Using Molecular Biology Techniques to Characterize the Diversity of Amazonian Ornamental Fishes.

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ABSTRACT

This paper briefly reviews the genetic studies already performed in Amazon fishes and propose some methods that may be addressed to the study of genetic diversity of ornamental fishes in the region. From a suggested list of approximately 200 fish species to be traded in the aquarium industry in the Amazon, the cardinal tetra (*Paracheirodon axelrodi*) and discus (*Symphysodon* spp.) are among the most prized specimens. Despite the intense exploitation of these resources for several decades, basic biological and ecological parameters, including the genetic structure of the populations and their renewal capability, are poorly known. Up to the present, genetic studies in the Amazon included cytogenetics, isozyme electrophoresis, and DNA sequencing. The diploid/haploid chromosome numbers have been determined for less than 20 ornamental fish species, isozymic studies were performed in about 5 species, and partial DNA sequences are available for less than 10 species. Most of these studies relate to fish evolution and biodiversity including phylogeny and of electric fishes (Gymnotiformes), catfish (Siluriformes), characins (Characiformes), and cichlids (Perciformes), and essentially no publication is available about population genetics or stock assessment. Therefore, as a continuum of Project Piaba, we propose that along with project Piaba 2000 chromosomal and molecular biology (microsattelites, PCR-RFLP, RAPD and DNA sequencing) should be used as molecular tools in order to characterize genetically the most relevant fish species being exploited in the Barcelos region. This will allow us to learn about their genetic diversity as well as the renewal capabilities of the stocks.

RESUMO

Este trabalho apresenta uma breve revisão dos estudos genéticos já realizados em peixes amazônicos e propõe alguns métodos genéticos que podem ser direcionados ao estudo da diversidade genética de peixes ornamentais desta região. De uma lista sugerida de aproximadamente 200 espécies de peixes ornamentais para serem

comercializadas na Amazônia, o cardinal (Paracheirodon axelrodi) e o acará-disco (Symphysodon spp.) são as espécies de mais destaque. Independente da intensa exploração destes recursos nestas últimas décadas, vários parâmetros biológicos e ecológicos, incluindo a estrutura genética de populações e sua capacidade de sustentação, ainda são pobremente conhecidas. Até o momento, os estudos genéticos em peixes amazônicos foram os cromossômicos, eletroforese de enzimas e proteínas e o sequenciamento de DNA. O número cromossômico diplóide/haplóide foi determinado para menos que 20 espécies de peixes ornamentais, aproximadamente 5 espécies para estudos isozímicos e menos que 10 quanto ao estudo do DNA. A maioria desses registros faziam parte de trabalhos relacionados com estudos evolutivos e biodiversidade, incluindo filogenia e biogeografia de peixes elétricos (Gymnotiformes), bagres (Siluriformes), caraciformes (Characiformes), e ciclídeos (Perciformes). Praticamente não há nenhuma publicação sobre genética de populações ou genética de estoques dos peixes ornamentais. Portanto, como uma continuidade do projeto Piaba, nós estamos propondo que ao longo do projeto Piaba 2000 estudos cromossômicos e moleculares (microsatélites, PCR-RFLP, RAPD e sequenciamento de DNA) sejam utilizados como ferramentas moleculares com o intuito de caracterizar geneticamente as espécies de peixes ornamentais mais relevantes na região de Barcelos para dessa maneira entender a diversidade genética existente e a capacidade de renovação dos estoques.

INTRODUCTION

Knowledge of the genetic diversity of an organism is one of the crucial aspects to be known for both basic and applied conservational purposes. The Amazon Basin is well known for his extreme biodiversity, which includes about 2,500 fish species, with approximately 10% of them having a potential to be exploited as ornamental fish. Traditionally, studies on fish genetics conducted in the region addressed mainly cytogenetics (chromosomes) or the final product of DNA (proteins and isozymes). The use of DNA sequence data and new types of molecular markers have opened a new perspective in the evolutionary and genetic studies of the ichthyofauna.

