these groups, it is not likely that the lower richness at the western side was associated with amphibian extirpation. Instead, the pattern of α-diversity may be explained by the historical disjunction of the North and South American fauna around the Isthmus of Panama.

The limited sampling effort may also affect the estimates of β-diversity, given our snapshot sampling over a 1-day

period that may be incomplete and therefore artificially increase the inferred species turnover. However, it is unlikely that a pattern of high endemicity would be created simply from undersampling, as it would favour the detection of widespread and common species, which we do not find. At the genus level, numerous taxa were recovered consistently among sites and their composition was comparable with other studies in this region (Colón-Gaud *et al.*, 2009, 2010a, b), and in the case of the locality at Chucanti, our repeated sampling over 3 years revealed a very similar community composition (Rugenski, 2013). This suggests that our sample is a good representation of the local biota, and therefore, the turnover within genera at the species and genetic level is likely to be an adequate reflection of the true change among sites. The detected turnover also may be a consequence of other factors such as seasonality and patchiness of macroinvertebrate communities associated with flow disturbances (e.g. the low diversity found at río Blanco was presumably affected by a recent flooding event) (Lake, 2000; Ríos-Touma *et al.*, 2011). However, even with limited sampling, the rarefied species and genetic diversities were remarkably similar at the 8 study sites, with the greatest proportion of species belonging to Ephemeroptera, approximately half as many species of Coleoptera and Trichoptera, and only a few species to Plecoptera. The largely uniform number of mayfly species, all of which are considered to be part of the grazer and collector guild, is a strong indication of their ubiquity and ecological significance, while at the species and genetic levels, there is strong turnover among sites.

The high interspecific and intraspecific turnover among sites supports the notion of long-term habitat stability in tropical highlands that resulted in limited dispersion and an increase in genetic differentiation among mountain sites (Cadena *et al.*, 2011; García-López *et al.*, 2013). Similarly, high community turnover at the levels of haplotypes, species and higher clades was found in terrestrial scarab beetles across several Neotropical highland forests, which was attributed to the long-term ecological stability of local populations that may track suitable habitats to accommodate climatic changes without long-distance dispersal (García-López *et al.*, 2013). The aquatic insects examined here showed even higher genetic structure among communities at a similar geographical scale, possibly because dispersal among streams, especially among headwaters, is even more limited than among the largely contiguous terrestrial rain forest habitats inhabited by scarabs (Bohonak & Jenkins, 2003; Finn *et al.*, 2011). Long-term habitat stability may be surprising given the undoubted effects of Pleistocene climatic fluctuations on species movements, especially in the Northern Hemisphere (Hewitt, 2000), but in the Tropics, the resulting shifts in habitat distributions are comparatively small and in case of highland habitats can be tracked by populations up and down mountain slopes with minimal movements (Rull, 2005; Graham *et al.*, 2006; Múrria & Hughes, 2008). Population persistence in unstable habitats requires high dispersal rate to ensure survival, and accordingly, species tend to exhibit lower genetic endemism and larger ranges than those in long-term stable habitats (Ribera *et al.*, 2003; Papadopoulou *et al.*, 2009). Habitat stability would amplify patterns of high β-diversity over geographical distance and the size of regional species pools in tropical rain forests (Graham *et al.*, 2006; Carnaval *et al.*, 2009) and also preserve biogeographical signatures at higher hierarchical levels.

Nucleotide diversity π was highly variable among and within lineages (except Plecoptera that had the lowest π) and among sites (Fig. S7). Distribution of π in mtDNA among species has been correlated with differences in relevant traits such as long-lived or low fecundity (Romiguier *et al.*, 2014), recurrent adaptive evolution in mtDNA genes (Bazin *et al.*, 2006) or geographical range and dispersal abilities of species (Fujisawa *et al.*, 2015). In our study, the disparity of π within and among lineages is not predicted from differences in geographical ranges, dispersal abilities and the correlated population size (Fujisawa *et al.*, 2015) because almost all species have narrow, isolated geographical distributions. The high disparity of π shown by closely related species that share functional traits and inhabit the same isolated stream (e.g. the two species *Farrodes* at Capira) may refute the hypothesis of differences in adaptive traits and the explanations based on habitat stability because these species presumably have similar population history associated with local abiotic factors such as flood events or biogeography. It is unlikely that these observations are explained by sampling artefacts, as the number of individuals sequenced per GMYC groups and π were not correlated (Fig. S7). The high disparity of π remains unexplained but may be the results of contingent factors affecting the long-term effective population size of each species, as species abundance or frequent bottlenecks affect comparatively small populations in a fluctuating environment (Fujisawa *et al.*, 2015), or may reflect the time since the last occurrence of a selective sweep for each species (Bazin *et al.*, 2006). Regardless of the determinant of the detected high variability of π, there was no significant shift among the pre- and post-decline sites, indicating that disparity in π is not an initial indication of declining populations following amphibian extirpation.

We did, however, find a correlation between number of GMYC groups and intraspecific diversity of the local species present at these sites (confirming the SGDC of Vellend, 2003). This correlation is usually explained by the action of uniform processes that generate patterns at both hierarchical levels, such as dispersal among sites that affect the total species counts as the product of immigration–extinction dynamics and affect genetic diversity as the result of genetic drift. The detected high turnover among sites at both hierarchical levels suggests that indeed extended isolation of local assemblages (i.e. a neutral process) is the main mechanism causing the observed macroecological pattern, rather than selection for particular species traits (e.g. those relevant to niches vacated by amphibians). This is further support for an evolutionary history of isolation and restricted migration that limits species ranges and the potential responses of aquatic

insects to amphibian declines. Just as the loss of endemic amphibian assemblages resulted in numerous species extinctions in the wake of chytrid infection (Crawford *et al.*, 2010), each Neotropical highland stream harbours a unique diversity of aquatic insects that is highly erodible and irreplaceable because of long-term isolation. Local extinctions caused by anthropogenic disturbances, for example from agriculture, logging, mining and wind farms, are not likely to be compensated for by recolonization because of limited dispersion among mountain ranges.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Geographical distances among sites.

Appendix S2 Taxonomical composition per site.

Appendix S3 Methods of molecular analysis.

Appendix S4 *Cox1* sequences downloaded from GenBank.

Appendix S5 Individual identification (BMNH number) of all *cox1* sequenced.

Appendix S6 Taxonomical information of each GMYC group and haplotype, and their location.

Appendix S7 Table S7: Number of individuals sequenced, Nucleotide Diversity (π) and genetic diversity (GD) per site; Figure S7: Relationship between number of individuals sequenced and Nucleotide Diversity (π).

BIOSKETCHES

Cesc Múrria is a postdoctoral researcher at Université de Sherbrooke (Quebec) where he examines macroecological patterns of diversity at the population and community level and their correlation.

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Author contributions: All authors conceived the study, C.M. and A.T.R. did the field work, taxonomic identifications and molecular analysis, C.M. performed all statistical and phylogenetic analysis, and figures. All authors discussed the results, C.M. and A.P.V. wrote a draft of the paper, and all authors contributed substantially to the revision.

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