

**COVER SHEET FOR PROPOSAL TO THE NATIONAL SCIENCE FOUNDATION**

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NAME OF ORGANIZATION TO WHICH AWARD SHOULD BE MADE <b>University of Puerto Rico-Rio Piedras</b>			ADDRESS OF AWARDEE ORGANIZATION, INCLUDING 9 DIGIT ZIP CODE <b>University of Puerto Rico-Rio Piedras PO Box 21790 San Juan, PR. 009311790</b>			
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		<input type="checkbox"/> FOR-PROFIT ORGANIZATION	<input type="checkbox"/> WOMAN-OWNED BUSINESS			
TITLE OF PROPOSED PROJECT <b>Center for Applied Tropical Ecology and Conservation: Biodiversity Conservation Under the Scenario of Climate Change</b>						
REQUESTED AMOUNT \$ <b>100,000</b>	PROPOSED DURATION (1-60 MONTHS) <b>0</b> months	REQUESTED STARTING DATE	SHOW RELATED PRELIMINARY PROPOSAL NO. IF APPLICABLE			
CHECK APPROPRIATE BOX(ES) IF THIS PROPOSAL INCLUDES ANY OF THE ITEMS LISTED BELOW						
<input type="checkbox"/> BEGINNING INVESTIGATOR (GPG I.G.2)		<input type="checkbox"/> HUMAN SUBJECTS (GPG II.D.7) Human Subjects Assurance Number _____				
<input type="checkbox"/> DISCLOSURE OF LOBBYING ACTIVITIES (GPG II.C)		Exemption Subsection _____ or IRB App. Date _____				
<input type="checkbox"/> PROPRIETARY & PRIVILEGED INFORMATION (GPG I.D, II.C.1.d)		<input type="checkbox"/> INTERNATIONAL COOPERATIVE ACTIVITIES: COUNTRY/COUNTRIES INVOLVED (GPG II.C.2.j)				
<input type="checkbox"/> HISTORIC PLACES (GPG II.C.2.j)		_____				
<input type="checkbox"/> EAGER* (GPG II.D.2) <input type="checkbox"/> RAPID** (GPG II.D.1)		<input type="checkbox"/> HIGH RESOLUTION GRAPHICS/OTHER GRAPHICS WHERE EXACT COLOR REPRESENTATION IS REQUIRED FOR PROPER INTERPRETATION (GPG I.G.1)				
<input type="checkbox"/> VERTEBRATE ANIMALS (GPG II.D.6) IACUC App. Date _____		_____				
PHS Animal Welfare Assurance Number _____						
PI/PD DEPARTMENT <b>Biology</b>		PI/PD POSTAL ADDRESS <b>Room 301-A Facundo Bueso Building, UPR-RP San Juan, PR 00931 United States</b>				
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## CERTIFICATION PAGE

### Certification for Authorized Organizational Representative or Individual Applicant:

By signing and submitting this proposal, the Authorized Organizational Representative or Individual Applicant is: (1) certifying that statements made herein are true and complete to the best of his/her knowledge; and (2) agreeing to accept the obligation to comply with NSF award terms and conditions if an award is made as a result of this application. Further, the applicant is hereby providing certifications regarding debarment and suspension, drug-free workplace, and lobbying activities (see below), nondiscrimination, and flood hazard insurance (when applicable) as set forth in the NSF Proposal & Award Policies & Procedures Guide, Part I: the Grant Proposal Guide (GPG) (NSF 09-1). Willful provision of false information in this application and its supporting documents or in reports required under an ensuing award is a criminal offense (U. S. Code, Title 18, Section 1001).

