

## The imperiled fish fauna in the Nicaragua Canal zone

Andreas Härer\*<sup>1</sup>, Julián Torres Dowdall<sup>1,2</sup> & Axel Meyer<sup>1</sup>

<sup>1</sup>Zoology and Evolutionary Biology, Department of Biology, University of Konstanz, Germany

<sup>2</sup>Zukunftskolleg, University of Konstanz, Konstanz, Germany

\*[axel.meyer@uni-konstanz.de](mailto:axel.meyer@uni-konstanz.de)

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### Abstract

Large-scale infrastructure projects commonly have strong effects on the environment. Thus, it is of highest importance to evaluate possible impacts on biodiversity and take measures to reduce these. The planned construction of the Nicaragua Canal will irreversibly alter the

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aquatic environment of Nicaragua in many ways. Two distinct drainage basins (San Juan and Punta Gorda) will be connected, fostering biotic homogenization due to establishment of non-native species, extirpation of local species and alteration of numerous ecosystems.

Considering the far-reaching impact of this project on Nicaragua's environment, too few studies on biodiversity have been performed in affected areas. This limits the ability to make solid environmental impact assessments. Here, we explore the geographic distribution of taxonomic and genetic diversity from representative freshwater fish species across the Nicaragua Canal Zone. We show that freshwater fish faunas likely differ substantially between drainage basins (Jaccard similarity = 0.33). Further, most populations from distinct drainage basins are highly differentiated based on mitochondrial *cytb*. Removing the geographic barrier between these basins will promote genetic homogenization and the loss of unique diversity. Moreover, we found species in areas where they were not known to exist and provide insights into the distribution of an undescribed, highly distinct clade of livebearing fish (*Poecilia*). Our results indicate that the Nicaragua Canal likely will have strong impacts on Nicaragua's freshwater biodiversity. However, knowledge about the extent of these impacts remains scarce, highlighting the need for more thorough investigations to inform more cogently on the status quo before the environment is altered irreversibly.

## Introduction

Human induced changes to the environment are now one of the major driving forces for evolutionary change (Palumbi 2001; Davies and Davies 2010). Alterations commonly have drastic effects on the integrity of natural populations and threaten the maintenance of

biodiversity (Carroll et al. 2014). This applies specifically if environmental changes occur too quickly for species to adapt to, as currently seen on a large scale in what has been called the Earth's sixth mass extinction (Barnosky et al. 2011). Particularly, biodiversity in freshwater environments is far more threatened than in terrestrial environments (Ricciardi and Rasmussen 1999; Sala et al. 2000). This is alarming because some freshwater ecosystems have exceptionally high species richness. Although freshwater habitats cover only 0.8% of Earth's surface (Gleick 1996), they harbor approximately 6% of all species described (Hawksworth 1995). Major threats are overexploitation, water pollution, water flow modification, destruction or degradation of habitat and invasion by exotic species (Dudgeon et al. 2006). The planned construction of the Nicaragua Canal might entail all of these major threats and adversely affect the aquatic fauna of Nicaragua (Meyer and Huete-Pérez 2014; Brett et al. 2015; Huete-Pérez et al. 2015).

In July 2013, the Hong Kong Nicaragua Canal Development Investment Co. Group of China (HKND) was granted the concession to build an interoceanic shipping canal, called the Nicaragua Canal, across the Central American isthmus through the country of Nicaragua connecting the Caribbean Sea and the Pacific Ocean (ERM 2014). The concession grants HKND rights to use any water body, including Lake Nicaragua, the largest freshwater lake in Central America (and 19<sup>th</sup> largest worldwide) with 8,400 km<sup>2</sup> of surface area (Schwoerbel 2013). Yet the lake's water volume is only 80 km<sup>3</sup> since it is rather shallow with an average depth of 15 meters and a maximum depth of 50 meters (Barluenga and Meyer 2010). The fish fauna of the lake is well characterized (Koenig et al. 1976; Barluenga and Meyer 2010) with more than 40 species described (Astorqui 1976; Bussing 1976), but knowledge about the fish fauna in the remote eastern regions of Nicaragua is relatively scarce. The prospective construction and operation of the canal are of major environmental concern, threatening the

fauna of Lake Nicaragua, adjacent rivers and several natural reserves (Huete-Pérez et al. 2013; Meyer and Huete-Pérez 2014). Moreover, freshwater fishes of Lake Nicaragua are of socioeconomic importance since fishing constitutes the livelihood of many Nicaraguans (Davies 1976; Huete-Pérez et al. 2013).

