Genetic characterization of Amazonian discus fishes of the genus *Symphysodon* based on microsatellite markers: implications for systematics and classification

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Abstract

Historical interest in the Neotropical cichlids of the genus *Symphysodon* has generated a number of studies and taxonomic revisions in both the professional and hobbyist literature. While these efforts have resulted in the recognition of phenotypically differentiated varieties and in the description of several species, they have at times generated unnecessary taxonomic uncertainty and produced contradictory classification schemes. In an attempt to contribute to the systematic knowledge of this group of fishes, and to resolve some of the the current classification controversies, we analyzed 336 individuals from 24 localities throughout the entire distributional range of the genus *Symphysodon*. We analyzed variation at 13 nuclear microsatellite markers, and subjected the data to Bayesian analysis of genetic structure. The results indicate that *Symphysodon* is composed of four genetic groups: group PURPLE – phenotype Heckel and abacaxi; group GREEN – phenotype green; group RED – phenotype blue and brown; and group PINK – populations of Xingú and Cametá. Although the phenotypes blue and brown are predominantly biological group RED, they also have substantial contributions from other biological groups, and the patterns of admixture of the two phenotypes are different. The two phenotypes are also characterized by distinct and divergent mtDNA haplotype groups. All phenotypic groups also show differing levels of mean habitat use. We therefore conclude that *Symphysodon* comprises five evolutionary significant units: *Symphysodon discus* (Heckel and abacaxi phenotypes), *S. aequifasciatus* (brown phenotype), *S. tarzoo* (green phenotype), *Symphysodon* sp. 1 (blue phenotype) and *Symphysodon* sp. 2 (Xingú group).

Introduction

Tropical regions contain many more species than do temperate and polar regions, however, explanations as to why remain unclear [e.g. 1, 2]. The Amazon Basin is particularly species rich, and harbors arguably the world's greatest terrestrial [3, 4] and freshwater [5] biodiversity. Numerous potential processes generating this diversity within the Amazon basin have been proposed [e.g. 6, 7, 8]. Testing of processes that have generated Amazonian biodiversity depends on solid taxonomy since species are the operational units in all studies of biodiversity [e.g. 9]. The taxonomy of many Amazonian groups is still poorly known [3, 5] and at least in fishes a number of instances of haplotype sharing between closely related species have been reported [e.g. 10, 11-14]. This makes it difficult to apply the phylogenetic species concept in species discovery and diagnosis. The delimitation of species of the genus *Symphysodon* has also been problematic in part due to sharing of mitochondrial DNA haplotypes between species [15-17] and a complete lack of resolution with nuclear DNA haplotypes [15, 17].

The discus fishes (genus *Symphysodon*) have a natural distribution is the Amazon basin. Traditionally five principal phenotypic, largely allopatrically distributed groups are recognized in popular literature [18, 19]. These groups are: 1) green phenotype – found in the western Amazon basin; 2) blue phenotype – found in the central Amazon basin; 3) brown phenotype – found in the eastern Amazon basin; 4) Heckel phenotype – found in the Negro and Trombetas River basins; and 5) abacaxi phenotype – found in the Abacaxis River, a blackwater tributary of the lower Madeira River.

Since the description of the type species and the genus by Heckel in 1840, there has been taxonomic uncertainty and confusing classification related to the genus *Symphysodon*. Considering descriptions and taxonomic revisions until the year 2006, two species were recognized in scientific literature [20, 21]: *Symphysodon discus* Heckel, 1840 and *Symphysodon aequifasciatus* Pellegrin, 1904, and four subspecies in popular literature: *S. discus willischwartzi* Burgess, 1981 (phenotype abacaxi), *S. discus tarzoo* Lyons, 1959 (phenotype green), *S. aequifasciatus haraldi* Schultz, 1960 (phenotype blue) and *S. aequifasciatus axeroldi* Schultz,

1960 (phenotype brown), with the nominal sub-species *S. discus discus* Heckel, 1840 (phenotype Heckel) and *S. aequifasciatus aequifasciatus* Pellegrin, 1904 (phenotype green) being restricted to just one major phenotype. Recently, Ready *et al*. [17] proposed the existence of three species: *S. discus* (phenotype Heckel and abacaxi), *S. aequifasciatus* (phenotype blue and brown) and *S. tarzoo* (green phenotype). Bleher *et al*. [16] also argued for the existence of three species: *S. discus* (phenotype Heckel and abacaxi), *S. aequifasciatus* (phenotype green) and *S. haraldi* (phenotype blue and brown), with *S. aequifasciatus* and *S. haraldi* occurring allopatrically, but throughout the Amazon basin (*i.e.* the species are not restricted to the western, and central+eastern Amazon basin). Farias and Hrbek [15] on the other hand argued that the genus *Symphysodon* is probably a biological complex in the process of speciation. Farias and Hrbek [15] also reported the existence of an additional deeply divergent mitochondrial lineage from the Xingú River basin.

All three studies found individuals of the green phenotype to form a monophyletic mtDNA group. Farias and Hrbek [15] also observed monophyly of the blue phenotype and the Xingú lineage. All three studies observed haplotype sharing between the Heckel, abacaxi and brown phenotypes, with Bleher *et al*. [16] considering the brown phenotype individuals to be blue phenotype individuals introgressed with Heckel phenotype mtDNA.

Inclusion of nuclear DNA sequence data did not identify monophyletic groups or clarify taxonomy of *Symphysodon*. Farias and Hrbek [15] analyzed the third exon the recombination activation gene one (RAG1) observing extensive haplotype sharing among all phenotypes. Ready *et al*. [17] included the Rhodopsin gene in their analysis, however, it showed no sequence variation within *Symphysodon*.

The two published isozyme studies of *Symphysodon* [22, 23] could not find any diagnostic marker that would separate *S. discus* and *S. aequifasciatus*, and Kokoscha and Greven [23] even observed that among-population divergences within *S. aequifasciatus* were larger than the interspecific genetic divergence of these populations and *S. discus*. Two mtDNA sequence studies by Zhang *et al*. [24, 25] of aquarium material also reported that interspecific divergence was smaller than among-population divergences within *S. aequifasciatus*. Results

from chromosomal studies of Gross and collaborators [26, 27] have reported extensive karyotypic variation within and among sampling localities, but have found no consistent karyotype differences between different species and populations of *Symphysodon*.

Genetic characterization of *Symphysodon* and identification of biological populations are essential for the understanding of evolutionary processes operating on the genus *Symphysodon* and for delimitation of evolutionary species. Neutral molecular markers with high mutation rates have the greatest ability to record signatures of recent evolutionary events.

Microsatellite loci have a mutation rate estimated at 2.5×10^{-3} in humans [28, 29] to 5.6×10^{-4} [30] mutations per generation with similar values observed in mice [31], and are commonly used for intra-specific population studies [e.g. 32, 33-35]. Since microsatellite flanking regions are generally conserved among closely related species, they are also often used for interspecific studies and studies of species complexes [e.g. 36, 37-41]. Microsatellite markers thus lend themselves well to delimiting taxonomic boundaries and identifying cases of hybridization [42-44].

The aim of the present study was to use microsatellite markers to genetically characterize phenotypic variants of *Symphysodon* sampled throughout their area of natural distribution, and to investigate association between genetic variants, geographic distribution and described species / subspecies. We also use the proxy variables of pH and conductivity to test for differences in mean habitats occupied by the different phenotypes and groups. Neither pH nor conductivity are necessarily the primary agents causing ecological separation, but they are likely to reflect differences in habitats occupied by the different phenotypes as correlates of other important environmental variables or differences in the geological history of the areas occupied by species of *Symphysodon*.

Material and Methods

Samples

A total of 336 individuals were sampled from 24 localities (Figure 1) throughout the known geographic distribution of the genus *Symphysodon*. All five principal phenotypes (Heckel, abacaxi, brown, green and blue) were sampled, with an average number of 13 individuals per

sampled locality. Individuals were assigned to phenotype following Farias and Hrbek [15]. Fishes were collected by cutting and submerging branches in appropriate habitats on the margins of lakes and small rivers for approximately a week to allow sufficient time for individuals to colonize this habitat (popularly known as “galhada”). After the fish colonized this habitat, they were collected with nets. Samples were also obtained from local ornamental fishermen. A small sample of tissue from the caudal peduncle or the left pectoral fin was removed and stored in 96% ethanol until processing in the laboratory, with remainder of the specimen preserved in 10% formalin, and after fixation transferred to 70% ethanol. Tissue and specimen samples are stored in the tissue collection of the Laboratory of Evolution and Animal Genetics (LEGAL) of the Federal University of Amazonas, Manaus, Brazil.

DNA extraction and amplification

Total DNA was extracted from tissue samples using the phenol/chlorophorm protocol [45]. For some samples the Genomic Prep Cells and Tissue DNA Isolation kit (GE Healthcare) was used.

To characterize *Symphysodon* individuals, we used 13 microsatellite pairs developed by Amado *et al*. [46]. Genotyping was done according to the economical method of Schuelke [47] that uses a 5’ tailed amplification primer, and then a third fluorescently-labeled primer in the genotyping reaction. This way just one labeled primer can be used to fluorescently label multiple microsatellite loci.

