Journal of the American Killifish Association

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January — March, 2021
Volume 54 Number 1.

Journal of the American Killifish Association

A quarterly publication of the American Killifish Association, Inc.
for distribution exclusively to members.

Volume 54, No. 1
Jan — Mar. 2021

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Photographic Acknowledgements: The American Killifish Association thanks the photographers who generously contributed to this issue: Jim Gasior, Greg Niedzielski, Charles Nunziata, Alf Persson, Rudolf Pohlmann, Fritz Rohde, Scott Smith, Tony Terceira & Frans Vermeulen.

The George Maier Fund: The Maier Fund was organized to support the study of killifish. Donations directly fund projects that investigate killifish reproduction, life cycle, maintenance, nutritional requirements and food sources, biology, ecology, habitat, conservation, nomenclatural and biological relationships. Applications are accepted from anyone regardless of race, ethnicity, gender, or religious affiliation. Donations can be made to donations@maierfund.org", either directly through www.paypal.com or from the American Killifish Association website; www.aka.org. Alternatively, make checks to the "George Maier Fund", mail to "The George Maier Fund, c/o Jack Heller, 14212 Trail Top Drive Chesterfield, MO. 63017. For more information, contact Charles Nunziata at epiplaty@tampabay.rr.com.

Cover: *Rivulus* sp. collected Northwest of Manacapura, Brazil. © Scott Smith

ISSN 0002–9674, USPS 533–940

Collecting *Rivulus* near Manaus, Brazil:
A George Maier Fund Award
Fritz Rohde, Scott Smith, and Dustin Smith

We have been surveying the fishes, focusing on *Rivulus*, around Iquitos and Pucallpa, Peru from 2014-2017 (four trips). We have taken photographs and fin clips from most. Several *Rivulus* experts said that some of our populations were *Rivulus ornatus*, but we were skeptical since the type localities were some 1,300 river miles downstream of Iquitos. In order to start making sense of this, we contacted Dr. Jean Huber and he recommended that we travel to Brazil and specifically to Silves, which he had designated as the official type locality for *Rivulus ornatus* (and recognized by Costa, 2003). So, we successfully applied for a Maier Grant from the American Killifish Association to partially fund our trip to Manaus, Brazil in August 2018.

The goal of this project was to sample, identify, photograph, and fin clip killifishes of the genus *Rivulus* from the vicinity of Silves and Manaus, Brazil, with special attention to recollecting *Rivulus ornatus* and *Rivulus obscurus* from their type localities.

Two of the leading experts on the genus *Rivulus* have been at odds on what these two species really are for at least 27 years. To quote Huber: "This is a complicated case and a confusion due to the simultaneous publications of two authors at the end of 1992 and the beginning of 1993, without knowing the project of each other." First, the author's Rivulus book (Huber, 1992) where lectotypes of *R. ornatus* and *R. obscurus* are designated; and second, Costa's paper in the DKG Journal (Costa, 1993) where he mentions that the true *R. ornatus* is *R. obscurus* according to material from near Manaus (3.130S, 60.020W). The aquarium *R. ornatus* is then an unknown species, without knowing the lectotype designation for *R. ornatus* from Silves, i.e. not in the area of Manaus.

This is unfortunate and lies only in the lack of communication between Costa and Huber. But the problem actually comes from the fact that in his descriptions, Garman (1895) used several lots for *R. ornatus* with three different and distant localities, and one lot for *R. obscurus* with a type locality near Manaus, identical with one of the three for *R. ornatus*.

For *R. ornatus*, the three localities are Silves, Lake Saraca, N. Brazil {2.880S, 58.350W}; Parana do Janauari, Brazil {3.200S, 60.080W} (near Manaus); Lago Cudajas, now L. Badajos, N. Brazil {3.250S, 62.780W}, and for *R. obscurus*, Lago Januaria, in the vicinity of Manaus, N. Brazil {3.200S, 60.080W}. By selecting Silves as the official type locality for *R. ornatus* without knowing Costa's research, Huber made Costa's publication inappropriate because Costa allocated *R. ornatus* to a fish we do not know to be identical with the fish from Silves.

Let's wait until live material is collected from the two fixed type localities, Silves for *R. ornatus*, and Januaria, near Manaus for *R. obscurus* to fix the issue. That, in turn, will address another issue, the identification of the aquarium

strains of *R. ornatus*: one the aquarium import from Obidos (?), lower Amazon (NSC-2), Brazil {1.920S, 55.520W} and the other from Padre Isla, Iquitos, Peru {3.620S, 73.700W}."