Fish genetics began to be studied in the Amazon region in the late 70s. In 1978, a series of preliminary studies were carried out with cytogenetics and isozymes of several fish species. This was the first effort to establish a laboratory at the Instituto Nacional de Pesquisas da Amazônia, which have been consolidated four years later. Since then, several papers have demonstrated the existence of a significant genetic diversity in the species analyzed and more recently we have seen reviews about the Amazonian fish genetics (Almeida-Val *et al.*, 1991), fish cytogenetics (Porto *et al.*, 1993), and fish physiology (Val & Almeida-Val, 1996). Lately, DNA sequence analysis

also began to be used and a series of papers, which have contributed to understanding the evolutionary relationships of some fish groups (Alves-Gomes *et al*., 1995; Ortí *et al*., 1996; Farias *et al*., 1999; Alves-Gomes, 1999) are currently available.

In this paper we review the studies performed with Amazonian fish species using only cytological (chromosomes) and molecular (Sequencing, RAPD, and PCR-RFLP) markers as a tool to screen fish diversity. The data derived from these studies have been employed in investigations of organism evolution, questions regarding phylogenetic relationships and in a lesser degree differences at the population level. In doing this we expect to lay out a framework that may serve as an preliminary proposal of using our current background to study the genetic structure of ornamental fish in the Rio Negro Basin by means of molecular markers.

CHROMOSOMES

Chromosomes are cytological units comprised of highly condensed DNA and associated with a series of proteins. When the chromosomes are ordered according to their size and shape and identified by differential staining, they constitute the karyotype, which is believed to be unique for the great majority of species. Karyotypic data have contributed to the studies of biology, genetics, and systematic of Amazonian fish fauna. This is particularly true in respect to diagnosis of sibling species (Nakayama, 1997), detection of polymorphism (Porto & Feldberg, 1993; Feldberg *et al*, 1999), detection of supernumerary chromosomes (Alves *et al*., 1999), and detection of natural triploid (Giuliano-Caetano & Bertollo, 1990). Besides, chromosomes are thought to be related with processes of speciation.

Porto *et al*. (1993) provided a checklist of karyotypic data of Amazonian freshwater fishes from where it was possible to show that more than 200 nominal species occurring in this region have had their chromosome number determined. However, no more than 20 ornamental fish species were karyotyped. As observed in Table 1, chromosome numbers in Amazonian fishes may range widely, such as from 2n=22 in a pencilfish (*Nannostomus unifasciatus*) to 2n=134 in an armored catfish (*Corydoras aeneus*). The characins (Characiformes) are the best represented in the checklist, followed by cichlids (Perciformes), catfishes (Siluriformes) and electric fishes (Gymnotiformes). Other fish groups such as sciaenids, Osteoglossiformes and Lepidosireniformes also are present. The chromosome number alone is sometimes sufficient to differentiate two species. However, there are some families or subfamilies whose chromosome number is very conservative (i.e., bryconins, thriportheins, anostomids, curimatids, prochilodontids, hemiodids, chilodids) although there are exceptions in each group.

At the present, despite the considerable development of cytogenetics, it is still a

challenge to study the chromosomes of ornamental fishes such as the tetras (Tetragonopterinae), the cardinal/neon tetras (Cheirodontinae), and the pencil fishes (Lebiasinidae) mainly because of their size. However, Europeans analyzed these species in the 60's and 70's, at the very beginning of cytogenetic studies, when chromosome preparations were still being developed (Post, 1965; Lueken & Foerster, 1969; Scheel & Christensen, 1970; Scheel, 1972; Scheel, 1973). The cardinal/neon tetras were a target of debate in past issues of Tropical Fish Hobbyists magazine more than 10 years ago: the questions at the time were: do they belong to the same genus? Do they belong to the same subfamily or evolutionary branch? In the debate, the chromosome features of the cardinal and neon tetras were claimed to corroborate the hypothesis of one group (Géry & Mahnert, 1986) but not by the other (Weitzman & Fink, 1987). The current belief is that cardinal/neon species are *incertae sedis* inside Characidae family.

Beyond characins, *Corydoras* catfish and cichlids have been considered as an appropriate group to be studied cytogenetically due to several chromosomal peculiarities. *Corydoras* and related genera have undergone events of gain and loss of DNA through events of polyploidization (Oliveira *et al*., 1993). This is the only record known for an Amazonian fish family. The cichlids were thought to be conservative in terms of chromosomal number. However, if we consider the morphology of chromosomes, the location of nucleo;ar organizer regions (NORs) and the pattern of heterochromatin (C-banding), the group cannot be considered so conservative. The two "discus" species, which belong to the genus *Symphysodon*, are one example. Both species (*S. discus* and *S. aequifasciata*) present the highest chromosome number recorded for cichlids, i.e., 2n=60. They also present a series of rarely found microchromosomes. The different pattern of NORs sites makes it possible to differentiate two species by their karyotypes (Salgado, et al.,1996a,b). Such karyotypic characteristics open a new perspective for the identification of additional chromosomal differences in the different subspecies and strains of "discus" which can enormously help the understanding of the genetic structure of this important resource.