The canal will start in the Caribbean Sea at Río Punta Gorda, follow Río Tule, cross Lake Nicaragua, head westward via Río Las Lajas and enter the Pacific Ocean close to the small village Brito (Fig. 1, solid line). Hence, the Punta Gorda and San Juan drainage basins will be connected (ERM 2014), promoting alterations in species' distribution and ecosystem structure. Multiple routes had been proposed, two of the rejected ones included the Escondido basin (Fig. 1, dashed lines). The final decision on the route via Río Punta Gorda, which was released in the course of the environmental and social impact assessment (ESIA) report in late 2015, was based mainly on technical and economic grounds (<http://hknd-group.com/portal.php?mod=view&aid=293>). However, the data presented in the ESIA report is, according to a panel of Nicaraguan and international scientists, not sufficient in many respects to properly evaluate effects of the Nicaragua Canal on Nicaraguan biodiversity (Huete-Pérez et al. submitted). Thus, there have been appeals to conduct independent studies on a broad taxonomic scale for different ecosystems in affected areas (Huete-Pérez et al. 2015).

Construction of the canal will break a geographic barrier between two distinct drainage basins, promoting homogenization of freshwater biotas across the affected regions. Biotic homogenization describes an increase in genetic, taxonomic and/or functional similarity of previously distinct biotas (Olden 2006) due to invasion of non-native species, extirpation of native species and habitat alteration (Rahel 2002). This has been observed for fish faunas (Rahel 2000; Vitule et al. 2012) and is in most cases associated with human actions (e.g.

canal building, ballast water discharge and introduction of non-native species for recreational use), which commonly leads to a decrease in biodiversity (McKinney and Lockwood 1999). Additionally, successful establishment of invasive species is mainly explained by human activities and consequential disturbance of the environment (Leprieur et al. 2008). One specific example can be seen in the Mediterranean Sea where species invasions occur via the Suez Canal, causing adverse ecological and social consequences (Galil et al. 2015).

The aquatic landscape of Nicaragua is characterized by a number of rivers and lakes. It has been suggested that its fish fauna is similar across distinct drainage basins of Nicaragua (Bussing 1976), indicating low levels of beta diversity. This has not been adequately tested, and, to this end, we surveyed geographic distribution and population structure of representatives of three teleost families (Supporting Information) across three basins: Escondido, Punta Gorda & San Juan (Fig. 1). Remarkably, the latter two will be part of the Nicaragua Canal Zone. The studied species belong to the most species-rich and widespread teleost families in Central America with 31 (Cichlidae), 7 (Characidae) and 8 (Poeciliidae) described species in Nicaragua (Froese 2014). Compared to other countries in Central America, the number of poeciliid and characid species appears relatively small (Supporting Information). For example, only one poeciliid species (*P. mexicana*) has been described for Nicaragua, compared to six in Guatemala, three in Costa Rica and six in Panama. This, in turn, indicates low species diversity across Nicaragua as previously depicted (Bussing 1976). On the other hand, recent biogeographic investigations restricted to the Pacific slope found several species of the *Poecilia sphenops* and *Poecilia mexicana* species complexes (Alda et al. 2013; Bagley et al. 2015). Moreover, Bagley et al. (2015) detected a third genetic cluster, referred to as *Poecilia* sp. “Tipitapa”, that fell outside the *P. sphenops* and *P. mexicana* species complexes. This suggests that there is an apparent lack of knowledge about the geographic