Costa (2006) examined the type specimens of both species and determined that *R. ornatus* consisted of males and *R. obscurus* were all females of the same species and thus synonyms. Huber (2020) mentions Costa's synonymizing of *obscurus* into *ornatus* but dismisses it since Costa did not address Huber's arguments and brings no new data to his 1993 move. Additionally, Huber (1992, 2020) has recognized several populations of *Rivulus* in Peru as *R. ornatus*, but Costa (2006) maintains that these are misidentified.

Based on pre-travel literature review (Huber 1992; Costa 2004, 2006; Hrbek et al. 2004) we expected to find anywhere from three to six species of *Rivulus* around Manaus, depending on how many of them might be synonyms. Huber (1992) listed *Rivulus atratus*, *R. micropus* (possible syn. *compressus*), *R. obscurus*, *R. ornatus*, and *R. strigatus* (synonym *R. geayi*) from the region. Costa (2004) described *R. kirovskyi* from Manaus, synonymized *R. compressus* with *R. micropus*, and inexplicitly added *R. taeniatus* to the region (its type locality is in Colombia) as well as synonymizing *R. obscurus* with *R. ornatus* (Costa 2006).

When Hrbek et al. (2004) described *R. duckensis* (synonym of *R. kirovskyi*) from Manaus, they said there were only three taxonomically valid species of *Rivulus* from the central Amazonian area. These are *R. obscurus* (synonym *R. ornatus* and probably *R. atratus*), *R. compressus* (probable synonym *micropus*), and *R. geayi* (probably *R. dibaphus*). And to further muddy the waters, Fernandes (2014) found *R. kirovsky*, *R. micropus*, and *R. obscurus* in streams in the Ducke Reserve in Manaus.

After four successful trips to Peru, we felt confident that we would be as equally successful in our trek to Brazil. One of the things that made Peru work so well was that we had contacts already there to assist us in our work. In February, we contacted an ichthyologist in Manaus and a past George Maier Grant recipient. He responded that he would get back with us soon and seemed interested, but after several months of not hearing from him, we got concerned. We tried several other researchers in Manaus but results were negative.

At this point, we even considered canceling the trip and returning the funds, but we contacted Frans Vermeulen in June and he came through. He has a British friend, Wayne, who has lived in Manaus for 16 years and is interested in tropical fishes and aquatic plants. We contacted Wayne via Facebook Messenger and he seemed eager to help, but he would be out of touch until we almost got to Manaus, which made us a little nervous since we tend to over plan things.

We arrived in Manaus late on July 31, 2018. Wayne met us the next morning at our hotel where we spent a considerable amount of time explaining what we wanted to do and where we wanted to go. He informed us that we should not go to Silves (the designated type locality for *R. ornatus*) for several reasons: primarily long distance and safety. However, he did have a friend with a boat so we could explore some areas around Manaus

that way (another type locality for *R. ornatus* as well as *R. obscurus*).

So not a good start to our trip, but it quickly got better. We went to Wayne's place to see his facilities and to have a sumptuous lunch of Arapaima, two rice dishes, beans, and salad prepared by his then girlfriend, now wife, Jane. Since we were eager to get started, Wayne drove us to a stream on the outskirts of Manaus (Site 1, Figures 1, 2). Instant success! We collected a handful of cichlids, *Macrobrachium* sp., tetras, and *Rivulus* aff. *micropus* (Figure 3).

August 2:

The next morning, we headed over to Igarapé Tarumã-açu, a tributary of the Rio Negro (Site 2) on the west side of Manaus. In that large body of water, we noticed small, almost transparent fish,