### Conflict of Interest Certification

In addition, if the applicant institution employs more than fifty persons, by electronically signing the NSF Proposal Cover Sheet, the Authorized Organizational Representative of the applicant institution is certifying that the institution has implemented a written and enforced conflict of interest policy that is consistent with the provisions of the NSF Proposal & Award Policies & Procedures Guide, Part II, Award & Administration Guide (AAG) Chapter IV.A; that to the best of his/her knowledge, all financial disclosures required by that conflict of interest policy have been made; and that all identified conflicts of interest will have been satisfactorily managed, reduced or eliminated prior to the institution's expenditure of any funds under the award, in accordance with the institution's conflict of interest policy. Conflicts which cannot be satisfactorily managed, reduced or eliminated must be disclosed to NSF.

### Drug Free Work Place Certification

By electronically signing the NSF Proposal Cover Sheet, the Authorized Organizational Representative or Individual Applicant is providing the Drug Free Work Place Certification contained in Exhibit II-3 of the Grant Proposal Guide.

### Debarment and Suspension Certification

(If answer "yes", please provide explanation.)

Is the organization or its principals presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from covered transactions by any Federal department or agency?

Yes

No

By electronically signing the NSF Proposal Cover Sheet, the Authorized Organizational Representative or Individual Applicant is providing the Debarment and Suspension Certification contained in Exhibit II-4 of the Grant Proposal Guide.

### Certification Regarding Lobbying

The following certification is required for an award of a Federal contract, grant, or cooperative agreement exceeding \$100,000 and for an award of a Federal loan or a commitment providing for the United States to insure or guarantee a loan exceeding \$150,000.

### Certification for Contracts, Grants, Loans and Cooperative Agreements

The undersigned certifies, to the best of his or her knowledge and belief, that:

- (1) No federal appropriated funds have been paid or will be paid, by or on behalf of the undersigned, to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with the awarding of any federal contract, the making of any Federal grant, the making of any Federal loan, the entering into of any cooperative agreement, and the extension, continuation, renewal, amendment, or modification of any Federal contract, grant, loan, or cooperative agreement.
- (2) If any funds other than Federal appropriated funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with this Federal contract, grant, loan, or cooperative agreement, the undersigned shall complete and submit Standard Form-LLL, "Disclosure of Lobbying Activities," in accordance with its instructions.
- (3) The undersigned shall require that the language of this certification be included in the award documents for all subawards at all tiers including subcontracts, subgrants, and contracts under grants, loans, and cooperative agreements and that all subrecipients shall certify and disclose accordingly.

This certification is a material representation of fact upon which reliance was placed when this transaction was made or entered into. Submission of this certification is a prerequisite for making or entering into this transaction imposed by section 1352, Title 31, U.S. Code. Any person who fails to file the required certification shall be subject to a civil penalty of not less than \$10,000 and not more than \$100,000 for each such failure.

### Certification Regarding Nondiscrimination

By electronically signing the NSF Proposal Cover Sheet, the Authorized Organizational Representative is providing the Certification Regarding Nondiscrimination contained in Exhibit II-6 of the Grant Proposal Guide.

### Certification Regarding Flood Hazard Insurance

Two sections of the National Flood Insurance Act of 1968 (42 USC §4012a and §4106) bar Federal agencies from giving financial assistance for acquisition or construction purposes in any area identified by the Federal Emergency Management Agency (FEMA) as having special flood hazards unless the:

- (1) community in which that area is located participates in the national flood insurance program; and
- (2) building (and any related equipment) is covered by adequate flood insurance.

By electronically signing the NSF Proposal Cover Sheet, the Authorized Organizational Representative or Individual Applicant located in FEMA-designated special flood hazard areas is certifying that adequate flood insurance has been or will be obtained in the following situations:

- (1) for NSF grants for the construction of a building or facility, regardless of the dollar amount of the grant; and
- (2) for other NSF Grants when more than \$25,000 has been budgeted in the proposal for repair, alteration or improvement (construction) of a building or facility.