distribution of aquatic biodiversity in Nicaragua and how it will be affected by the Nicaragua Canal. Therefore, we aimed at shedding more light on species' distribution and patterns of population structure across three distinct drainage basins, two of which will be directly affected by the canal. Our approach was to cover a broad taxonomic range of freshwater fishes by including species from three teleost families. Additionally, we chose our study species to differ in maximum size and trophic level as well as reproductive features (Supporting Information). Our results show that species assemblages are quite dissimilar among drainage basins and that populations from distinct basins differ significantly based on mitochondrial DNA. These results contribute toward a better understanding of the effects of artificially connecting distinct basins on the freshwater fauna of Nicaragua and will help to evaluate the route via Punta Gorda and Lake Nicaragua from a conservation standpoint.

## Methods

### Study area & sample collection

The species included in this study are two poeciliids (*Poecilia* spp. "Cluster 2", *Poecilia* spp. "Cluster4"), two cichlids (*Amatitlania siquia*, *Hypsophrys nematopus*) and two characids (*Brycon guatemalensis*, *Roeboides bouchellei*). Study species were chosen based on their geographic distribution across Nicaragua and on ecological characteristics (maximum size, trophic level & reproductive aspects, Supporting Information). Information about species' distribution was obtained from the literature and based on empirical findings in the field. We aimed to collect 25 specimens per species at each location. However, this was not achieved for all populations. Samples for interdrainage comparisons were from single sample sites for the Punta Gorda and Escondido basins and six sites from the San Juan basin (Supporting Information). We collected fishes with seines (6 m length; 1 cm mesh size), gill nets (mesh sizes between 1 and 4 cm) and cast nets during a field trip to Nicaragua in August 2014. We

labeled each specimen with a specified identification number, took standardized pictures and stored muscle tissue/fin clips in ethanol for later DNA extraction. We identified species in the field by visual examination and subsequently by *cytochrome b* (*cytb*) sequencing.

#### DNA extraction & amplification, Sanger sequencing & Data analysis

Mitochondrial *cytb* was PCR amplified by DreamTaq DNA Polymerase (Life Technologies, Carlsbad, USA). The size of the amplified PCR products was approximately 1200 basepairs (bp), primers are listed in the Supporting Information. Primer annealing temperatures were 54°C (*Poecilia* spp.) or 58°C (*A. siquia*, *H. nematopus*, *B. guatemalensis*, *R. bouchellei*). We sequenced purified templates on an ABI 3130xl Genetic Analyzer (Life Technologies). The quality of sequencing reads was manually checked, reads were trimmed and assembled with SeqMan Pro (DNASTAR Lasergene). The trimmed DNA sequences used for all analyses constituted total lengths of 1086 bp (*Poecilia* spp.), 1044 bp (*A. siquia*), 1065 bp (*H. nematopus*), 973 bp (*B. guatemalensis*) and 991 bp (*R. bouchellei*). *cytb* sequences for phylogenetic analyses were obtained from NCBI GenBank (Supporting Information). We aligned *cytb* sequences and built Maximum Likelihood phylogenetic trees with SeaView Version 4 (Gouy et al. 2010) using the GTR model of nucleotide substitution for phylogenetic tree reconstruction. We created Median Joining haplotype networks with PopART version 1.7 (<http://popart.otago.ac.nz>). Hierarchical clustering of genetic diversity was investigated using AMOVA in Arlequin v3.5.2.1 (Excoffier et al. 2005). Measures of population differentiation ( $F_{ST}$ ), haplotype diversity and nucleotide diversity were calculated with DnaSPv5 (Librado and Rozas 2009) and Arlequin v3.5.2.1.