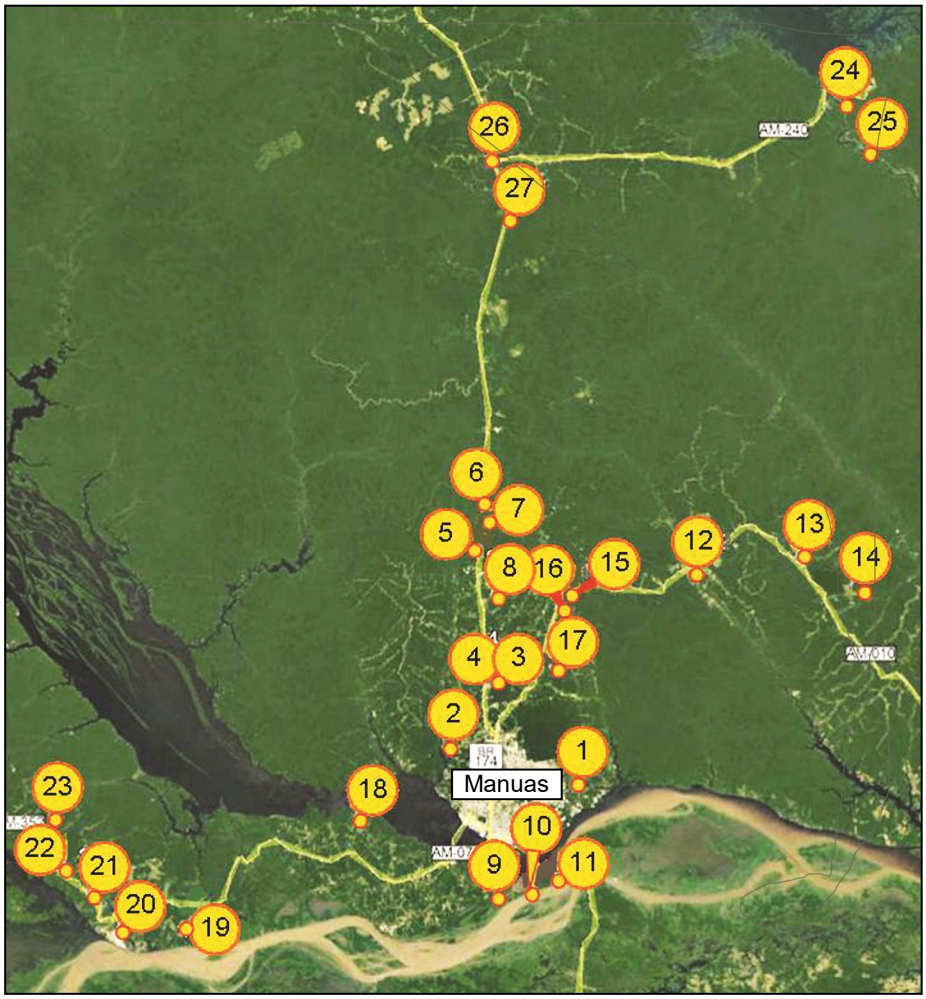

Figure 1: Collecting locations in the vicinity of Manuas, Brazil.
Large symbol: Site location number referenced in text. Small symbol: Site location.
[Google map: Region around Manuas, Brazil, 2020]

Figure 2: Site 1: A stream on the outskirts of Manaus. © Fritz Rohde.

Figure 3: Site 1: *Rivulus* aff. *micropus* (See Addendum) © Scott Smith.

Figure 4: *Fluviphylax* species (possibly *F. pygmaeus*) © Scott Smith.

which turned out to be *Fluviphylax pygmaeus* (or *obscurus*) (Figure 4). Unfortunately we didn't have the right equipment with us to take a quality photograph of such a small species.

Adjacent to the swimming beach was a small spring where we collected several specimens of a very attractive *Rivulus*, which we later determined via DNA to be *R. ornatus* (Figure 5). See Addendum for genetic results.

We next headed north to a sizeable lake (Site 3) on the Rio Igarapé Leao, notable mainly because of its outdoor toilet with the best views in all of Amazonia (Figure 6). We did not anticipate catching any *Rivulus* in the lake, but we did get 11 species of characins and cichlids. From here, we walked some 600 yards into the forest where Wayne knew there was a small stream in a tiny village (Site 4, Figure 7). We caught what we believe to be *R. compressus* (Figure 8).

We then headed 14 miles north on BR 174 to Site 5, an outlet of another lake (Figure 9). We collected two species of *Rivulus*, including one we think is somewhat distantly (genetically) related to *R. kirovskyi* (Figure 10), and the other was a female *R. ornatus (*Figure 11).

The next two sites (6 and 7) were busts but we caught a *Rivulus* at Site 8

Figure 5: *Rivulus ornatus* male © Scott Smith.

Figure 6: Amazonian bathrooms. © Fritz Rohde.

Figure 7. Site 4. © Fritz Rohde.