PCR reactions for all primer pairs were carried out in a final volume of 10 µL containing 4.5 µL of ddH_2O, 0.7 µL of $MgCl_2$ (25 mM), 0.8 µl of dNTPs (10 mM), 1.0 µL of 10x PCR buffer (100mM Tris-HCl, 500mM KCl), 0.5 µL of forward primer with M13(-21) 5’ tail (2.0 µM), 1.0 µL of reverse primer (2.0 µM), 0.5 µL of fluorescently-labeled M13(-21) primer (2.0 µM), 0.2 µL of Taq DNA Polymerase (5 U/µL) and 1 µL of DNA (concentration varied between 50 ng and 100 ng). PCR reactions were performed in two stages, an amplification stage, and labeling stage. For the microsatellite loci Sd04 and Sd05 the amplification reaction consisted of denaturation at 94°C for 60 seconds, followed by of 25 cycles of denaturation at 93°C for 5 seconds, primer annealing at

65°C for 5 seconds, and primer extension at 68°C for 10 seconds. For the microsatellite loci Sd08 and Sd10 the amplification reaction consisted of denaturation at 94°C for 60 seconds, followed by of 35 cycles of denaturation at 93°C for 20 seconds, primer annealing at 65°C for 20 seconds, and primer extension at 68°C for 30 seconds. For the microsatellite loci Sd11, Sd12, Sd14, Sd15, Sd22, Sd23, Sd25, Sd27 and Sd30 the amplification reaction consisted of denaturation at 94°C for 60 seconds, followed by of 35 cycles of denaturation at 93°C for 20 seconds, primer annealing at 55°C for 20 seconds, and primer extension at 68°C for 30 seconds. The PCR labeling stage consisted of 25 cycles of denaturation at 94°C for 5 seconds, primer annealing at 53°C for 10 seconds, and primer extension at 68°C for 30 seconds, followed by a final extension for 20 minutes at 68°C. Subsequent to the genotyping reaction, the PCR product was diluted 1:10 to 1:50 depending on the microsatellite used, and 1 µL of the diluted product was resuspended in 9 µL Hi-Di formamide / ET 400 size standard mix (GE Healthcare), and resolved on a MegaBace automatic sequencer (GE Healthcare). The programs Genetic Profiler and Fragment Profiler were used to extract raw fragment data, and infer fragment sizes.

Statistical analyses

The programs GenAlEx 6.41 [48] and ARLEQUIN version 3.11 [49] were used for the calculation of allelic frequencies and number of alleles, observed (H_O) and expected (H_E) heterozygosities, linkage disequilibrium between pairs of loci, and to test for Hardy-Weinberg equilibrium within sampling localities. In cases of multiple comparisons, significance was adjusted using the serial Bonferroni method proposed by Rice [50].

To estimate the degree of genetic differentiation between sampling localities and phenotypes, we calculated F_{ST}-like values [51, 52] and tested their significance via 10,000 bootstrap replicates. Hierarchical analysis of molecular variance (AMOVA) [53] was used to test four hypotheses: 1) grouping of localities into two species [21]; 2) grouping of localities into three species [17]; 3) grouping of localities into five phenotypes [18, 54]; and grouping of localities into five phenotypes and the Xingu group [15].

The data were analyzed in the program STRUCTURE version 2.3.2 [55, 56] with the goal of assigning individuals into groups, given a specific number groups (K). We used the 'admixture' and 'correlated-allelic-frequencies' models. Assignment space was explored with 1,000,000 MCMC chains, preceded by 100,000 MCMC chains discarded as burn-in. Each analysis was repeated ten times from a different randomly selected starting point, and independent runs summarized in the program CLUMPP 1.1.2 [57]. Results were visualized in the program DISTRUCT 1.1 [58]. The most likely number of biological groups (K) was inferred using the methodology of Evanno *et al*. [59] implemented in Structure Harvester 0.6.1 [60]. Since not all geographical groups and not all individuals were genetically pure, *i.e.* composed of just one biological group, we analyzed differences in genetic composition and admixture among phenotypic groups using a MANOVA where individual q values were the dependent and phenotypic groups the independent variables. Genetic composition of individuals was summarized in the form of principal components, and heterogeneity of genotypic composition of phenotypes was calculated from weighted eigenvalues of all contributing principal components. When the data permitted, we used a logistic regression to investigate differences in the pH and conductivity of water sampled from the habitats occupied by the different phenotypes of *Symphysodon* [16]. The distribution of phenotypes in localities was based on our understanding of the geographic distribution of phenotypes (see online supplement S1). All analyses were performed in the software R 2.11.1 (cran.r-project.org).

To identify possible Evolutionary Significant Units (ESUs) comprising the genus *Symphysodon*, we used the methodology proposed by Crandall *et al*. [61]. The methodology is based on testing if geographically separated populations are genetically and ecologically exchangeable or were in the recent past.

Results

Genetic diversity

The total number of alleles encountered across all the loci was 150, with an average of 11.5 ± 9.0 alleles per locus. The highest allele number was observed in the locus Sd30, and the

lower number in the loci Sd10 and Sd22. Allele numbers per locus are: Sd04 (9 alleles), Sd05 (10 alleles), Sd08 (10 alleles), Sd10 (5 alleles), Sd11(10 alleles), Sd12 (8 alleles), Sd14 (12 alleles), Sd15 (25 alleles), Sd22 (5 alleles), Sd23 (8 alleles), Sd25 (4 alleles), Sd27 (8 alleles) and Sd30 (36 alleles).

Observed heterozygosity varied from 0 to 1 while expected heterozygosity varied from 0.01 to 0.96 (Table 4). Majority of the sampled localities had low observed heterozygosities (0.4 – 0.5) while the lowest observed heterozygosities (0.2 – 0.3) were observed in Demini, Novo Airão, Buibui and Xingú. There were seven cases of Hardy-Weinberg disequilibrium. Four loci (SD04, SD08, SD11 and SD30) were at Hardy-Weinberg disequilibrium in the locality Trombetas, while the loci SD08, SD08 and SD15 were in Hardy-Weinberg disequilibrium in the localities Tabatinga, Nhamundá and Tefé, respectively.

Genetic differences among species and hierarchical groups

Analysis of molecular variance [52] revealed that a majority of genetic variance was encountered within sampling localities rather than at higher hierarchical levels when hierarchical levels represented species or phenotypes (Table 2). All hierarchical levels were significant. Grouping reflecting the two [21] vs. three [17] species classification system explained 24.80% vs. 21.67% of total variance. Grouping individuals into six phenotypes [61] or five ESUs (this study) vs. five phenotypic clusters [18, 54] explained more variance (22.42% and 22.41% vs. 19.54%). When six phenotypic clusters or five ESUs were considered, relatively more variance was explained by among phenotype differences rather than by differences among localities within phenotypes, than in any other hierarchical grouping scheme (Table 2). In general, pairwise differences between localities (online supplement S2) were significant in majority of comparisons with the exception of most pairwise comparison involving localities of the green phenotype (Tabatinga, Jutaí, Jurua, Tefé and Japurá) and the blue phenotype (Coari, Purus, Manacapuru, and Iranduba/Mamuri). Pairwise differences between ESUs were also significant (online supplement S3).

9

Biological groups

The most likely number of biological groups inferred in the program STRUCTURE was four (Figure 2). The majority of individuals had q > 0.9, *i.e.* had >90% probability of belonging to a particular biological group, and phenotypes were composed of individuals belonging to the same biological cluster. The phenotype green was present at the localities Tabatinga, Jutaí and Juruá where 100% of individuals had q > 0.9 and in the Tefé and Japurá localities where 88% and 84% individuals, respectively, had q > 0.9; fishes in all five localities belonged to the biological cluster GREEN. The phenotypes Heckel and abacaxi comprised the same biological cluster (cluster PURPLE), and 100% of individuals sampled from Buibui, Novo Airão, Demini and Abacaxis had q > 0.9. In the Trombetas locality where individuals also belong to the Heckel phenotype and are predominantly comprised of the biological cluster PURPLE, 80% of individuals had q > 0.9 while the remaining 20% had q > 0.8. In the Nhamundá and Nova Aripuana localities some individuals had the Heckel and abacaxi phenotypes, and of these individuals 3 of 12 and 3 of 3, respectively, had q > 0.9. Biological cluster PINK was composed of individuals from the localities Xingú (100% of individuals with q > 0.9) and Cametá (53% of individuals with q > 0.9). This biological cluster is not recognized as a distinct taxon or phenotype in professional or popular literature, but individuals from this cluster were identified as belonging to the Xingú clade in the study of Farias and Hrbek [15]. The phenotypes blue and brown composed of individuals whole genomes were predominantly the biological cluster RED. Majority of these individuals had q > 0.9, although there was a relatively large number of individuals with lower q values. The low q values were largely due to sharing of genome portions principally with fishes of biological cluster PURPLE (phenotype Heckel+abacaxi) and to a lesser extent with cluster PINK (Xingú group) and cluster GREEN (phenotype green). Population level q values are summarized in Table 1 and Figure 2b.

Viewed through the prism of the traditional two species taxonomy [21], all biological samples representing *Symphysodon discus* were within biological cluster PURPLE, while *Symphysodon aequifasciatus* was divided into three biological clusters (cluster RED, GREEN and PINK). Cluster GREEN represents phenotype green found in the western Amazon basin, cluster RED represents phenotypes blue and brown from the central and eastern Amazon basin, while

cluster PINK represents fishes of phenotype brown from the Brazilian Shield tributaries of the Amazon River in the eastern Amazon basin.