## Results

Geographic distribution of the freshwater fauna in the Nicaragua Canal Zone

Based on our field data and data collected during the ESIA (ERM 2015), at least 27 species occur in both affected drainage basins (compared to 24 species exclusive to Punta Gorda and 31 species exclusive to San Juan), representing a Jaccard similarity coefficient of 0.33 (Supporting Information). We found two non-native species recently introduced to Lake Nicaragua, the Nile Tilapia (*Oreochromis niloticus*) and the devil fish (*Hypostomus panamensis*), to be widespread across the San Juan basin (unpublished data). Among our target species, we found the characids *R. bouchellei* and *B. guatemalensis* and the cichlid *A. siquia* in all three drainage basins. On the other hand, the cichlid *H. nematopus* was found in multiple locations within the San Juan basin. For *Poecilia*, we assigned samples to known species based on *cytb* sequences (Fig. 2, Supporting Information) according to a recent phylogeny (Bagley et al. 2015). *Poecilia* formed four genetic clusters that grouped with *Poecilia gillii* (*P. sp.* “Cluster 1”), *Poecilia mexicana* “Clade 8m” (*P. sp.* “Cluster 2”), *Poecilia mexicana* “Clade 8a” (*P. sp.* “Cluster 3”) and *Poecilia sp.* “Tipitapa” (*P. sp.* “Cluster 4”) (Fig. 2, Supporting Information). *P. sp.* “Cluster 1” is restricted to the Punta Gorda basin, *P. sp.* “Cluster 3” and *P. sp.* “Cluster 4” to the San Juan basin. In contrast, *P. sp.* “Cluster 2” occurs in all three basins. In the small creek Río Caño Chiquito, a tributary to Río Punta Gorda, two genetic clusters, *P. gillii* (Cluster 1) and *P. mexicana* “Clade 8m” (Cluster 2), co-occur. In Río Las Lajas, three different clusters co-occur, *P. sp.* “Cluster 2”, *P. sp.* “Cluster 3” and *P. sp.* “Cluster 4”. The still undescribed *P. sp.* “Tipitapa” (Cluster 4) from Río Tipitapa at the northern shore of Lake Nicaragua (Bagley et al. 2015) appears to be widespread across the San Juan drainage basin and shows high levels of haplotype diversity (Fig. 3f and Table 2).

Hierarchical analysis of genetic diversity

Hierarchical clustering analyses revealed that a large proportion of genetic diversity is explained by drainage basin origin in most species (Fig. 3 and Table 1). For the cichlid *A. siquia* and the poeciliid *P. sp.* “Cluster 2” (*P. mexicana*) a highly significant proportion of genetic diversity, 71% and 89% respectively, is attributable to drainage basin origin (Table 1). Notably, the *A. siquia* population from Punta Gorda is strongly differentiated from those at Escondido and San Juan, which are less divergent (Fig. 3a and Supporting Information). Moreover, haplotype diversity varies strongly across drainage basins and both haplotype and nucleotide diversity are much lower in Punta Gorda (Supporting Information). In contrast, genetic diversity is less variable in the other species (Table 2). Differences in drainage basin explain a high (63%), but only marginally significant ( $p = 0.065$ ), proportion of genetic diversity for the characid *B. guatemalensis*. Populations from Escondido and Punta Gorda possess a single shared haplotype, whereas haplotype diversity is higher in populations from San Juan (Fig. 3b and Supporting Information). On the other hand, for *R. bouchellei*, the crystal tetra, 96% of diversity is attributable to variation within populations, compared to 1% among populations within the same basin and 3% among populations from distinct basins (Table 1). This pattern of low differentiation among populations from the same drainage basins also applies to the poeciliid *P. sp.* “Cluster 4” (*P. sp.* “Tipitapa”) indicating low levels of genetic differentiation within the San Juan basin. However, the cichlid *H. nematopus*, found at three locations within the San Juan basin, shows higher levels of differentiation among populations (Table 1).