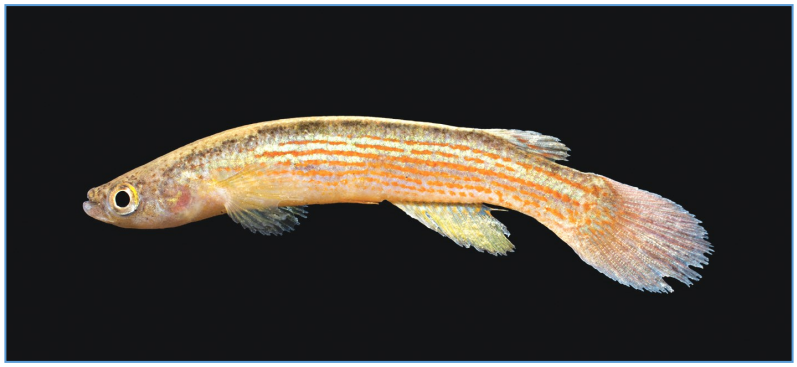

Figure 8. *Rivulus compressus*. © Scott Smith.

Figure 9. Collecting at Site 5. © Fritz Rohde.

Figure 10. *Rivulus* sp. © Scott Smith.

Figure 11. *Rivulus ornatus* female. © Scott Smith.

Figure 12. *Rivulus ornatus* male. © Scott Smith.

(Figure 12) similar to the ones caught at Site 2. All in all, a good day.

August 3:

When planning this trip, we wanted to re-visit the type localities of *R. obscurus* (Lake Januaria near Manaus) and *R. ornatus* (Silves, Lago Badajos, Lago Alexo, and Lake Januaria). Since Silves was out, we decided to spend several days around Lake Januaria, which is south of Manaus across the Rio Negro. Piece of cake. Wayne has a British friend, John, who had a boat, and they both were eager to do this. The boat looked seaworthy (Figure 13) so we headed out.

In our experience in Peru, we knew that the water should be fairly low in August, but we had not factored in how far downstream we were from Peru. So, after an uneventful crossing of the Rio Negro, we entered a flooded forest (Figure 14).

John and Wayne decided they knew a short cut to our destination, so we veered

Figure 13. Lake Januaria. © Fritz Rohde.

Figure 14. The flooded forest. © Fritz Rohde.

away from the main channel into the forest. Wayne stood up in the bow and emphatically yelled "John, this way!", which John promptly ignored and went the other way, often bouncing off trees and raining all kinds of insects and branches upon us. What is life without a little adventure? A complicating factor in all this was that he could not get the engine into reverse since his seat placement was preventing the shift from engaging. We eventually went aground so we hopped out and started dipping at Site 9. Bingo! Another male *R. ornatus*. (Figure 15). It was about this point that we began noticing Wayne's uncanny ability to locate *Rivulus*.

After getting the boat unstuck we ventured further south into the Rio Solimões (which is what the Amazon is called above the confluence with the Rio Negro) where we caught a female *R. ornatus*. This was considered by many to be *R. obscurus*, but mitochondrial DNA analysis proved that it was genetically identical to the male pictured in (Site 10, Figure 16) (See Addendum).

Since the day was getting shorter we decided to head back to Manaus via the Amazon River. We did make a collec-

Figure 15. *Rivulus ornatus* male, caught at site 9. © Scott Smith.

Figure 16. *Rivulus ornatus* female from Site 10 is genetically identical to male shown in Figure 15. © Scott Smith.

tion at Site 11 along the banks of the river but caught only characins, catfishes, and cichlids. As we neared Manaus, we managed to hit the only log floating in the river and lodge it between the transom and the motor's low unit. After a bunch of grunting and yanking, Scott and Dustin got it loose. The ride back up the Amazon and Rio Negro was rough and slow as the wind had shifted creating a serious chop. When we finally made it back safely to our landing, we decided that one day of boating was enough.

August 4:

Back on dry land, we headed northeast from Manaus on AM-010 (Figure 17), first to Rio Preto de Eva (Site 12) where we dip netted along the shore of the river and in some swampy areas. We caught several nice-looking *R. compressus* (Figure 18) and a female similar to that from Site 10 (Figure 19).

Site 13 was a bust, but we got a beautiful male (Figure 20) at Site 14, similar in appearance to *R. compressus*. After site 14, we were caught in one of those famous tropical rain showers, which made for a scary ride while dodging potholes and hydroplaning. This was the easternmost site we collected on this trip.

Site 15 was interesting in that after scaring off a group of monkeys we had to slide down an almost vertical drain into a

Figure 17. Heading northeast from Manaus on AM-010 to Rio Preto de Eva, Site 12. © Scott Smith.