In summary, in approximately two decades of chromosome study in the Amazonian fish species we have learned how to deal with the tiny chromosomes, when compared to chromosomes of other vertebrates, and how to extract cytosystematic information of these cytological units. Despite the fact that cytogenetics have been used for such long time, there still is space for development of the technique because published karyotypes of many species are still lacking. However, we are confident to say that chromosomes may tell us many important things but, depending of the question being addressed, other technique that can be more informative must be considered.

Table 1. The range of diploid number of selected groups of Amazon fishes.

Order	Family	Genus	Species	2n
Osteoglossiformes				
	Arapaimidae	1	1	56
	Osteoglossidae	1	2	54-56
Characiformes				
	Anostomidae	7	21	54
	Characidae	21	61	32-54
	Chilodontidae	2	2	54
	Ctenolucidae	1	1	36
	Curimatidae	7	14	46-102
	Erythrinidae	3	4	48-50
	Gasteropelecidae	2	2	48-54
	Hemiodidae	4	10	54
	Lebiasinidae	4	11	22-44
	Prochilodidae	2	3	54
	Serrasalmidae	11	26	54-64
Siluriformes				
	Ageneiosidae	1	2	54-56
	Auchenipteridae	1	1	58
	Callichthyidae	5	16	44-132
	Doradidae	5	7	58
	Loricariidae	2	2	52-62
	Pimelodidae	4	5	50-58
	Trichomycteridae	1	1	32
Gymnotiformes				
	Apteronotidae	1	1	24
	Gymnotidae	1	1	42-48
	Hypopomidae	2	3	36-50
	Rhamphichthidae	1	1	52
	Sternopygidae	2	3	28-46
Perciformes				
	Cichlidae	17	28	38-60
	Sciaenidae	1	2	48-50
Lepidosireniformes				
	Lepidosirenidae	1	1	38

DNA

Comparative examination of DNA across different taxa shows classes of sequences common to many species and classes of sequences totally divergent. For this reason, DNA analysis has been responsible for one of the best options for testing evolutionary hypothesis and estimating population structure (Avise, 1994). Both mitochondrial DNA (mtDNA) and nuclear DNA may be used (Meyer, 1994). The main feature of mtDNA molecule is that it is clonally inherited from the maternal line. Thus, because the lack of recombination (in most cases), the mtDNA genes have been used in many evolutionary studies (reviewed by Meyer, 1994).

Nuclear DNA sequences also represent a potentially valuable tool for population analysis because many non-coding DNA sequences with high mutation rates are available. The internal transcribed spacer 1 region (ITS 1) of ribosomal DNA, for example, has been used successfully to reveal phylogenetic relationships among members of closely related species such as salmonids (Pleyte *et al.*, 1992). Protein coding genes such as growth hormone (Bernardi *et al.*, 1993) and ependymine (Ortí & Meyer, 1996) also have been used in Teleostei evolutionary studies. Besides sequence data, the use of molecular techniques such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Ramdom Amplified Polymorphism DNA), STR (Short Tandem Repeat), VNTR (Variable Number of Tandem Repeat), SSCP (Single Strand Conformation Polymorphism) has provided DNA fingerprints and allelic variation suitable to assess genetic population structure of many fish groups (Park & Moran, 1994).

Table 2. Number of selected groups of Amazon fish sequenced for 12s and 16s ribosomal mitochondrial tDNA (mtDNA) genes by the fish genetics group

Order	Family	Genus	Species
Characiformes			
	Serrasalmidae	13	22
Siluriformes			
	Aspredinidae	2	2
	Auchenipteridae	4	4
	Callichthyidae	2	3
	Cetopsidae	3	4
	Doradidae	3	3
	Loricariidae	5	5
	Pimelodidae	8	9
	Trichomycteridae	3	4
Gymnotiformes			
	Apteronotidae	17	40
	Eigenmanniidae	4	11
	Electrophoridae	1	1
	Gymnotidae	1	8
	Hypopomidae	4	12
	Rhamphichthyidae	5	14
	Sternopygidae	1	2
Perciformes			
	Cichlidae	31	46