To summarize, populations within the same basin show almost no genetic differentiation whereas comparisons across basins exhibit higher differentiation; the characid *B. guatemalensis* (no differentiation between Punta Gorda and Escondido) and the cichlid *H. nematopus* (high differentiation between Río Las Lajas compared to other San Juan

populations) being exceptions to this pattern. These results indicate that populations from distinct drainage basins might represent independent evolutionary significant units (ESUs, sensu Fraser and Bernatchez 2001) as there is apparently only little gene flow among drainage basins.

## Discussion

Nicaragua is part of the Mesoamerican biodiversity hotspot, showing particularly high levels of vertebrate endemism (Myers et al. 2000). Moreover, the Neotropics harbor a large proportion of freshwater fish's global taxonomic and functional diversity (Toussaint et al. 2016). Functional diversity plays a major role in maintaining ecosystem functioning and services relevant to humans (Diaz and Cabido 2001). The Nicaragua Canal will have strong impacts on the environment and it has been claimed that it poses a threat to Nicaragua's biodiversity (Meyer and Huete-Pérez 2014). One major concern is that connecting the Punta Gorda and San Juan drainage basins will substantially affect the freshwater fauna in multiple ways. Based on the "Freshwater Ecoregions of the World" (FEOW) map, the San Juan and Punta Gorda basins are grouped together (Ecoregion 205) in the San Juan ecoregion (Abell et al. 2008). Ecoregions are defined as areas harboring distinct assemblages of species communities and are viewed as conservation units. Hence, it is assumed that the two drainage basins have more or less similar freshwater faunas. Yet, data from the ESIA reveals a Jaccard similarity coefficient of 0.33 for freshwater fishes (ERM 2015), suggesting that both basins have only one third of species in common (Supporting Information). After the canal has been built, species assemblages are likely to become more similar due to biotic homogenization (Olden 2006). Yet the scope of these effects remains dubious since there is still a lack of knowledge about the distribution of taxonomic and genetic diversity of freshwater fishes in

affected areas. Here, we discuss several threats to freshwater biodiversity associated with the Nicaragua Canal.

#### Habitat alteration & destruction

Water pollution, destruction or degradation of habitat and water flow modification are among the major threats to freshwater biodiversity (Dudgeon et al. 2006). The canal will likely lead to increased water pollution, e.g. by construction works, oil spills or ship traffic itself and it will cause significant alteration of habitat along its route. Multiple rivers in the Canal Zone, representing unique and distinct ecosystems, will be altered in their water flow regime and turned into more lake-like environments. This homogenization of habitats and connection of distinct drainage basins can increase the likelihood of biotic homogenization caused by widespread, generalist species replacing locally adapted species in affected areas (Rahel 2000; Vitule et al. 2012). Further, destruction of habitats along the route will affect populations of numerous species, possibly posing a risk to biodiversity in these areas.

For example, three different genetic clusters of *Poecilia* co-occur in Río Las Lajas.

According to a recent phylogeny on fishes of the genus *Poecilia* in Central America (Bagley et al. 2015), these refer to *P. mexicana* “Clade 8a”, *P. mexicana* “Clade 8m” and *P. sp.* “Tipitapa” (Fig. 2, Supporting Information). This small river on the eastern shore of Lake Nicaragua will be part of the canal and therefore disappear in its current form. Notably, Río Las Lajas harbors elevated nucleotide and haplotype diversity for most of the species investigated (Table 2, Supporting Information). Dredging of the river during construction works will irreversibly destroy this habitat, putting the persistence of these populations at risk. In the Punta Gorda basin, the *Poecilia* sp. “Cluster 1” from Río Caño Chiquito shows only one *cytb* haplotype. This clade might be highly threatened by rapid environmental changes since reduced genetic diversity negatively affects population’s adaptive potential to

environmental challenges (Sgro et al. 2011) and decreases survival chances under stress conditions (Frankham 2005).