Although the blue group formed a well supported mtDNA clade [15], microsatellite nDNA profile indicated that individuals of the blue and brown phenotypes predominantly belong to the biological cluster RED (most individuals have q > 0.9). However, individuals of the blue and brown phenotypes were genetically the most admixed (Table 3), and the patterns of admixture were different between the blue and brown phenotypes (MANOVA of q values; Pillai's trace = 0.06324, df = 1, p = 0.0397).

Environmental variables

Differences in mean habitat use between phenotypes were tested using water type [62] and water characteristics [16]. Logistic regression indicated significant differences in pH or conductivity or both in all pairwise comparisons of phenotype except the pairwise comparison of the green (*S. tarzoo*) and the blue (*Symphysodon* sp. 1) phenotype. The ranges of pH and conductivity individually or in combination did not overlap involving comparisons of the Heckel (*S. discus*) and other phenotypes.

Discussion

Although the east African rift lakes contain some of the most spectacular, recently evolved assemblages of cichlid fishes [63, 64], the insular environments of the Caribbean are well known for their *Anolis* and *Eleutherodactylus* radiations [65-67], and the Hawaiian islands harbor spectacular radiations of *Drosophila* [68], the Amazon basin has the highest species diversity across the broadest taxonomic scope of any known region on this planet [3]. Just in the last 10 years, over 2000 new species have been described from the Amazon basin [69]. Reis *et al*. [5] report 4475 species described for the Neotropical region and estimate another 1550 undescribed species known from ichthyological collections.

The Amazon basin also has a complex history that reflects a mix of Miocene geomorphological events and Plio-Pleistocene climatic oscillations [70]. All these events have left

290 an impact on Amazonian ichthyofauna and the fauna and flora of the region, in general. The
291 Amazon basin is also very large, encompassing over 6.87 million km^2. Probably no species has a
292 basin-wide distribution, but there are a large number of broadly distributed fish species and
293 species complexes [5]. This inevitably results in large census sizes, and in many cases also in
294 large effective population sizes [e.g. 71]. From a population genetic perspective, the time to
295 speciation, *i.e.* reciprocal monophyly, is directly proportional to effective population sizes, having
296 a 95% probability of occurring within 2.2 *Ne* generations for mtDNA to upwards from 8.7 *Ne*
297 generations for just one nDNA locus [72]. Assuming that many of the fish species of the Amazon
298 basin have large effective population sizes, one is poised with an additional difficulty of
299 recognizing species that are non-monophyletic, and distinguishing these species from intra-
300 specific geographic variants.

301 Haplotype sharing appears to be a relatively common phenomenon in Amazonian fishes.
302 Examples include extensive interspecific haplotype sharing in the genera *Cichla* [10, 11],
303 *Potamotrygon* [12], *Symphysodon* [15], *Serrasalmus* [13] and *Piaractus* [14]. Hybridization and
304 incomplete lineage sorting have been invoked as explanations of the pattern of haplotype
305 sharing, but irrespective of the ultimate cause, interspecific haplotype sharing makes recognition
306 and delimitation of species and evolutionary significant units (ESUs) difficult. Monophyly is a
307 convenient operational criterion for recognition of species [73] and ESUs [74], but monophyly is
308 neither necessary nor sufficient for inference of species or ESUs. Broadly encompassing or
309 primary species concepts such as the evolutionary species concept are difficult to apply due to
310 lack of an all-encompassing operational criterion. However, incorporating both evolutionary as
311 well as ecological information in inferring species and ESUs is crucial. To this end, Crandall *et al.*
312 [61] proposed to test for recent as well as historical genetic and ecological exchangeability as a
313 criterion for inferring cohesion between studied groups sensu Templeton [75]. The flexibility of
314 this approach allows the identification of intraspecific structuring, of ESUs, of species
315 experiencing different degrees of evolutionary isolation, as well as cases where formerly distinct
316 species have recently lost their evolutionary distinctness.

Among the different ways to distinguish between recent and historical genetic exchangeability, Crandall *et al.* [61] propose to use mtDNA to test historical genetic exchangeability and microsatellite loci to test recent genetic exchangeability. We use this recommendation since analyses of the mtDNA data were phylogenetic [15] and thus conveyed information about lineages and history of these lineages, while analyses of microsatellite data focused on current system of mating of the studied groups (this study). For recent exchangeability we analyzed the microsatellite data collected in this study in the program STRUCTURE [55]. The number of biological groups observed in our study sample was inferred using the methodology of Evanno *et al*. [59] and we inferred four biological groups. The algorithm in STRUCTURE takes into account both Hardy-Weinberg equilibrium and linkage disequilibrium among loci (correlated allelic frequencies within biological groups); it therefore makes inferences about recent patterns of mating. The methodology of Evanno *et al*. [59] takes into account that most natural populations exist as metapopulations, *i.e.* that species comprise partially differentiated groups, which themselves are composed of smaller but much weakly if at all differentiated groups. In our analyses we assumed that individuals could be admixed, *i.e.* individuals' genetic composition could be the result of the contribution of more than one biological group. Phenotypic groups could then be composed of admixed individuals, but different phenotypic groups could have different patterns of admixture. Analyses of recent genetic exchangeability indicated the presence of four biological groups, two of which corresponded to recognized phenotypes (green and Heckel+abacaxi; the Heckel and the abacaxi groups belong to the same biological group), and one of which corresponded to the Xingú group (Xingú clade [15] and the Cametá locality). The fourth biological group formed the majority portion of genomes of individuals comprising the blue and brown phenotypes, but many individuals of both phenotypes were admixed with other biological groups. However, the patterns of admixture between the blue and the brown groups were different ($p = 0.0397$).

For historical exchangeability we used the mitochondrial DNA results of Farias and Hrbek [15]. The phylogenetic results reported in that study reflect the evolutionary history of the *Symphysodon* species complex on the time scale of the coalescent, and therefore were used to

345 infer historical exchangeability among the groups. Historically differentiated groups corresponded
346 to the green, the blue and the Xingú groups, while the brown and the Heckel groups showed
347 extensive haplotype sharing, but significant differences in allelic frequencies [15]. All abacaxi
348 individuals had common brown haplotypes.

349 The mitochondrial and microsatellite DNA genetic patterns observed in the blue and
350 brown phenotypes are not concordant. The blue phenotype forms a distinct mitochondrial clade
351 supported by numerous molecular synapomorphies [15]. The blue and brown phenotypes also
352 have subtle differences in color and color patterns, yet from the microsatellite nDNA perspective,
353 they are only weakly divergent from each other as a result of different patterns of admixture of the
354 RED biological group with other biological groups. However, in spite of potentially ongoing gene-
355 flow between the blue and the brown phenotypes at the nuclear DNA level, this geneflow has not
356 impacted the cohesiveness and evolutionary distinctness of the two phenotypes.

357 The mitochondrial and microsatellite DNA genetic patterns observed in the
358 Heckel+abacaxi and the brown phenotypes also are not concordant. However, in this case, the
359 Heckel+abacaxi and the brown phenotypes represent distinct biological groups, PURPLE and
360 RED, respectively, but with extensive mtDNA haplotype sharing between the phenotypes [15] and
361 the presence of admixed individuals.

362 Inference of potential recent ecological exchangeability was based on the types of water
363 inhabited by different groups of *Symphysodon*. *Symphysodon* species live in lentic habitats
364 associated with major bodies of water. They inhabit all three major types of Amazonian waters
365 [62], however, because of the lentic character of the waters inhabited, the white-water type has
366 little suspended sediment. The chemical characteristics of the tree principal water types are very
367 different [62], and also contain distinct fish faunas. Furthermore, the chemistry of white-water of
368 the Amazon is different from that of the Solimões. The Amazon River is formed at the confluence
369 of the black-water Negro River and the white-water Solimões where the Solimões contributes
370 49%, the Negro 14% and other Guyana and Brazilian Shield rivers the remaining 27% of the
371 volume of the Amazon [76]. The confluence of the Solimões with the Negro, and the formation of
372 the Amazon corresponds to the boundary between the blue, the Heckel and the brown

Symphysodon groups, respectively, and there are significant differences in pH and conductivity of *Symphysodon* habitats occupied in these three rivers. The Xingú clade together with the Cametá locality occur in the clear water type of the Brazilian Shield, and thus are likely ecologically differentiated from other groups. The southern and northern tributaries of the Amazon are further differentiated by hydrological regimes, which potentially create a temporal reproductive barrier. The parapatrically distributed green and blue phenotypes occur in the lentic habitats of the Solimões that based on their pH and conductivity appear not to be different; however, it is likely there are other ecological differences separating the green and blue phenotypes. The Heckel and the abacaxi groups both inhabit lentic black-water habitats, however, they occur in Guyana and Brazilian Shield drainages, respectively. It is unclear whether these groups are ecologically exchangeable, however, it is worth noting that the geographic distribution of the abacaxi group is restricted to few affluents of the lower Madeira River. These same affluents contain ichtiofauna shared with the Negro River, e.g. *Cichla temensis* [10], contain at least one species of *Rivulus* (TH pers. obs.) from the Guyana Shield clade of *Rivulus* [77], which may indicate historical connection of the lower Madeira River region with the Negro River basin.