If we consider only the Amazonian ichthyofauna, probably the first record for using DNA was the paper published by Rennó *et al*. (1991), who used RFLPs to assess intraspecific differentiation of the anostomid *Leporinus fasciatus* from Guiana Rivers. Later on, a series of papers focused on phylogenetic studies and using the ribosomal subunits of mtDNA, (rRNA), were published. Many of the taxa analyzed are endemic of Amazon basin. Alves-Gomes *et al*. (1995) and Ortí (1997), provided phylogenetic analysis of two important orders: the electric fishes (Gymnotiformes), and the characins (Characiformes), respectively. Some fish families, such as serrasalmids (Ortí *et al*., 1996), loricariids (Montoya-Burgos *et al*., 1997), and cichlids (Farias *et al*., 1999), were also analyzed using mtDNA rRNA genes (Table 2). All of them tested previous hypothesis based on morphological analyses and proposed a new hypothesis for the relationship of each group.

Studies are currently being conducted in the Amazon in order to establish intraspecific differentiation among Amazonian fish species. One of the projects being executed combines PCR and RFLPs to search for variations in mtDNA genes of serrasalmids which occurs only in rivers with rapids and waterfalls and that drain the Guianean and Brazilian shields (Porto, 1999). Another portion of our research encompass the use of RAPDs to search for differences of catfishes (Bastos & Alves-Gomes, 1999) and electric fishes (Formiga & Alves-Gomes, 1999) along the Amazon River and its tributaries. However, no more than 10 ornamental fish species were included in this kind of analysis. In the near future, besides an enhancing in the number of ornamental fishes to be studied, the establishment of genomic libraries to isolate repetitive DNAs to be used in STR (microsatellites) and VNTR (minisatellites) will be needed to assist this work.

PROJECT PIABA 2000 AND THE PERSPECTIVES

Despite the intense exploitation of ornamental fishes for several decades, the basic parameters about their biology and ecology, including the genetic structure of the populations and their renewal capability, are poorly known. For this reason, we conceived Project Piaba as a integrated research program (recently approved by PRONEX, a Brazilian Program for Excellence Groups) to continue the former efforts of Project Piaba. The main goal of the fish genetics group is to develop a framework to characterize population genetics of wild stocks of ornamental fishes in the Rio Negro basin and apply the data to practical management and conservation issues.

When compared to ongoing molecular genetic studies in fish species from the Amazon few ornamental fishes have been included in these analyses. However, we must highlight some analysis in *Symphysodon* spp., using the mtDNA gene 16S and

nuclear loci (I.P.Farias at University of Amazonas), and in the *Paracheirodon axelrodi*, using D-loop sequences (Harris & Petry, in this volume). In our point of view, to maximize the potential use of genetic information of the Amazon fishes we still need to pay attention to several details. First, work together with local fisherman in order to know the exact location of capture of the ornamental fishes. Second, take into account the biogeography of fish species. Third, work together with taxonomist. Fourth, cross all data with the different interfaces present in the Project Piaba. These include socioeconomy, ecology, physiology, and others. Only this way will be possible to determine what genetic characters may contribute to taxonomy, evolutionary, species management and conservation issues.

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Q & A for Harris/Petry /Porto

Carmark: Since the mitochondrial DNA is maternally inherited, the variations occur through random mutations, it's not through the sexual process?

Harris: That's right. However, sexual reproduction tends to dampen down the rates of

mutation, which is why mitochondrial DNA evolves at least four times faster than nuclear DNA.

Carmark: Will this give any kind of picture as to gene flow through the populations?

Harris: It potentially can. It may not be immediate gene flow but what it can give is a picture of the hierarchical gene flow of these populations. For instance, between two adjacent tributaries, you may find that those may come out to be closely related. Therefore, you could postulate that gene flow is occurring, or has occurred in the recent pasts among those tributaries. From that then you may be able to go back and identify some nuclear markers like these micro satellites, which may actually be able to give us an estimate of effective population sizes and the gene flow. In fact, from the mitochondrial DNA, you can get some estimate of the effective population size but it's the effective population size of females in the population. Once we have an idea of the overall diversity of haplotypes throughout the Rio Negro basin, we can come up with some idea of what we think is the effective population size of females. You can then multiply that by 2 to maybe get an total effective population size and then go on from there.