Figure 18. One of several *R. compressus* caught at Rio Preto de Eva (Site 12). © Scott Smith.

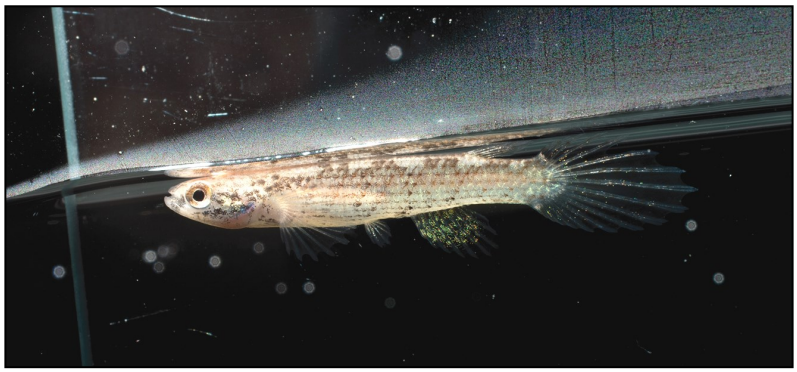

Figure 19. *R. ornatus* female similar to those caught at Site 10. © Scott Smith.

Figure 20. This beautiful male is similar to *R. compressus* caught at Site 14. © Scott Smith.

swampy palm grove. Wayne always managed to find water for us to sample. He would often look down a side road and muse, "Now, that looks interesting!" and it always was! We collected more *compressus*-looking fish and a female *ornatus* (Figure 21, 22).

Site 16 consisted of a culvert and ditch full of scour holes and was out in the open. Again, more *compressus*-looking fish (Figures 23, 24).

We wrapped up the day with a bang at Site 17. Wayne followed his nose and took us down several side roads where we finally parked and then walked down a trail to a tiny stream, which also served as a water supply for several nearby houses. It was an interesting road, freshly cut into virgin jungle and lined with palm trees whose leaves rose roughly 40 feet and curled over the road. You can imagine our excitement when we caught tiny bright orange and yellow *Rivulus* plus *compressus* (Figures 25-28).

August 5:

Once again, we crossed over the Rio Negro, this time via the beautiful bridge on AM-070 on our way to Manacapura (Figure 29). We first headed towards the historic ruins along the river at Vial de

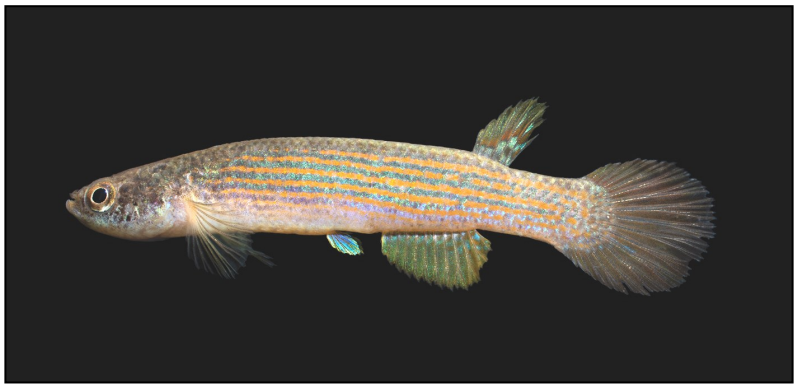

Figure 21. An *R. compressus* looking fish caught at Site 15, a swampy palm grove. © Scott Smith.

Figure 22. A female *R. ornatus* also caught at Site 15. © Scott Smith.

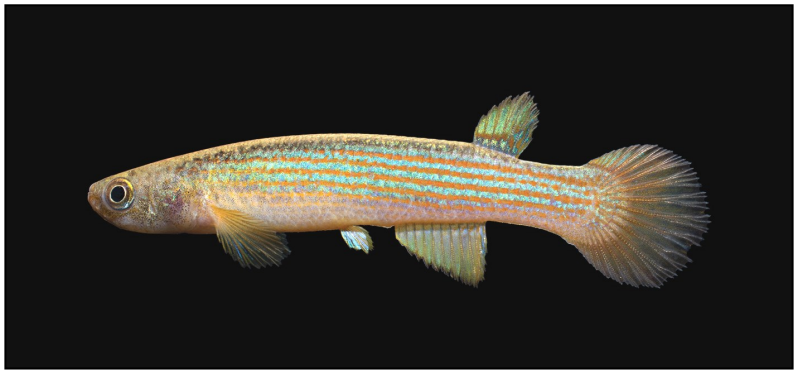

Figure 23. This *R. compressus* looking fish caught at Site 16, an open culvert and ditch. © Scott Smith.