No data exists on historical ecological exchangeability of the different *Symphysodon* groups, however, actual courses of Amazonian rivers and their headwaters in the three main geological formations of the Amazon basin, the Andes mountains, and the Guyana and Brazilian shields, have assumed their current forms at least six million years ago [78], although on a more regional scale, there has been much dynamism throughout the Pleistocene [79]. One can also argue from the principle of uniformitarianism and phylogenetic niche conservatism that current patterns of ecological association reflect historical patterns of ecological associations.

Analyses recent and historical ecological and genetic exchangeability permits us to diagnose ESUs and infer intraspecific population structure [61]. A summary of the diagnosis of *Symphysodon* phenotypes is presented in Figure 3. Based on the criteria of Crandall *et al.* [61] all comparisons resulting in Case 1 differentiation should be considered distinct species. Case 1 [61] is observed between the green and all other phenotypes but blue, between the brown and all other phenotypes, between the Heckel and all other phenotypes, between the abacaxi and all

401 other phenotypes but Heckel, and between the blue and all other phenotypes but brown.
402 Differentiation was weak between the Heckel and abacaxi phenotypes (Case 8) suggesting that
403 both phenotypes belong to the same species, but represent distinct populations. Whether the
404 blue vs. brown phenotype comparison represents Case 1 or Case 3 depends on how one
405 quantifies recent genetic exchangeability. One can either consider this hypothesis not rejected
406 (both phenotypes are predominantly biological cluster RED) or as rejected (patterns of genetic
407 admixture are different). We opt for rejecting the null hypothesis of recent genetic exchangeability
408 given that the mtDNA haplotypes of both phenogroups are geographically restricted and non-
409 overlapping, and therefore there is either no ongoing geneflow, there is no ongoing geneflow at
410 adaptive loci, or selection removes the "wrong" mtDNA haplotype if gene-flow occurs. Based on
411 the proxy variables of pH and conductivity, there appear to be no barriers to ecological
412 exchangeability between the green and blue phenotypes (Case 7), however, both groups
413 maintain their evolutionary distinctness and are parapatrically distributed, suggesting that there
414 likely are other ecological barriers not analyzed in this study (unobserved Case 1).

415 The results of the genetic and ecological exchangeability tests are summarized in Figure
416 3, and indicate the presence of five evolutionary species comprising the genus *Symphysodon*.
417 The Heckel, Xingú, green, blue and brown phenotypes represent full-fledged evolutionary
418 species. The blue and the brown phenotypes probably are experiencing gene-flow but are
419 maintaining their independent evolutionary trajectories, while the brown group has undergone
420 historical admixture with the Heckel+abacaxi and the Xingú group. Despite being allopatric, the
421 Heckel and the abacaxi phenotypes do not represent independent biological entities, possibly due
422 to recent geographic separation.

423 The notion that the five phenotypes represent biological species rather than intraspecific
424 variation is also supported by the amount of genetic divergence between the phenotypes. We
425 observed inter-phenotype F_{ST} values between 0.02 and 0.38 (online supplement S3). All intra-
426 phenotype F_{IS} values averaged at 0.09. These *F* values are comparable to values reported in
427 other studies of fish that used microsatellite markers to analyze recently diverged species groups.
428 For example, Barluenga *et al*. [80, 81] observed in the Central American Midas cichlid complex

(*Amphilophus citrinellus*, *A. labiatus*, *A. zaliosus*) interspecific F_{ST} values ranging from 0.01 to 0.35, and intra-specific values no larger than 0.08. Similarly, an analysis of a *Sebastes* species complex (*S. fasciatus, S. mentella, S. marianus* and *S. viviparus*) resulted in interspecific pair-wise F_{ST} values ranging from 0.12 to 0.50 [82], while inter-populational pair-wise F_{ST} values were never larger than 0.04 [82].

Taxonomy of the genus *Symphysodon*

Our analyses and diagnoses using the criteria of Crandall *et al.* [61] indicate the genus *Symphysodon* is comprised of five ESUs. There are several described species, and several specific names available, however, throughout the taxonomic history of the genus, there has been substantial confusion. Therefore, we re-evaluate existing classification.

The first described species, and the type species of the genus is *Symphysodon discus* Heckel, 1840. The type specimen was collected at Barra do Rio Negro, and it represents the phenotype Heckel. *Symphysodon discus* occurs in the Negro River basin, and the Trombetas River [21], however, based on this study as well as previous analyses [15], *S. discus* also occurs in the Nhamunda and Uatuma River basins. The Nhamunda and Uatuma Rivers are the two principal drainages geographically located between the Negro and Trombetas Rivers, and all these rivers drain the Guyana Shield. In 1981 Burgess described a sub-species from the Abacaxis River (*Symphysodon discus willischwartzi* Burgess, 1981). Analyses of the microsatellite data, and diagnoses of genetic and ecological exchangeability also indicate that the phenotype abacaxi represents the same taxon as *Symphysodon discus* Heckel, 1840. This conclusion is also supported by Kullander [21]. The abacaxis phenotype is allopatric to the Heckel phenotype and parapatric with the brown phenotype. Its distinguishing characteristic is its yellowish-reddish background body color [83] which has led some authors to suppose that the abacaxi phenotype is a hybrid between *S. discus* and *S. aequifasciatus* [84]. From a nuclear DNA perspective, the abacaxi phenotype sampled from the type locality is nearly pure *S. discus* while some individuals of the abacaxi phenotype from the region of Novo Aripuaña show a signature of genomic admixture with the brown phenotype. In spite of instances of probable hybridization, the

genomic composition of the abacaxi phenotype is no different than that of the Heckel phenotype. However, the mitochondrial genome of the abacaxi phenotype has been replaced by the mitochondrial genome of the brown phenotype, most likely via introgressive hybridization with the brown phenotype. The Heckel phenotype/ESU is *Symphysodon discus*.

The second species of *Symphysodon* was described over sixty years later as *Symphysodon discus* var. *aequifasciatus* Pellegrin, 1904. Three individuals were used in its description, two from Tefé and one from Santarém. Tefé is within the geographic distribution of the green phenotype, while Santarém is within the geographic distribution of the brown phenotype. These differences reported in the original description of Pellegrin were used by Schultz [18] as basis for elevating *Symphysodon discus* var. *aequifasciatus* to the species level (*Symphysodon aequifasciatus*).

In 1959/1960 Lyons described the sub-species *Symphysodon discus tarzoo* that differed from *S. aequifasciatus* by the presence of red spots on its fins and body. Its description was based on specimens from Letícia, Colombia, and its characteristics are those of the green phenotype. Short time later Schultz [18] revised the genus *Symphysodon*, rejecting the name 'tarzoo' and describing three sub-species of *Symphysodon aequifasciatus*. Schultz [18] described the sub-species *S. aequifasciatus axelrodi* (brown phenotype) designating a type from Belém (eastern Amazon), the sub-species *S. aequifasciatus haraldi* (blue phenotype) designating a type from Benjamin Constant (western Amazon), and restricted the nominal sub-species *S. aequifasciatus aequifasciatus* to the green phenotype designating a type locality as Tefé (western Amazon). The geographic distribution of the blue phenotype is the central Amazon, however. There both Bleher [19] and Kullander [21] doubt that the type specimens of *S. aequifasciatus haraldi* were collected at Benjamin Constant by Axelrod and Schultz as reported in Schultz [18].

There is little recent controversy with respect to the taxonomy of the green phenotype. It forms a well supported mtDNA clade [15-17], and our microsatellite DNA data indicate that it represents a biological entity that is clearly differentiated from other phenotypes. The classification of the western Amazon green phenotype is contentious, however.

Following the revision of Schultz [18], most authors [e.g. 20, 21, 85] did not recognize the sub-specific classification of Schultz [18]. Ready *et al.* [17] based on mtDNA and morphometric evidence recognized the green phenotype as a species, revalidating the name *Symphysodon tarzoo* Lyons, 1959, and designating a neotype (INPA 25960). However, Bleher *et al.* [16] rejected the name *S. tarzoo* in favor of *Symphysodon aequifasciatus* Pellegrin, 1904. These differences in classification of the green taxon have several sources.

First, the magazine in which Lyons' article was published is dated 1960 (*Tropicals* – Holiday Issue – 1960, Vol. 4, No. 3) rather than 1959, and therefore *Symphysodon discus tarzoo* Lyons, 1960 would be a junior synonym of *Symphysodon aequifasciatus aequifasciatus* Schultz, 1960 published in the June issue of the Tropical Fish Hobbyist. However, the publication of Lyons' article must have preceded that of Schultz since Schultz [18] cites the Lyons' article as "Holiday issue 1960" followed in parentheses by the date "November 28, 1959", and then goes on to reject Lyons' description on the grounds that it does not satisfy standards for species descriptions by the International Rules of Zoological Nomenclature, now the International Code of Zoological Nomeclature (ICZN). However, whether Lyons' description was sufficient to meet ICZN standards is subjective since Lyons does have a description which includes diagnostic characters, and does provide a photograph of the new sub-species even if not of the type specimen, and therefore Ready et al. [17] consider Lyons' description as valid. Bleher *et al.* [16] also make a second argument for rejecting the name *Symphysodon tarzoo* Lyons, 1959 on the grounds that Lyons did not explicitly name the new species. It is true that the description is not explicit and scientifically rigorous, however, according to the regulations of the ICZN, only after 1999 do species descriptions have to be explicit and intentional. Therefore, even if Lyons did not explicitly state that the name *tarzoo* referred to a new sub-species, the ICZN rule requiring to do so did not yet exist in 1959.