Byatt: I understand why it is that you use mitochondrial DNA as opposed to genomic DNA. Is there a danger, given that the mitochondrial genome is so condensed and it's so small, it's at its smallest functional size, that you're not going to see much variation in the mitochondrial genome even within that nontranslated region?

Harris: The answer to your question is we don't seem to have found that yet, so that does not seem to be a problem. The mitochondrial DNA has been used to study the relationships of life on Earth down to the relationships of populations with a lot of gene flow. Selection of the proper region of the mitochondrial genome is quite critical in this particular case. For instance, with Jorge Porto's fish, the Amazon is a very, very old ecosystem and things like that, he's well justified in examining these two ribosomal genes which tend to evolve very, very slow. In fact these are the genes that I use when I'm examining relationships among genera within families. Right now I'm working on a couple different projects, one of which is examining the relationships of suckers and there are a huge number of species of suckers, they're in different families and we're using these slowly evolving genes to look at the relationships among the genera there. For instance, with the minnows that I worked on for my dissertation in the West, that may have only been isolated from each other since the Pleistocene, so about 10,000 years ago, but certainly much longer than that. Then something like cytochrome b which has an intermediate rate of evolution is a more appropriate question to ask or a more appropriate gene to use for the question that we're asking. Finally, something like this where the potential for gene flow exists, and it can be quite high, then something very, very fast like this seems to be the appropriate region. In fact, we now know that all of these protein coding genes, not so much these intervening transfer RNA, but all of these protein coding genes evolve at their own rate. Even though the mitochondrial DNA is inherited as a unit, within that unit, these things seem to be changing at their

own rate. There's no one calibration for any one gene as yet.
Byatt: Right. The question is given that the size of the mitochondrial genome is so small the amount of informativeness within there is going to be a lot more limited than you have within the fish itself.
Harris: I would say in theory, that's right, in application we have not yet encountered that. Have you?
Byatt: I would say that it's a clean information, and DNA would have a lot of information, a lot of duplication. In mitochondrial DNA we have a clean information about what's happening so it's free to evolve, there's no recombination so it can go there and just pick up information.
Petry: I think that is the main question, we are after on the bottom line. We know that the fisherman don't go out randomly and catch fish, they have areas where they like to go, where the fish are more abundant therefore they go there to maximize their time and effort. The importance of getting the information on the population structures is that we can actually come up with policies that would maintain the viability of these evolutionary units. If we have several areas where there seems to be a lot of uniqueness, we can create, or we can suggest special treatments from the harvest part for that particular area. I'm not saying that we would block completely from harvest areas that have a unique haplotype, but we have to have a special concern to maintain those haplotypes viable. We don't know what the effects would be in a long run if some diversity be eliminated by over fishing. They have been around about 8 million years or so at least and I hope they stay much longer after we are gone. From the practical standpoint, I think it's very important that we consider the fact that each evolutionary unit that we might detect is a very important player in keeping the mosaic intact. If you pull apiece out, you don't know how much you'll lose. This mosaic has been evolving for over millions of years and I guess the question is, essentially you're putting the management issue in the context of more than one goal. We must have two goals: to sustain the populations which are economically important in the region; and to preserve biodiversity in another region, which is a conservation more than an economic issue. But they don't have to be mutually exclusive. They can be inclusive.
Byatt: They can be, but you have to convince us that maintaining these genetically distinct units is essential to maintaining the unit they're exploiting. Over millions of years, maybe so. I think the question is complicated. You're saying that these sub-populations may have been isolated for millions of years genetically and how are doing away with one going to affect the other? That's my question.
Harris: Well, to be very blunt, the answer to your question is it won't. If there's no gene flow among some of these populations, then getting rid of one of the populations will probably not effect the populations whatsoever. However, at this point we don't know. We don't know if it's one large panmictic population or if it's a series of smaller isolated populations, if gene flow is, or is not occurring, we have no idea about the potential for local adaptations, the local environment which often accompanies

population subdivision or population regulation. To take a particular example, from the Western United States, each of those particular enteric basins from the great basin tend to have their own form of trout, they tend to have their own form of *Plecostomus*, sucker and my own personal favorite, the pinnacle of minnow evolution the **Tooey** Chub which is what I worked on and each one of these things have distinct characteristics associated with them yet the potential time of isolationism for some of them has not been that great. That's a very extreme example where you have a very arid environment driving some of the selection for local adaptation. That may or may not be occurring. You just don't know until you start somewhere and you start asking some of these questions.