### Invasion by non-native species

The canal will facilitate colonization of novel habitats by non-native species, as seen in similar scenarios (Kolar and Lodge 2000; Holeck et al. 2004). This might happen either by species' range expansion after connecting the two drainage basins or by introduction of new species via ballast water discharge. Studies from Brazil have shown that establishment of non-native species has detrimental effects on ecosystems, e.g. eutrophication, extirpation of native species and biotic homogenization (Figueredo and Giani 2005; Menezes et al. 2012; Vitule et al. 2012). Altered environments, as it is expected for the Punta Gorda region after construction of the canal, face a higher risk of invasive species becoming established (Moyle and Light 1996; Johnson et al. 2008). Moreover, the expected homogenization of the environment is predicted to further increase the speed of species invasions (Garcia-Ramos and Rodriguez 2002). Additionally, changes in abiotic factors, especially water flow regime, might increase the risk of successful establishment of invasive species (Baltz and Moyle 1993). In Californian rivers, non-native species tend to replace native species after reduction of water flow velocity and variability caused by the construction of a dam (Marchetti and Moyle 2001). Another determinant of successful establishment of introduced species is the propagule pressure (Williamson 1996; Lockwood et al. 2009) which reduces effects of demographic stochasticity. Propagule pressure is determined by propagule size (number of individuals) and propagule number (number of release events) (Simberloff 2009). Since the canal will form a permanent connection between distinct drainage basins, it can be assumed that propagule pressure will be high, facilitating colonization and establishment.

Our study shows that even within the well-studied genus *Poecilia*, too little is known about the geographic distribution of species in Nicaragua, specifically in the Punta Gorda region. For example, out of the two genetic clusters found in this region, only one was previously described for Nicaragua. The other cluster groups with a clade of *P. gillii* from Costa Rica (Clade 5b in Bagley et al. 2015). This cluster was not found in the San Juan and Escondido drainage basins. On the other hand, a recently discovered undescribed species (*P. sp.* "Tipitapa"; Bagley et al. 2015) is known only from the San Juan basin. Our data shows that *P. sp.* "Tipitapa" is widespread across the San Juan basin and occurs at least in Lake Nicaragua (El Tule and Isletas), Lake Managua and three adjacent rivers; Río San Juan, Río Las Lajas and Río Oyate, but was not detected in the Punta Gorda basin. An artificial connection of the two basins will enhance the potential for range expansions and contact between previously allopatric species. This, in turn, might cause extinctions due to competitive exclusion between ecologically similar species (Hardin 1960). Since two thirds of freshwater fish species in the San Juan and Punta Gorda basins are restricted to one or the other of the two basins, removing the geographic barrier could cause strong shifts in ecological dynamics for the better part of Nicaragua's freshwater fauna.

Moreover, several non-native species have been introduced to Lake Nicaragua; most notably, three species of Tilapia (*Oreochromis niloticus*, *O. mossambicus* and *O. aureus*) and the devil fish (*Hypostomus panamensis*). Tilapia has been listed as one of the most invasive species in the world (Lowe et al. 2000) and its occurrence in Lake Nicaragua is correlated with a decrease in abundance of native cichlid fishes (McKaye et al. 1995). Likewise, based on accounts of local fishermen, the occurrence of the devil fish is accompanied by a decrease in abundance of native species (personal communication), especially of the commercially important Guapote (*Parachromis managuensis*). However, the ecological impacts of this

species have not been formally investigated. All of the aforementioned species appear to be highly invasive in Nicaragua and have spread across the whole San Juan drainage basin within a few years (McKaye et al. 1995, personal observation) but have not been reported in the Punta Gorda basin to date. Hence, the construction of the canal will facilitate colonization of new habitats by removing biogeographic barriers (Rahel 2002), most likely posing a threat to the local fauna.