Further Bleher *et al.* [16] argue that when Pellegrin [86] was describing *Symphysodon discus* var. *aequifasciatus*, he intended to associate this name with the green phenotype since two of the three specimens in the type series are from Tefé (green phenotype) and were described by Pellegin before the one specimen from Santarém (brown phenotype). Still further,

the authors argue that because Schultz [18] restricted *S. aequifasciatus aequifasciatus* to the green phenotype selecting lake Tefé as the type locality, and that since the revision the name *aequifasciatus* has always been associated with the green phenotype and never with the blue or brown phenotypes, the name *Symphysodon aequifasciatus* should be the scientific name used for the green phenotype.

However, taxonomic rules are clear with respect to homonimies (Article 23.1 of the ICZN). If Pellegrin in 1904 described the variety *aequifasciatus* based on two different phenotypes (green and brown) which are now recognized as two species, and posteriorly Lyons in 1959 described the subspecies *tarzoo* using individuals of only the green phenotype, Lyons became the the first reviser. Therefore, we follow the precedent of the first reviser of this taxon and adopt the name *Symphysodon tarzoo* Lyons, 1959, following the classification proposed by Ready *et al.* [17], for the green phenotype/ESU identified in this study.

A third and fourth biological species identified in our analyses are the brown and blue phenotypes. Both the brown and blue phenotypes are not genetically pure. In the case of the brown phenotype, many of its individuals are admixed principally with Heckel phenotype (*Symphysodon discus*) and the Xingú group phenotype, while in the case of the blue phenotype one observed admixture with the green phenotype (*Symphysodon tarzoo*). The blue phenotype forms a distinct mtDNA clade [15], while there is some haplotype sharing between the brown and Heckel phenotypes [15]. Bleher *et al*. [16] also observed that a small number of individuals of the blue/brown phenotype, all from the eastern Amazon, past the confluence of the Negro and Solimões Rivers, shared haplotypes with the Heckel phenotype. The authors considered these individuals to be old hybrids, or more correctly be blue/brown individuals with introgressed Heckel mtDNA. The study of Ready *et al.* [17] sheds no information on the species status of the blue phenotype since individuals of this phenotype were not included in their analysis.

The classification of the brown and blue phenotype is also apparently controversial. This controversy stems directly from the controversy surrounding the classification of the green phenotype expounded on previously, the fact that Ready *et al.* [17] did not include the blue phenotype in their analysis, and that Bleher *et al*. [16] considered the brown phenotype to be the

blue phenotype introgressed with mtDNA of the Heckel phenotype. Ready *et al.* [17] propose the name *Symphysodon aequifasciatus* for the brown phenotype and designate the Santarém individual from Pellegrin's type series (MNHN 1902-130) as lectotype, restricting the species *Symphysodon aequifasciatus* to the brown phenotype. However, since Bleher *et al.* [16] adopted the name *Symphysodon aequifasciatus* for the green phenotype, they then adopted the name *Symphysodon haraldi* for the blue and brown phenotypes. Although the type locality of *Symphysodon aequifasciatus haraldi* Schultz, 1960 (USNM 00179829) was reported as Benjamin Constant, this is highly doubtful based on several lines of evidence [19, 21] and material discussed in Bleher and Géry [87], leading Bleher and Géry [87] to propose Lake Berurí of the lower Purus River system as the correct type locality for Schultz's type. If Schultz's type (USNM 00179829) really originated from Lake Berurí in the lower Purus River system, the revisions of Ready et al. [17] and Bleher et al. [16] can be viewed as largely non-contradictory; however, ultimately the true type locality of Schultz's type is unknowable at this point in time.

The results of our study indicate that while both the brown and blue phenotypes are derived from the same biological group, as phenotypic groups they show different patterns of admixture. The brown and blue phenotypes are also clearly differentiated at the mtDNA level [15]. Therefore *Symphysodon aequifasciatus* Pellegrin, 1904 (*apud*. Ready et al. [17]) should be restricted to the brown phenotype/ESU occurring in the eastern Amazon, downstream of the confluence of the Solimões and Negro Rivers, while the blue phenotype/ESU occurring in the central Amazon upstream of the confluence of the Solimões and Negro Rivers and east of the Purus Arch likely represents a new species of *Symphysodon*.

We also identified a fifth ESU in our analysis. This ESU is comprised of individuals from the Vitoria do Xingú (Xingú River) and the Cametá (Tocantins River) localities, both situated at the northern margins of the Brazilian Shield. The presence of this evolutionary entity was already observed by Farias and Hrbek [15], but neither the study of Ready *et al.* [17] nor that of Bleher *et al.* [16] include fishes from the Xingú or Tocantins drainages, and therefore neither study observed the presence of this group. No potential scientific name seems to exist for this taxon. Schultz in 1960 described the sub-species *Symphysodon aequifasciatus axelrodi* from Belém;

however, Belém is a city on the southern Amazon River delta from which fish were exported, and *Symphysodon* species do not occur in the vicinity of the city. Geographically the closest region where the genus *Symphysodon* occurs is in the lower Tocantins River represented by the Cametá locality in our study, and drainages in the Portel region west of Belém. According to Bleher [19] the type specimen of *Symphysodon aequifasciatus axelrodi* Schultz, 1960 (USNM 00179831) looks most like fishes from Breves, Marajó Island (another region from which fish are exported but not collected), while Bleher and Géry [87] – within the book of Bleher [19] – postulate that the most likely region from where the type was collected is the lower Tapajós River where the brown phenotype occurs. If the type locality is the lower Tapajós River this would make *S. aequifasciatus axelrodi* a junior synonym of *Symphysodon aequifasciatus* Pellegrin, 1904 (*apud*. Ready et al. [17]). Rest of the type series is listed as having been collected from the lower Urubu River where the brown phenotype occurs. Again, the true location of the type locality is unknowable at this point in time, but ultimately has no bearing on the taxonomic status of the Xingú phenotype/ESU.

In summary, we conclude that the genus *Symphysodon* is comprised of five ESUs:

- *Symphysodon discus* Heckel, 1840 (Heckel and abacaxi phenotypes – western Guyana Shield – Negro and upper Uatuma, Nhamunda and Tombetas Rivers; western Brazilian Shield – Abacaxis River and some other blackwater affluents of the Madeira River)

Synonym: *Symphysodon discus willischwartzi* Burgess, 1981

- *Symphysodon tarzoo* Lyons, 1959 (green phenotype – western Amazônia – river drainages west of the Purus Arch)

Synonym: *Symphysodon discus* var. *aequifasciatus* Pellegrin, 1904 in part

Symphysodon Discus Tarzoo Lyons, 1959

Symphysodon aequifasciatus aequifasciatus Schultz, 1960

593 *Symphysodon aequifasciatus haraldi* Schultz, 1960? (in the unlikely assumption
594 that Schultz's account of the type locality – Benjamin Constant – is correct)
595 *Symphysodon aequifasciatus* in Bleher *et al.* [16]
596
597 • *Symphysodon aequifasciatus* Pellegrin, 1904 (brown phenotype – eastern Amazônia –
598 lower reaches of Amazon River and affluents east of the confluence of the Negro and
599 Solimões Rivers)
600 Synonym: *Symphysodon discus* var. *aequifasciatus* Pellegrin, 1904 in part
601 *Symphysodon aequifasciatus axelrodi* Schultz, 1960? (if actual type locality is
602 lower Tapajós River [87])
603 *Symphysodon haraldi* in Bleher *et al*. [16] in part
604
605 • *Symphysodon* sp. 1 (blue phenotype – central Amazônia – river drainage systems east of
606 the Purus Arch and west of the Negro and Solimões River confluence)
607 Synonym: *Symphysodon haraldi* in Bleher *et al*. [16] in part
608 *Symphysodon aequifasciatus* in Ready and Kullander [17] in part
609
610 • *Symphysodon* sp. 2 (the Xingú group – eastern Brazilian Shield – lower Tocantins and
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612

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832

32

Figures

Figure 1 – Distribution and collecting areas of phenotypes of the genus *Symphysodon*. Numbers represent: 1) Tabatinga (Calderão River), 2) lower Jutaí River, 3) Lake Santa Maria (Japurá River), 4) Bauana (Juruá River), 5) Igarapé Bowona (Tefé River), 6) Lake Coari (Coari River), 7) Lake Castanho (Purus River), 8) Lake Manacapuru (Manacapuru River), 9) Iranduba/Mamuri (Solimões River), 10) lower Demini River, 11) Igarapé Bui-Bui (Negro River), 12) Novo Airão (Negro River), 13) Novo Aripuanã – Acari (Madeira River), 14) Nova Olinda do Norte (Madeira River), 15) central Abacaxis River, 16) Maues (Maués River), 17) Itapiranga (Uatumã River), 18) Nhamundá (Nhamundá River), 19) Porto Trombetas (Trombetas River), 20) Belterra (Tapajós River), 21) Lake Grande (Amazonas River), 22) Igarapé Arapiranga (Jari River), 23) Vitória do Xingu (Xingu River), 24) Cametá (Tocantins River). Colors correspond to the phenotypes analyzed in this study: green = green phenotype; blue = blue phenotype; black = Heckel phenotype; yellow = abacaxi phenotype; red = brown phenotype; and pink = Xingu clade. Localities 13/14 and 18 contained the 'abacaxi' and 'brown' phenotypes, and the 'Heckel' and 'brown' phenotypes, respectively.