Figure 24. Another *R. compressus* looking fish caught at Site 16. © Scott Smith.

Figure 25. Unidentified *Rivulus* species, small and brightly colored, caught in a small stream at site 17. © Scott Smith.

Figure 26. Another unidentified *Rivulus* species caught at site 17. © Scott Smith.

Figure 27. Another unidentified *Rivulus* species caught at site 17. © Scott Smith.

Figure 28. *R. compressus* caught at Site 17. © Scott Smith.

Paricatuba. This was more of a sightseeing detour than a fishing one, but it was a neat stop. Our first site (18) was nearby where we caught what we believe is *R. micropus* (Figure 30).

Site 19 was further up the highway and in an open creek/swampy area (Figure 31) where we caught some attractive cichlids and a *R. micropus* (Figure 32).

Site 20 was behind Wayne's friend's hotel in Manacapura. While it looked promising, we did not catch any *Rivulus*. Heading northwest from there, we collected two new and beautiful *Rivulus* at Site 21, genetically similar to that collected at Site 5. (Figures 33-35).

Site 22 (Figure 36) was below a water park that was undergoing construction and two *Rivulus* were present. One was *R. micropus* (Figure 37) and is genetically identical to the one we collected at Site 19. The other species (Figure 38) is genetically similar to that collected at Site 21.

While it looked promising, Site 23 was bust with no fishes present so we headed back to Manaus.

August 6 – last day

With high hopes we headed north once again on BR 174 towards Presidente

Figure 29: Rio Negro, AM-070 bridge on the way to Manacapura. © Fritz Rohde.

Figure 30. This specimen, thought to be *R. micropus,* was caught at site 18. © Scott Smith.

Figure 31 Site 19, cichlids were found in this open creek/swampy area. © Fritz Rohde.

Figure 32: *R. micropus* found at site 19. © Scott Smith.

Heading northwest from site 20, two new and beautiful *Rivulus species,* Figures 33, 34 and 35 were collected at Site 21. These specimens were found to be genetically similar to the fish collected at Site 5.

Figure 33. © Scott Smith.

Figure 34. © Scott Smith.

Figure 35. © Scott Smith.

Figure 36: Site 22: Two *Rivulus,* Figures 37 and 38, were found at this site, located below a water park. © Fritz Rohde.

Figure 37: *R. micropus,* is genetically identical to the fish collected at Site 19. © Scott Smith.

Figure 38: The other species collected is genetically similar to the fish collected at Site 21. © Scott Smith.

Figueiredo. Our first stop (Site 24) was east of that city near the Represa de Balbina on the Uatumã River. Here we caught a beautiful *Rivulus uatuman* (Figure 39) as well as the non-*Rivulus* highlight of the trip, *Poecilocharax weitzmani* (Figure 40). Farther up that dirt road, we caught two additional species at Site 25 (Figure 41, 42).

Site 26 was in a waterfall park (Parque Urubui) in Pres. Figueiredo on the Igarapa Santa Cruz (Figure 43). We collected two different species, *R.* aff. *compressus* and one which Frans Vermeulen says is in the *agilae-geayi-strigatus* group and genetically aligns with *R. dibaphus* (see Addendum), in a seepage area in the woods adjacent to the park (Figures 44, 45, 46).

The final collection of the trip was at Asframa Falls off BR 174 (Site 27, Figure 47) where we collected *R.* aff. *compressus* (Figure 48)

During those 5-1/2 days, we visited 27 sites (Figure 1) and caught *Rivulus* at 20 of them. What species we got we are still unsure of, but we estimate that we caught at least 8 and up to 11 different species, several of which appear to be undescribed.

Addendum: Genetic Results

We collected with dip nets at 27 sites around Manaus, Brazil from August 1

Figure 39: *Rivulus uatuman* caught at site 24. © Scott Smith.

Figure 40: The non-*Rivulus* highlight of the trip, *Poecilocharax weitzmani*. was also caught at site 24. © Scott Smith.

Figure 41 above and 42 below are unidentified species caught at site 25. © Scott Smith.

Figure 43: Site 26: This site is in a waterfall park, Parque Urubui in Pres. Figueiredo on the Igarapa Santa Cruz © Fritz Rohde.