Longmore: In terms of management of a single genetic group, the genetic diversity which has some sort of phenotypic expression within that one group might be more important than the differences between the groups. I'm thinking let's see if there are forms more resistant to drought than others and we have these El Nino events which occur in different 4 year sessions, or schemes. So you may have some extreme events which on an ecological time scale are extremely important to the survival of one genetic group. Having some genetic diversity within that group, let's say, forms which are more resistant than others to drought or low oxygen or high oxygen levels might be more important than just the differences between the groups and that might be a place to focus as well.

Petry: Yes, I agree with you. One of the major concerns is when you have these types of catastrophic events, if you have a wipe out of one subunit or partial wipe out that you still have the rest to recolonize. The system works pretty much in our minds as a very large metapopulation scheme. We don't know exactly where the barriers are and where the conduits are. The way for us to address this question is we need to increase our precision of information to then flip the model over and look from the other side to really be able to make any concrete inference.

Harris: I just thought of something while you guys were talking. The fact is that most of the adaptations that you're talking about are part of the nuclear genome. Because the nuclear genome evolves four times slower than the mitochondrial genome, the mitochondrial genome may actually reflect population subdivisions earlier. So we may actually get this hierarchical structure that actually reflects something that is being trailed by the nuclear DNA. So you may get these units you can identify and you can later go back and test some of these physiological parameters too. In many cases mitochondrial DNA, probably the subdivision of mitochondrial DNA or the hierarchical structure probably precedes the nuclear DNA. But who knows?

Walker: I have a question for Dr., Harris. What is non-duplicated DNA? Is it just single strand, a loop, not a double helix?

Harris: No, it in fact is double stranded. When I say it's non-duplicated, it doesn't contain any tandem repeats that we found. What's duplicated is the number of tandem repeats throughout that and then you get into this area where you do not have any

tandem repeats, it's just simply normal.
Porto: In this region, the control region in some vertebrates they acted as some kind of promoter. Basically, on the other slide...
Walker: One domain is the promoter for the other.
Porto: Yes
Walker: I'm very glad to hear for once that sexual reproduction is a conservative mechanism mainly. You know, it really gets twisted. In general, evolutionary theories sexual reproduction is always seen as something for creating variation. It's just the opposite, it's to maintain the information within the population.
Carmark: There's a lot of cardinals captured in a given year and I'm sure a significant percentage of them escape from the fishermen during their water changes. Are the sampling taking this into account, are they going far enough up the tributaries to eliminate this random pollution by other genotypes?
Petry: The samples that we have right now are, what we consider RAP. It's a very rapid assessment of the situation, just to give a very blurry big picture. From now on we're going to start to get finer resolution. The samples that we have are all bound to specific locations but we cannot account if there is genetic pollution? based on dumping. I don't think this happens because normally what you see is that the fishermen fish in on particular part of river. They stop the fish there and then load up the boat and leave. They don't go from one river to next river, to next river and so on, at least that's not the normal practice. Even on the Rio Negro when they are changing the water I don't think the Rio Negro is a good habitat for cardinals and they probably get wiped out even before they make it to the shore.
Carmark: We caught some cardinals in Salt Creek (Igarapé Tarumã) right in the middle of the city of Manaus.
Petry: Yes. You go to the Tarumã Basin where it is a completely artificial situation because there is leakage from the exporters. They have a stream where they have the water and some of the loose fish go down to the streams and have an artificial invasion there. All of populations there we did not even think about using because we know it is a complete artificial situation.
Carmark: What about natural disposition during catastrophic flooding events? What about inadvertent downstream transfer of genetic material due to catastrophic flooding events, like what we saw in Guatemala this last year as during the hurricanes, the worst flood in 100 years. We have not seen that in recordable of time but we could.
Petry: I think that's part of the ball game because that is a natural disturbance that if the fauna can put up with it, it stays; if it doesn't, it's gone. It's that simple. Natural disturbance and natural catastrophes are not that rare. They happen all the time. The frequency may vary and intensities may vary. It's not a smooth, easy-going world out there.
Forsberg: Ilse and I made some observations that might be of some interest at some point. I don't know if you remember, Ilse, but when you and I were once at the Jaú River