Figure 2 – Graphical representation of results of STRUCTURE analyses generated in the program DISTRUCT [58]. Figure 2a represents individual level variation, while Figure 2b represents population level variation.

Figure 3 – Diagnosis of phenotypes of *Symphysodon* using the methodology and criteria of Crandall *et al*. [61]. Lower diagonal – tests of hypotheses of genetic (left column) and ecological (right column) exchangeability during recent (upper row) and historical (lower row) times: + = null hypothesis rejected; - = null hypothesis not rejected. Upper diagonal – Inference of ESU categories: Case 1 = long separated species; Case 2 = distinct species; Case 3 = distinct populations (recent admixture and loss of genetic distinctness); Cases 7 and 8 = single population.

33

Tables

Table 1 – Results of the program STRUCTURE assuming K = 4 clusters, with proportion of individuals with q values > 0.9 and 0.8 in each locality, and proportion of genomes in locality estimated in each of the four clusters. q values < 0.050 are not shown.

Table 2 – Analysis of molecular variance (AMOVA) of *Symphysodon* species, ESUs, phenotypes and sampling localities. * = significant at P = 0.05.

Table 3 – Proportion of each biological group (PURPLE, GREEN, RED and PINK – see Figure 2) in each of the five ESUs. Heterogeneity – heterogeneity in genetic composition.

Table 4 – Indexes of genetic diversity at the 13 microsatellite loci used for the analysis of the 24 localities of *Symphysodon* phenotypes. A – number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity. P = probability that H_E and H_O are not different.

Table 5 – Analysis of differences in physico-chemical properties of water in which different groups of *Symphysodon* occur. Data were taken from Tables 3 and 4 of Bleher *et al*. [16]. Data are in online supplement 1. The abacaxi and Xingu phenotypes were not included in analyses due to small sample sizes. Because of linear separation of the independent variables (pH and conductivity) in tests involving the Heckel phenotype, it was not possible to include both variables in the same model, and therefore variables were analyzed separately. Linear separation also occurred in pH due to non-overlapping pH values for the Heckel and blue phenotypes.

34

Online supplement

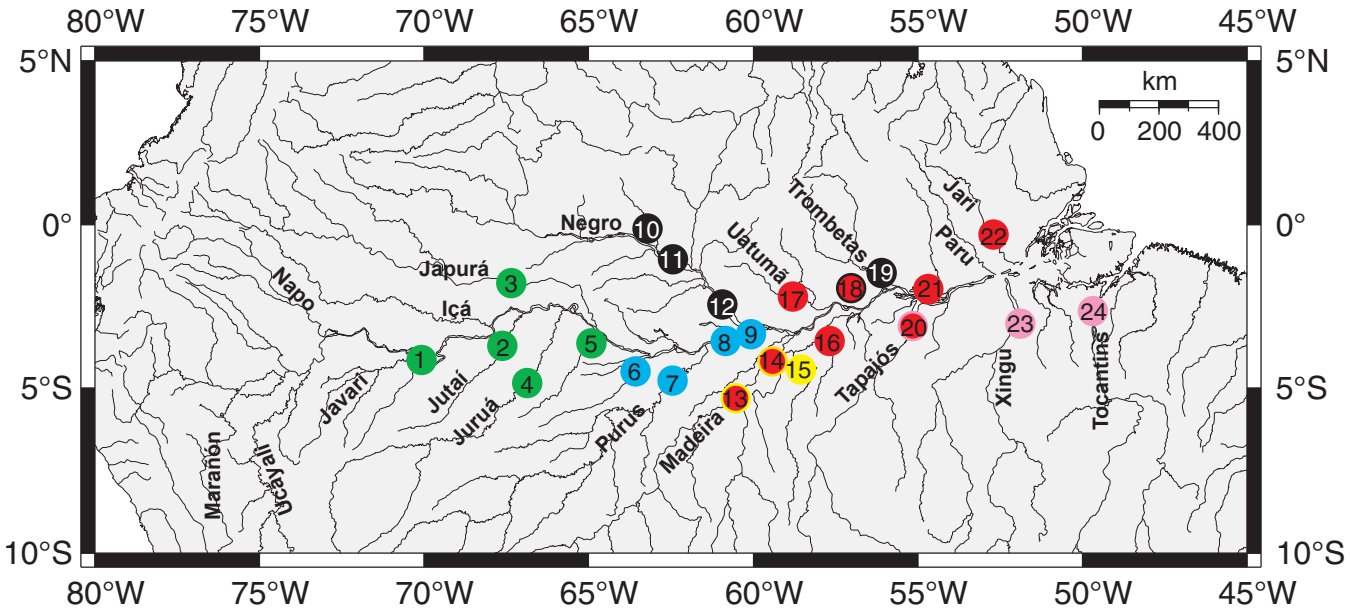
Supplement 1 – Table of pH and conductivity measured in *Symphysodon* habitats. Table based on Bleher *et al*. [16].

Supplement 2 – Table of pairwise F_{ST} differences between all pairs of sampling localities of *Symphysodon* (above below diagonal) and their significance (above diagonal).

Supplement 3 – Table of pairwise F_{ST} differences between all pairs of ESUs of *Symphysodon* identified in this study (above below diagonal) and their significance (above diagonal).

35

Figure 1


80°W
75°W
70°W
65°W
60°W
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45°W
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Negro
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Içá
Napo
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Jutaí
Juruá
Purus
Madeira
Maranon
Ucayali
Uatumã
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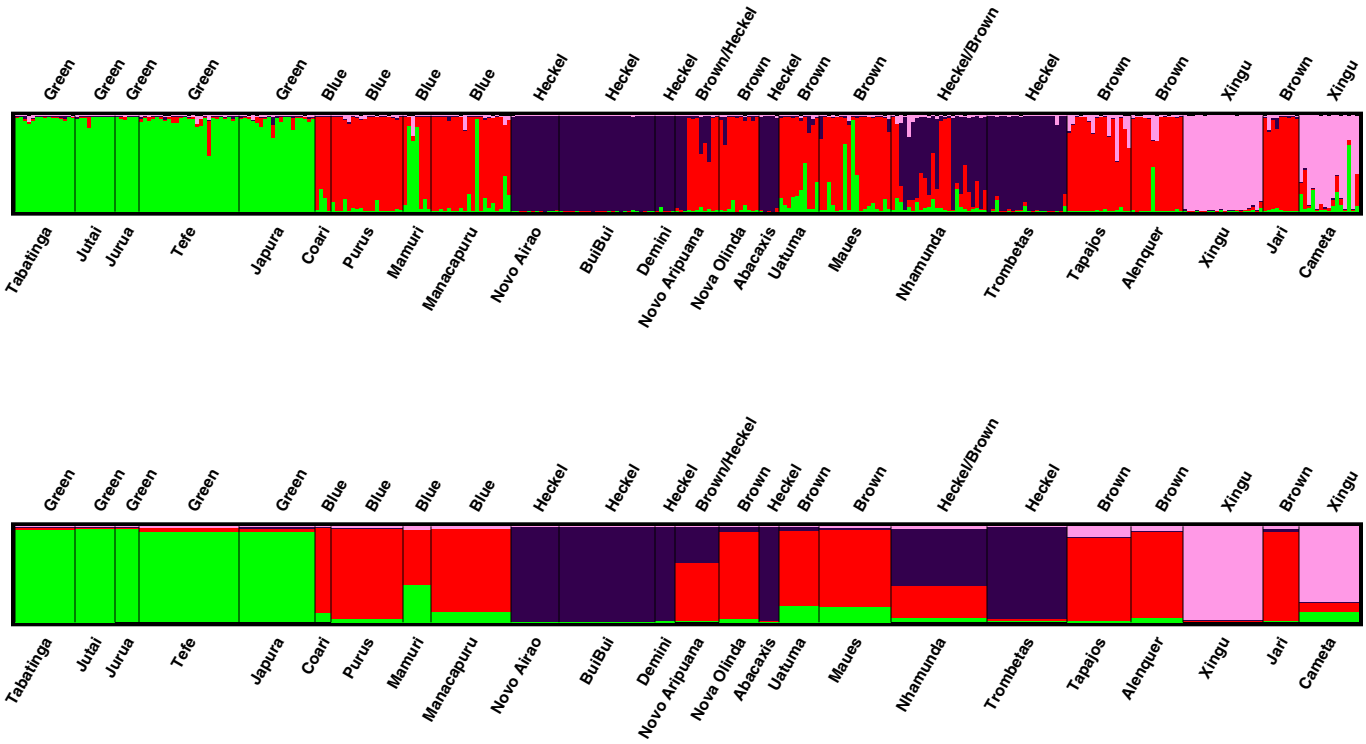
Figure 2