Figure 44: *R.* aff. *compressus* caught in a seepage area in the woods adjacent to the park. © Scott Smith.

Figure 45 (top) male, belongs to the *R. agilae-geayi-strigatus* group.
Figure 46 (above) female *R.* aff. *compressus*: Collected with Figure 44.
© Scott Smith.

Figure 47: Site 27: This is the last collection site of the trip;
Asframa Falls off BR 174. © Fritz Rohde.

(afternoon) through August 6, 2018 (Figure 1). Fin clips were taken from 23 individuals collected at 17 different locations. Tissue for genetic assay was harvested from fin clips that had been stored in 95% ethanol. Total genomic DNA (gDNA) was extracted following the manufacturers' protocol (Qiagen, Inc.). The presence of high molecular weight template DNA was confirmed by electrophoresing 5 µL of each extraction in 1.5% agarose gels and visualizing with ultraviolet light. Extracted DNA was used as template for the polymerase chain reaction (PCR), with each reaction consisting of ~10 ng template DNA. We

Figure 48: *R.* aff. *compressus* collected at Site 27. © Scott Smith.

amplified a portion of the 12S rRNA mitochondrial locus using primers 12sar and 12sbr described in Palumbi (1996). Successful PCR amplifications were prepared for direct sequencing by incubating 10 µL of PCR product with 1 µL of a 3:1 ratio of shrimp alkaline phosphatase (1 unit/µL): exonuclease I (20,000 units/mL) at 37°C for 30 min, followed by a deactivation step at 80°C for 20 minutes.

Preparations were then cycle-sequenced in both the forward and reverse directions using Applied Biosystems Big Dye Terminators (v. 3.1) and each of the original PCR primers in separate sequencing reactions. DNA sequences were obtained from an Applied Biosystems 3130xl genetic analyzer. Results of forward and reverse DNA sequencing reactions were edited in Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) and then aligned using the default settings in Clustal W (Thompson et al. 1994).

Phylogenetic hypotheses were constructed using the Neighbor-Joining method (Saitou and Nei 1987) as implemented in MEGA X (Kumar et al. 2018). Neighbor-joining trees were constructed using pairwise Maximum Composite Likelihood (Tamura et al. 2004) distances calculated with the default settings. Bootstrapping (Felsenstein 1985) was used as an indicator of support for individual branches in the Neighbor-Joining phylogenies (500 replicates).

We also added nine sequences (eight species) obtained from GenBank, which were sequenced by Hrbek et al. (2004). The 12s tree is presented in Figure 49.

Rivulus ornatus versus *R. obscurus*

Based on our genetic analysis, Costa (2006) was correct. The description of *R. obscurus* was based on female *R. ornatus.* As seen in Figure 49, *R. ornatus* shows reciprocal monophyly; from a geographical standpoint one monophyletic group is from localities north and northeast of Manaus, and the second monophyletic group is found across the Rio Negro, near Lake Januaria, one of the type localities. Interestingly, *R. ornatus* from the Rio Purus, over 100 miles west of Manaus aligns with the second group. How significant the difference is between the two groups needs further study.