#### Hybridization between genetically distinct populations

Contact between genetically distinct populations could lead to (intra- and interspecific) hybridization and cause genetic homogenization of previously differentiated gene pools (Olden et al. 2004). This would become evident as a decrease in allelic composition and an increased similarity in allelic frequencies and possibly compromising local adaptation (Storfer 1999). We detected high levels of differentiation among drainage basins for taxonomically and ecologically diverse species. Assuming that our results are generally applicable across a broad taxonomic range, populations from distinct basins might represent evolutionary significant units (ESUs, *sensu* Fraser and Bernatchez 2001). However, the extent of gene flow among populations would have to be further investigated. On the other hand, populations within the same drainage basin show no significant genetic differentiation (Fig. 3d – f), even across large geographic distances of up to 180 km (e.g. Lake Managua – El Tule or Río Las Lajas – Río San Juan). These results suggest that connecting the two basins will most likely be accompanied by genetic homogenization for multiple species in the Nicaragua Canal Zone. At least 27 fish species occur in both basins (Supporting Information), emphasizing the large scale on which unique genetic diversity might be lost across Nicaragua’s freshwater fauna.

#### Political issues concerning the Nicaragua Canal

Unfortunately, environmental concerns are commonly not addressed properly in the course of political decision making processes. There are many cases of artificial waterways connecting distinct drainage basins in which associated risks for biodiversity were not taken into account (Zhan et al. 2015). For instance, the Brazilian government decided to transfer water between isolated river basins to combat the effects of a severe drought without accounting for environmental consequences (Vitule et al. 2015). Nicaraguan freshwater biodiversity will face serious threats once construction of the canal begins. The concession to build and operate the canal was given to the infrastructure development firm (HKND) by the Nicaraguan government without competitive bidding. Moreover, the environmental and social impact assessment (ESIA) was commissioned by HKND to the environmental consultancy ERM after the concession was already assigned. In general, the ESIA lacks comprehensive data in many areas, e.g. stratigraphy of the lake, seismic risk assessment or water quality concerns. These issues have been pointed out by a panel of Nicaraguan and international scientists in the framework of a workshop in Managua organised by the Academy of Sciences of Nicaragua in November 2015 (Huete-Pérez et al. submitted). Additionally, the ESIA does not properly take into account effects of the canal on distinct localized faunas in the two affected drainage basins and the detrimental effects of biotic homogenization for these regions. High risks for biodiversity in multiple ecosystems are associated with this project and the expected impacts for Nicaragua's freshwater biodiversity are profound and irreversible. Thus, a sustainable management concept should be framed. Still, the associated effects on biodiversity remain unclear. Hence, the precautionary principle, stating that the burden of proof that an action is not harmful to the environment is upon the one taking action, should be applied. This concept has already been endorsed in the UN World Charter for Nature (United Nations 1982) and has been advocated to become international common practice, especially in the context of comparable mega projects.

However, its efficacy to counteract the current loss of biodiversity and the proper incorporation of the precautionary principle into political decision making remain controversial (Myers 1993; Cooney 2004; Kanongdate et al. 2012).

#### Concluding remarks

We investigated the geographic distribution and population structure of representative species from three well-studied families of freshwater fishes. Our results clearly show that populations from distinct drainage basins are genetically highly differentiated and that species assemblages differ substantially between basins, highlighting the risk of genetic homogenization and biotic invasion. Breaking down the geographic barrier between the Punta Gorda and San Juan basins, as would be done through the construction of the canal, is worrisome from a conservation standpoint and might have drastic consequences for Nicaragua's freshwater fauna, putatively leading to loss of unique genetic diversity on a large scale. Evidently, too little is known about biodiversity, biogeography of species and the phylogeographic distribution of genetic variation within the Nicaragua Canal Zone, which will surely imperil the status quo ante. Due to this lack of knowledge, it is currently impossible to adequately predict consequences of the canal on Nicaragua's biodiversity. Therefore, it is of great urgency to determine which endemic species or genetically unique populations might be threatened by the construction. Hence, we call for more thorough and independent investigations of biodiversity in the ecosystems affected by the Nicaragua Canal. Otherwise, many more, putatively still unknown, species might be threatened by extinction.

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### Supporting Information

Supplementary tables (Appendix 1) and supplementary figures (Appendix 2) are available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author.