during this early extreme high water, just starting to fall where the river main channel was almost completely anoxic and all of the flooded forest was anoxic on the sides. What we found were just a very slow current going down the river and we saw enormous trails of detritus flowing down the river and they flowed by the boat and Ilse said those are full of fish. She started catching them all and there were all sorts of tropical fish. I don't know if you caught any of the tetras or whatever, but they were just flowing down the river, the main channel going out to the Negro for sure and I'm sure flowing down the stream. Once they got down to the Negro, I'm sure had more oxygen in it compared to the Jaú and it might be a mode of genetic drift. It's a very cyclic annual phenomenon.

Petry: We know that the river potentially is a conduit for jumps between basins but we cannot escape the fact that interfluvial is probably very efficient as well. You might have transport or mixing both directions or mixing across the channel so to be able to design this model we have to have at least some preliminary data to look at them and then see what kind of situation we can build in as some probabilities of gene mixing based on this. One thing we know for a fact is the fishes go up into the marshes when the water is high and they move down to the lower reaches of the river when it is getting low. There is this longitudinal movement and probably a lot of lateral movement of the fishes as well but we don't know the extension and how these movements might be influencing how the fish might jump over the next basin.

Prang: I wonder if you could put up the slide of the basin that shows the marsh areas you're talking about and I'll give some information I've learned dealing with this question from the fishermen. I just want to give an example of what I learned to give a better idea what this genetic mixing could be about. This is the big marsh. This is where we've been working. These are a bunch of little streams that come between. These areas here, many of these small igarapés go into this large area here. Now what I've found with many of the fishermen that move around to different patrons along these igarapés. One of them told me that during the high water season he can go up to the headwater of this igarapé, in this marsh area Paulo's talking about, and he found himself going down another igarapé where another large fishery is. If he can go up one river through the marsh and come down another river, then why can't the fish come down another river?

Byatt: I have another question for Jorge (Porto). It relates to the phylogenetic trees you showed within different species and within a family. You had phylogenetic trees for karyotype as well as for RFLP markers, I think. Have you ever compared the two to see if you get the same kind of tree with karyotype and with RFLP's? The thing is that when I looked at the karyotype and the number of chromosomes, although you've clustered together species which have the same number of chromosomes, the chromosomal duplications which led to that increased number might not have been the same in each of those cases. As a matter of fact, some may have had duplication of chromosomes, then lost chromosomes subsequently. I'm interested to see how the karyotype compares with the RFLP's.

Porto: Yes, I compared the two trees. Well, the main problem in using chromosomes for

inferring phylogeny is how to establish the homology of chromosomes. First, I search for the modal chromosome number of Characids, that's supposed to be the group most related to Serrasalmids. The chromosome number found was 48, and for the Serrasalmids varied between 54 and 64 chromosomes. So, the first guess was "they do not belong to the same family". So, we work with this hypothesis and this was in 1989. Well, since 54 chromosomes was the lower diploid number for serrasalmids and is close to the 48 chromosomes of the Characids then 54 could be the basal number. We tried to put the chromosome number over the available phylogenetic tree (based on morphology). What was the picture? Based on this tree, the basal number would be 58 or 60. So our guess was "there is something wrong!". The years passed, the PCR and the technology of DNA sequencing was used and to our surprise the phylogenetic tree obtained using mitochondrial DNA was similar to our hypothesis using chromosome number. I mean, the genera with 54 chromosomes were in the basal position in the tree. Thus, what I showed in my presentation was a comparison of the chromosome tree against the morphological and molecular trees. But I emphasize, I should not work with chromosome data just using the diploid number. Is necessary to use chromosomal banding in order to establish what is the true pair of chromosomes within and among species to be able to infer whether these chromosomes have the same origin. As I said before, this is our main problem or limitation in Cytogenetics because fish chromosomes are so tiny, and chromosomal banding is not resolutive, yet. Meanwhile, I can just use a sub-set of chromosomes characters and say, "Ok! Let's see what happen! Let's cross this data with DNA and morphology data". This is exactly how we worked. Now, our perspective is to use a chromosomal technique that uses fluorescence and *in situ* hybridization. Perhaps, that way, we will be able to establish the homology of chromosomes.