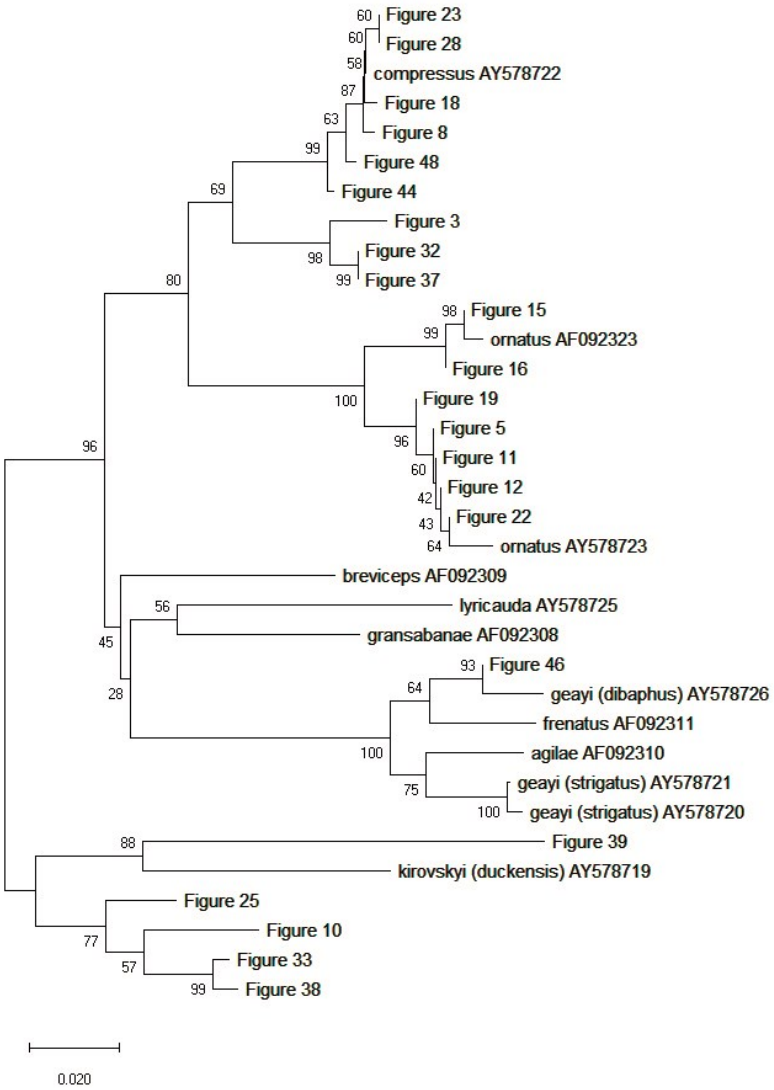

Figure 49: Phylogenetic tree illustrating results of the genetic analysis.

As mentioned earlier, Garman (1895) described. *R. ornatus* from three widely separated localities. He states "Pectorals elongate, as long as the head, reaching nearly to the ventrals. Caudal elongate, pointed. The markings of this fish are made up of puncticulations; they form transverse blotches along the back, streaks along the sides, series of dots across the fins, a dark band on the lower lip, and a dark streak backward from below each eye." All of which match the fish in Figure 50.

Relationship of *R. kirovskyi* (*duckensis*) Hrbek et al. (2004), based on their analyses, said that *R. duckensis* was most likely sister taxon to *R. rectocaudatus* from Peru and two additional species from Venezuela. Our analysis indicates that *R. uatuman* (Figure 39, Site 24(is closely related to *R. kirovskyi* and these two are a sister taxon to a group, which are likely composed of undescribed species (Sites 5, 17, 21, 22).

Rivulus compressus versus *R. taeniatus* The type locality for *R. compressus* is Manaus, Brazil while *R. taeniatus* was described from Morelia, Rio Caqueta drainage, Colombia (upper northwest

Figure 50: The original description of *R. ornatus,* Garman (1895) matches the characteristics of this specimen. © Scott Smith.

Amazon basin). Costa (2006) gave diagnostic characters for *R. taeniatus* that came from specimens collected at five widely scattered areas in central and western Brazil, including Manaus. These characters include short jaws, often with red pigment, a blunt snout, tip of anal fin rounded in the male, and pelvic-fin tip usually not reaching the anus and never the anal fin base. Many of these characters are evident in the fishes we are calling *R. compressus*. Until we can get genetic material from the type locality of *R. taeniatus* or from the western locations that Costa studied, we believe the ones near Manaus are *R. compressus*.

Rivulus micropus

One clade that consisted of Sites 1, 19, and 22 is sister to the *R. compressus* clade. We are calling them *R. micropus* since this species was initially described from somewhere in the Rio Negro, Brazil. Several characters that Costa (2006) used to diagnose this species are slightly elongated jaws, pointed snout, tip of anal fin slightly pointed in the male, and pelvic fin tip reaching anal fin base. These characters are particularly evident in Figure 30. We never collected *R. compressus* and *R. micropus* together at the same site. Fernandes (2014) included a photograph of what she was calling *R. micropus*. Based on our work, it more closely resembles *R. compressus*.

Anablepsoides and *Laimosemion*

Costa (2011) used a combination of mitochondrial and morphological data to elevate seven subgenera of *Rivulus* to full generic status. Although this elevation has not been universally accepted, our

analysis indicates that the species in the upper half of the tree are all included in *Anablepsoides* while those in the bottom half of the tree are all included in *Laimosemion*.

Acknowledgements:

The authors greatly appreciated the help and friendship of their new Brazilian friends, Wayne and John, who made the trip a success. We thank Joseph M. Quattro for graciously sequencing the fin clips and offering genetic advice.

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