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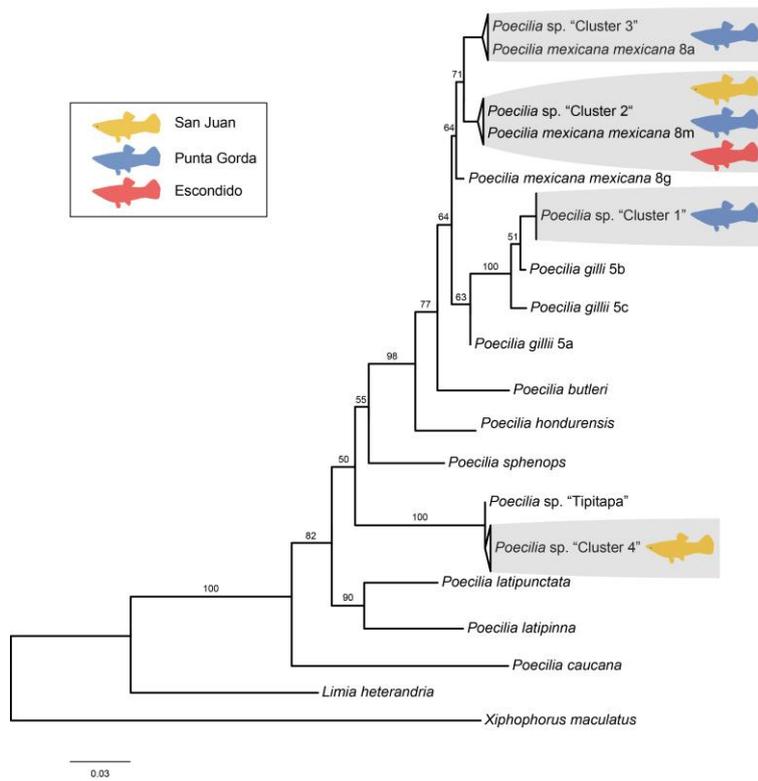
**Table 1.** Hierarchical analyses of genetic diversity in six fish species from the Nicaragua Canal Zone. For the first four species populations from all three drainage basins (San Juan, Punta Gorda and Escondio) are compared. Additionally, the last four species are represented by multiple populations within the same basin. Significance levels: † < 0.1, \* < 0.05, \*\* < 0.01, \*\*\* < 0.001.

Species	Percentage of diversity attributable to variation			
	Among			
	Within populations	populations within basin	Within basins	Among basins
<i>A. siquia</i>	-	-	29	71***
<i>B. guatemalensis</i>	37	0	-	63†
<i>R. bouchellei</i>	96	1	-	3
<i>P. mexicana</i> “Clade 8m”	-	-	11	89***
<i>H. nematopus</i>	79	21***	-	-
<i>P. sp</i> “Tipitapa”	97	3	-	-

**Figure 1** Map of Nicaragua showing the proposed route (solid line) and alternative routes (dashed lines) of the Nicaragua Canal. The three drainage basins involved are San Juan (red), Punta Gorda (blue) and Escondido (yellow). Sampling localities are marked with open diamonds.



**Figure 2** *Poecilia* phylogeny based on *cytb* sequence data (1086 bp). Samples collected during this study are indicated in grey boxes. Collected specimens formed four distinct clusters corresponding to different *Poecilia* species: *P. gillii* (*P. sp.* “Cluster 1”), *P. mexicana* 8m (*P. sp.* “Cluster 2”), *P. mexicana* 8a (*P. sp.* “Cluster 3”) and the undescribed species *P. sp.* “Tipitapa” (*P. sp.* “Cluster 4”). Species were assigned based on Bagley et al. (2015).



**Figure 3** Median joining haplotype networks for populations from distinct drainage basins (a - c, e) and within the San Juan drainage basin (d - f). For three investigated species, *A. siquia* (a), *B. guatemalensis* (b) and *P. mexicana* (c), strong differentiation can be observed for interdrainage comparisons. Circle sizes represent sample sizes for each haplotype.