Green
Green
Green
Green
Green
Blue
Blue
Blue
Blue
Heckel
Heckel
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Brown/Heckel
Brown
Heckel
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Brown
Heckel/Brown
Heckel
Brown
Brown
Xingu
Brown
Xingu
Tabatinga
Jutai
Jurua
Tefe
Japura
Coari
Purus
Mamuri
Manacapuru
Novo Airao
BuiBui
Demini
Novo Aripuana
Nova Olinda
Abacaxis
Uatuma
Maues
Nhamunda
Trombetas
Tapajos
Alenquer
Xingu
Jari
Cameta

Green
Green
Green
Green
Green
Blue
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Brown/Heckel
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Xingu
Tabatinga
Jutai
Jurua
Tefe
Japura
Coari
Purus
Mamuri
Manacapuru
Novo Airao
BuiBui
Demini
Novo Aripuana
Nova Olinda
Abacaxis
Uatuma
Maues
Nhamunda
Trombetas
Tapajos
Alenquer
Xingu
Jari
Cameta

Figure 3

	Heckel	abacaxi	green	blue	brown	Xingu
Heckel		Case 8	Case 1	Case 1	Case 2	Case 1
abacaxi	- | - + | -		Case 1	Case 1	Case 2	Case 1
green	+ | + + | +	+ | + + | +		Case 1/7	Case 1	Case 1
blue	+ | + + | +	+ | + + | +	+ | +/- + | +/-		Case 1/3	Case 1
brown	+ | + - | +	+ | + - | +	+ | + + | +	+/- | + + | +		Case 1
Xingu	+ | + + | +	+ | + + | +	+ | + + | +	+ | + + | +	+ | + + | +	

Table 1 – Results of the program STRUCTURE assuming K = 4 clusters, with proportion of individuals with q values > 0.9 and 0.8 in each locality, and proportion of genomes in locality estimated in each of the four clusters. q values < 0.050 are not shown.

Locality	(q>0.9)	(q>0.8)	*Cluster* PINK (q)	*Cluster* GREEN (q)	*Cluster* RED (q)	*Cluster* PURPLE (q)
Tabatinga	1.000	1.000		0.970		
Jutai	0.900	1.000		0.971		
Jurua	1.000	1.000		0.977		
Tefe	0.880	0.960		0.943		
Japura	0.842	0.947		0.945		
Coari	0.500	0.750		0.103	0.888	
Purus	0.83	1.000			0.946	
Mamuri	0.429	0.857*		0.396	0.566	
Manacapuru	0.700*	0.900*		0.109	0.864	
Novo Airao	1.000	1.000				0.985
Bui-Bui	1.000	1.000				0.987
Demini	1.000	1.000				0.980
Nova Aripuana	0.727†	0.727†			0.604	0.373
Nova Olinda	0.700	0.900			0.916	
Abacaxis	1.000	1.000				0.977
Uatuma	0.200	0.700		0.167	0.791	
Maues	0.556*	0.778*		0.161	0.800	
Nhamunda	0.333†	0.500†		0.052	0.328	0.591
Trombetas	0.800	1.000				0.955
Tapajos.	0.563	0.813	0.109		0.871	
Alenquer	0.846	0.846	0.051		0.900	
Xingu	0.950	1.000	0.975			
Jari	0.778	1.000			0.930	
Cameta	0.467	0.600	0.790	0.111	0.092	

* = includes individuals from clusters GREEN and RED; † = includes individuals from clusters RED and PURPLE

Table 2 – Analysis of molecular variance (AMOVA) of *Symphysodon* species, ESUs, phenotypes and sampling localities. * = significant at P = 0.05.

Source of variation	**SS**	**%**
Among localities	711.61	29.43*
Within localities	1549.17	70.57*
Among species*	279.33	24.80*
Among localities within species	448.99	15.64*
Within localities	1578.28	59.56*
Among species†	374.83	21.67*
Among localities within species	353.49	13.69*
Within localities	1578.28	64.64*
Among phenotypes‡	416.82	19.54*
Among localities within phenotypes	311.50	13.48*
Within localities	1578.28	66.98*
Among phenotypes◦	497.78	22.42*
Among localities within phenotypes	230.54	10.12*
Within localities	1578.28	67.47*
Among ESUs◊	484.96	22.41*
Among localities within ESUs	243.36	10.36*
Within localities	1578.28	67.27*

* = species *Symphysodon aequifasciatus* and *S. discus* (sensu Kullander, 1986)
† = species *Symphysodon tarzoo*, *S. aequifasciatus* and *S. discus* (sensu Ready *et al*., 2006)
‡ = phenotypes Heckel, abacaxi, green, blue and brown (sensu Schultz, 1960 and Burgess, 1981)
◦ = phenotypes Heckel, abacaxi, green, blue and brown, and Xingu (sensu Farias and Hrbek, 2008)
◊ = ESUs of *Symphysodon* identified in the present study

Table 3 – Proportion of each biological group (PURPLE, GREEN, RED and PINK – see Figure 2) in each of the five ESUs. Heterogeneity – heterogeneity in genetic composition.

	PURPLE	GREEN	RED	PINK	Heterogeneity
Heckel+abacaxi *Symphysodon discus*	0.886	0.025	0.073	0.016	0.309
Green *Symphysodon tarzoo*	0.011	0.948	0.029	0.013	0.104
Blue *Symphysodon* sp. 1	0.010	0.142	0.821	0.026	0.473
Brown *Symphysodon aequifasciatus*	0.030	0.087	0.836	0.047	0.409
Xingu *Symphysodon* sp. 2	0.009	0.064	0.055	0.872	0.369

Table 4 – Indexes of genetic diversity at the 13 microsatellite loci used for the analysis of the 24 localities of *Symphysodon* phenotypes. A – number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity. P = probability that H_E and H_O are not different.

		Locus													
POP		**Sd04**	**Sd05**	**Sd08**	**Sd10**	**Sd11**	**Sd12**	**Sd14**	**Sd15**	**Sd22**	**Sd23**	**Sd25**	**Sd27**	**Sd30**	**Total**
	A	2	3	2	2	4	2	4	12	3	2	1	3	4	43
TB	Ho	0.800	0.133	0.933	0.067	0.467	0.600	0.333	0.933	0.400	0.133		0.400	0.533	0.491
15	He	0.515	0.131	0.515	0.067	0.467	0.508	0.402	0.903	0.441	0.129		0.432	0.559	0.450
	P	0.019	0.994	0.001	0.894	0.837	0.390	0.789	0.332	0.320	0.782		0.810	0.966	
	A	2	2	2	1	5	2	3	10	4	3	2	1	3	39
JT	Ho	0.800	0.200	0.900		0.600	0.400	0.300	0.800	0.400	0.100	0.100		0.500	0.418
10	He	0.526	0.189	0.521		0.558	0.442	0.279	0.911	0.500	0.100	0.100		0.484	0.402
	P	0.058	0.725	0.010		0.868	0.880	0.958	0.426	0.094	0.868	0.868		0.675	
	A	2	1	2	1	4	3	2	6	3	2	2	3	2	33
JR	Ho	0.667		1.000		0.500	0.500	0.333	0.833	0.667	0.333	0.333	0.333	0.500	0.454
6	He	0.545		0.545		0.561	0.591	0.303	0.818	0.545	0.303	0.303	0.318	0.530	0.461
	P	0.414		0.014		0.466	0.828	0.624	0.913	0.682	0.624	0.624	0.971	0.944	
	A	4	5	4	4	7	2	5	16	4	4	3	2	7	66
TF	Ho	0.522	0.360	0.917	0.080	0.560	0.520	0.440	0.680	0.417	0.320	0.200	0.480	0.360	0.480
23	He	0.545	0.323	0.570	0.079	0.574	0.458	0.372	0.920	0.357	0.290	0.187	0.444	0.442	0.465
	P	0.897	1.000	0.009	0.998	0.523	0.428	0.996	0.001	0.948	0.989	0.958	0.607	0.615	
	A	4	4	3	3	4	3	2	13	3	5	2	3	11	60
JP	Ho	0.579	0.211	0.947	0.105	0.368	0.526	0.421	0.789	0.368	0.263	0.105	0.211	0.579	0.454
19	He	0.599	0.201	0.536	0.104	0.371	0.421	0.341	0.883	0.317	0.248	0.102	0.351	0.569	0.444
	P	0.489	1.000	0.002	0.996	0.771	0.489	0.245	0.065	0.809	1.000	0.809	0.224	0.929	
	A	4	1	2	1	4	2	1	6	1	1	1	2	5	31
CO	Ho	0.500		0.250		0.750	0.250		1.000				1.000	1.000	0.432
4	He	0.643		0.250		0.750	0.250		0.929				0.571	0.893	0.409
	P	0.227		0.775		0.677	0.775		0.679				0.046	0.629	
	A	4	2	3	1	8	3	2	15	2	2	2	4	14	62
PU	Ho	0.647	0.188	0.471		0.813	0.111	0.111	0.944	0.167	0.077	0.056	0.722	0.722	0.437
18	He	0.635	0.175	0.551		0.843	0.110	0.108	0.922	0.157	0.077	0.056	0.640	0.852	0.470
	P	0.780	0.679	0.710		0.472	0.996	0.803	0.080	0.700	0.885	0.904	0.545	0.060	
	A	4	2	3	2	5	2	3	8	4	3	2	2	10	50
IRM															
M	Ho	0.429	0.143	0.667	0.143	0.429	0.143	0.429	0.857	0.429	0.286	0.143	0.429	1.000	0.450
7	He	0.495	0.143	0.591	0.143	0.670	0.143	0.385	0.912	0.571	0.275	0.143	0.363	0.934	0.522
	P	0.361	0.839	0.421	0.839	0.064	0.839	0.914	0.676	0.827	0.978	0.839	0.471	0.633	
	A	4	3	3	1	10	3	4	16	2	4	3	3	14	70
MN	Ho	0.684	0.400	0.263		0.900	0.100	0.167	0.895	0.211	0.200	0.105	0.211	0.833	0.423
20	He	0.593	0.337	0.240		0.888	0.099	0.162	0.933	0.193	0.191	0.104	0.383	0.903	0.458
	P	0.450	0.741	0.933		0.334	0.997	1.000	0.140	0.608	1.000	0.996	0.064	0.308	
	A	2	3	1	2	2	3	3	7	2	2	2	1	8	38
NA	Ho	0.500	0.333		0.250	0.000	0.250	0.417	0.833	0.182	0.100	0.083		0.750	0.312
12	He	0.464	0.301		0.228	0.429	0.236	0.359	0.848	0.173	0.100	0.083		0.848	0.406
	P	0.665	0.923		0.621	0.046	0.970	0.842	0.871	0.740	0.868	0.880		0.876	
	A	3	5	3	3	4	3	4	11	3	4	3	2	9	56
BB	Ho	0.174	0.565	0.083	0.087	0.500	0.250	0.250	0.958	0.250	0.136	0.083	0.125	0.625	0.341
24	He	0.240	0.565	0.082	0.086	0.563	0.228	0.233	0.873	0.230	0.132	0.228	0.120	0.730	0.375
	P	0.185	0.845	0.997	0.997	0.075	0.921	0.998	0.518	0.921	0.990	0.002	0.744	0.006	
	A	3	2	1	1	2	1	3	8	2	1	1	1	4	30
DM	Ho	0.400	0.400			0.000		0.400	1.000	0.200				1.000	0.291
5	He	0.378	0.356			0.533		0.378	0.956	0.200				0.733	0.356
	P	0.958	0.576			0.083		0.958	0.628	0.804				0.544	