Petry: I just wanted to add something to what Jorge just said. Parallel to mapping the morphology of chromosomal data on the DNA, we mapped on the top of the phylogeny of highly host specific monogenoid parasites, and the degree of congruence between the three data sets was very high. Completely against what Marchado Allison (an expert in Serrasalmidae phylogeny), had done in his thesis based on morphology. One very interesting aspect of this, the way how Marchado Allison decided that *Colosoma, Mylossoma* and *Piaractus* were the most derived taxa was by assuming reversal of 10 out of the 19 characters, in relation to *Acnodon* (which he said was very basal). In our hypothesis *Acnodon* is not basal but *Colosoma* is, which makes perfect sense in our data. It also goes against his own argument because he had to assume the reversal of the characters to make his tree to fit in the first place.

Dawes: You mentioned earlier on Phil (Harris) that you were now beginning to think that there were at least two cryptic species (of cardinal tetra) but there might be more. How do you plan to investigate that' Have you taken your thinking that far?

Harris: The short answer is no. The long answer to that if I said two I really only mean one. It may be that short striped thing or it may be something else. My philosophy in all that is that you simply allow the data to tell the whole story and so you allow the data

to guide your actions. If the molecular data says we might have what we would tentatively identify as a unique evolutionary lineage among all these haplotypes, then what we have to do is go back and collect morphological data and life history data to incorporate into that. From my perspective, the molecular work is the first step in describing any of these new species or in elucidating patterns of biodiversity. You have to follow it up with ecological studies or life history studies or the morphological, systematic and taxonomic studies and those things can actually be fairly fast once you've identified, you use your molecular data as the hypothesis and then you can just go back and collect color, size and spawning and things like that.

Porto: Just to make a small note. Using *Mylesinus* populations, as an example, I showed that the sequence divergences between them are pretty higher compared to the most divergent cardinals, which is 3 to 4 percent. Isn't that right Phil?

Harris: Yes. It's about 3.9 to 4.3 percent. I know these are not perfectly representations of the fish but look at this. Look at the origin of the anal fin here and the insertion of the dorsal fin here. Then look at the origin of the anal fin here and the insertion of the dorsal fin there. Also look at the separation on the caudal peduncle, the insertion of the adipose fin. Triangular shape here, rounded shape there. This in particular is very striking in addition to the banding. Is that real?

Harris: Yes. In fact I took one slide out of the talk that I should've saved for the end. It was talk about when you asked *What are we going to do about this?* One of the things that I've been working on is doing quantitative morphology analysis. I've been talking to several people about this, especially Barry Chernoff and with Bill Fink, to use partial warps analysis to look at quantitative morphology of these things and look how you have pure shape changes in geometric space that can be used as a diagnosis to recognize these groups on a morphology basis. We got to get away from this teeth business because it has just created confusion. Not only do we have the computer power and we have this very powerful analytical geometric morphological methodologies, I think we can do a very nice pilot work with this and then move on to other groups even more complex groups like bleeding hearts, *Plecostomus* and so on, which is a lifetime project but someone has to start to tackle it. I'd be willing to bet there are more dorsal fin rays on that one.

Petry: Well, I can give you Weitzman and Fink original data where they made several counts and there is a bit of variation and they actually managed to get a little difference for a few of the characters. They concluded that this variation was inconclusive. Again, the problem is we have to find out what the short-striped guy is and where it is distributed. This fish also came from the trade and it was given to me by a German aquarist who said, Did you see this?? and I said ?Nope. I'd never seen it.? Then when I read Geissler's paper, Schwartz had seen this in the '60's and nobody ever paid any attention to him. That's what's so striking. The information has always been available and I've talked to several people and they've never seen it.

Q: This has been our dream project for years. In the middle, the little fat one there

actually I looked at the same collection I find some like this size and most of them are females. If they have the sharper snout, they are male. The females, when they mature, have a much more blunt snout. Also, the collections here, I couldn't find, I didn't have a chance to take a picture all the time. We put this in alcohol and all the blue went away, only the red left. You see the red base color is turning to black. The blue was not preserved in the collection that's why there's a problem with collecting. We cannot trace everyone back. It can be one thing from the Czech breeder.

Petry: One thing that is different with this case here is that you find this blond form in nature. We have this blond form in our fish tank and at night everybody goes blond. Everybody turns blond. The question is, maybe the blond form lost the ability to turn back blue and nothing else. Because there's such a lack of baseline information, we have to assume that there is some difference between these forms.