	A	4	2	5	1	3	3	4	12	2	2	2	4	8	52
NAR	Ho	0.300	0.375	0.455		0.400	0.364	0.545	0.909	0.100	0.250	0.091	0.200	0.600	0.484
11	He	0.595	0.325	0.775		0.600	0.537	0.593	0.931	0.100	0.500	0.091	0.537	0.821	0.534
	P	0.024	0.514	0.399		0.644	0.297	0.680	0.425	0.868	0.187	0.875	0.107	0.476	
	A	4	3	3	2	6	2	2	9	2	3	2	3	6	47
NO	Ho	0.900	0.250	0.100	0.111	0.667	0.100	0.400	0.900	0.333	0.375	0.100	0.400	0.700	0.434
10	He	0.668	0.433	0.416	0.111	0.680	0.395	0.337	0.884	0.294	0.492	0.100	0.647	0.758	0.599
	P	0.608	0.042	0.018	0.860	0.159	0.020	0.429	0.772	0.549	0.767	0.868	0.246	0.945	
	A	2	2	3	1	2	3	2	8	1	2	3	1	4	34
AX	Ho	0.750	0.400	1.000		0.200	0.400	0.200	1.000		0.000	0.400		0.500	0.405
5	He	0.536	0.356	0.644		0.200	0.378	0.200	0.956		0.356	0.378		0.750	0.464
	P	0.230	0.576	0.172		0.804	0.958	0.804	0.628		0.025	0.958		0.544	
	A	4	2	4	2	6	3	2	11	2	2	1	3	9	51
UA	Ho	0.600	0.200	0.400	0.100	0.800	0.300	0.600	0.900	0.200	0.200		0.400	0.800	0.482
10	He	0.600	0.189	0.489	0.100	0.858	0.279	0.442	0.942	0.189	0.189		0.568	0.879	0.545
	P	0.002	0.725	0.930	0.868	0.074	0.958	0.175	0.433	0.725	0.725		0.343	0.100	
	A	6	3	5	1	7	2	4	15	3	4	2	5	11	68
MA	Ho	0.611	0.111	0.529		0.706	0.278	0.389	1.000	0.278	0.278	0.056	0.667	0.778	0.486
18	He	0.743	0.110	0.631		0.795	0.322	0.459	0.927	0.252	0.257	0.056	0.756	0.890	0.552
	P	0.598	0.996	0.832		0.828	0.631	0.067	0.461	0.926	0.998	0.904	0.711	0.355	
	A	3	4	3	1	7	3	3	14	4	2	4	4	12	74
NH	Ho	0.278	0.444	0.652		0.500	0.652	0.391	0.875	0.318	0.364	0.227	0.238	0.958	0.441
17	He	0.427	0.611	0.592		0.884	0.590	0.531	0.924	0.289	0.312	0.215	0.443	0.879	0.610
	P	0.179	0.890	<0.001		0.201	0.918	0.586	0.077	0.992	0.780	0.999	0.022	0.564	
	A	4	3	2	1	4	4	5	11	2	4	2	2	10	54
TR	Ho	0.350	0.450	1.000		0.188	0.200	0.650	0.800	0.056	0.316	0.056	0.050	0.500	0.409
20	He	0.406	0.535	0.513		0.546	0.345	0.526	0.868	0.056	0.360	0.056	0.050	0.777	0.469
	P	<0.001	0.710	<0.001		<0.001	0.274	0.914	0.461	0.904	0.978	0.904	0.909	<0.001	
	A	4	3	2	1	7	3	4	12	2	2	3	5	10	58
TP	Ho	0.813	0.188	0.125		0.688	0.375	0.500	1.000	0.133	0.063	0.125	0.688	0.750	0.472
16	He	0.647	0.179	0.226		0.808	0.401	0.421	0.879	0.129	0.063	0.123	0.718	0.738	0.482
	P	0.492	0.982	0.086		0.772	0.940	0.939	0.995	0.782	0.897	0.995	0.917	0.882	
	A	5	2	4	1	7	2	3	9	3	2	1	5	10	54
AL	Ho	0.385	0.077	0.231		0.846	0.231	0.154	0.769	0.250	0.154		0.692	0.846	0.399
13	He	0.566	0.077	0.566		0.831	0.409	0.151	0.880	0.236	0.148		0.662	0.828	0.504
	P	0.776	0.885	0.122		0.889	0.136	0.993	0.165	0.970	0.764		0.247	0.034	
	A	2	4	3	2	2	4	4	12	2	4	2	2	8	51
XI	Ho	0.421	0.350	0.350	0.053	0.150	0.100	0.300	0.800	0.050	0.200	0.100	0.200	0.600	0.320
20	He	0.444	0.314	0.456	0.053	0.142	0.191	0.276	0.931	0.142	0.276	0.097	0.185	0.697	0.385
	P	0.911	0.989	0.608	0.906	0.717	0.003	0.996	0.502	0.004	0.211	0.814	0.619	0.970	
	A	3	2	1	1	3	2	2	7	3	4	1	3	3	35
JA	Ho	0.667	0.333			0.444	0.333	0.333	0.889	0.111	0.889		0.444	0.889	0.475
9	He	0.582	0.294			0.386	0.529	0.503	0.850	0.307	0.608		0.386	0.569	0.445
	P	0.606	0.549			0.865	0.317	0.370	0.691	0.029	0.451		0.865	0.124	
	A	4	2	3	1	4	3	3	9	2	2	2	4	6	45
CA	Ho	0.867	0.133	0.933		0.667	0.467	0.333	0.800	0.333	0.286	0.067	0.333	0.733	0.505
15	He	0.641	0.129	0.605		0.614	0.384	0.453	0.887	0.287	0.254	0.067	0.306	0.648	0.462
	P	0.438	0.782	0.008		0.857	0.708	0.627	0.185	0.439	0.533	0.894	0.996	0.969	

Table 5 – Analysis of differences in physio-chemical properties of water in which different groups of *Symphysodon* occur. Data were taken from Tables 3 and 4 of Bleher et al. [16]. Data are in online supplement 1. The abacaxi and Xingu phenotypes were not included in analyses due to small sample sizes. Because of linear separation of the independent variables (pH and Conductivity) in tests involving the Heckel phenotype, it was not possible to include both variables in the same model, and therefore variables were analyzed separately. Linear separation also occurred in pH due to non-overlapping pH values for the Heckel and blue phenotypes.

	Heckel *Symphysodon discus*	Green *Symphysodon tarzoo*	Blue *Symphysodon* sp. 1
Green *Symphysodon tarzoo*	pH: p = 0.022 Cond: p = 0.158		
Blue *Symphysodon* sp. 1	pH: p < 0.001 Cond: p = 0.144	pH: p = 0.514 Cond: p = 0.549 pH:Cond: p = 0.546 Wald test: p = 0.770	
Brown *Symphysodon aequifasciatus*	pH: p = 0.019 Cond: p = 0.004	pH: p = 0.013 Cond: p = 0.059 pH:Cond: p = 0.059 Wald test: p = 0.038	pH: p = 0.005 Cond: p = 0.021 pH:Cond: = 0.020 Wald test: = 0.019