AUTHORIZED ORGANIZATIONAL REPRESENTATIVE		SIGNATURE	DATE
NAME <b>Gladys Escalona de Motta</b>		<b>Electronic Signature</b>	<b>Feb 27 2009 12:12PM</b>
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\* EAGER - EARly-concept Grants for Exploratory Research

\*\* RAPID - Grants for Rapid Response Research

# AN EVALUATION OF PLEISTOCENE CLIMATE CHANGE ON THE DEMOGRAPHIC HISTORY OF AMAZONIAN FISHES

**Overview:** The University of Puerto Rico, Rio Piedras Campus (UPR-RP) proposes to submit a CREST Partnership Supplement to our currently funded CREST grant. The CREST grant supports the Center for Applied Tropical Ecology (CATEC) which serves as a catalyst for systemic change in educational, research development and structural institutional transformation in a Hispanic minority serving institution. CATEC's mission is to promote a) training of human resources at the graduate, undergraduate and post-doctoral levels, b) state-of-the-art research in conservation biology and environmental issues, c) infrastructure improvement, and d) production of relevant results for policy and conservation management.

The unifying research theme is biodiversity conservation under the scenario of climate change. The questions addressed were chosen for their potential to be applied at the scale of the Caribbean Basin, or to the Tropics in general, and to serve as foundations for projects at the regional or international level. The Molecular Ecology, Evolution and Genetics (MEEG) area address conservation issues using molecular markers to understand the phylogeny, the spatial distribution of genetic diversity, and species interactions in the Puerto Rico and the Caribbean. The Species and Population Management (SPM) area focuses on impact of interactions between exotic and native species, and understanding patterns of regional diversification and distribution of endangered species and species of economic importance. The Ecosystem Processes (EP) area addresses the question of how species, both terrestrial and marine, respond to climate variability, by comparing the effect of paleoclimatic change with the impact of temporal and spatial variation in climate as well as historical anthropogenic changes in land use on ecosystem structure and function. All projects foster inter- and trans disciplinary synergies and interactions among research fellows within each thrust area, with other thrust areas, the University of Puerto Rico, and the local, national and international scientific communities.

The goal of the current supplement is to expand and enhance our STEM educational capacities through the fostering of long-lasting international partnerships for research and human resource capacity building in aquatic biodiversity studies and our ability to understand the genetic basis of adaptive responses of organisms to climate change. To accomplish this goal, we propose to study the genetic signatures of past climate change in a group of fishes in the Amazon basin by establishing collaborations with the Amazonian Ichthyology Research Network (RIIA – Red de Investigaciones Ictiológicas de la Amazonía), and formalizing relationships with the Genetics, Conservation and Evolutionary Biology (GCBEV) graduate program of the National Institute of Amazonian Research (INPA), and the Biological Diversity (DB) graduate program at the Federal University of Amazonas (UFAM). The *objective of this study* is to identify historical and ongoing processes that are responsible for generating and maintaining aquatic biodiversity in the Amazon basin. The *central hypothesis* to be tested is that the central and eastern Amazon basin is ecologically young, and that it has only recently been colonized from refugia located on the Brazilian and Guyana Shields, and in the western Amazon basin. This is a test of the refugium theory applied to aquatic fauna, and it tests the importance of past climatic events on shaping of current demographic patterns. It also provides information on location of aquatic refugia, and data for predicting the effects of future climate change on Amazonian aquatic fauna.

The **intellectual merits** of this supplement are fourfold: 1) develops an understanding on how past and present climate change affect biodiversity in the Neotropics, one of the topmost world biodiversity hotspots, 2) develops an understanding the genetic basis of response to changing environmental conditions, 3) develops an understanding of how fast organisms can track environmental changes via adaptive responses and 4) provides higher visibility for applied ecology and conservation research as a science career option to Hispanic students by providing competitive and attractive research training opportunities.

The **broader impacts** of the proposed activities are based on CATEC's leadership as a Hispanic science community where faculty and students lead significant synergic and integrative activities in research, education and knowledge-transfer in applied ecology and conservation of natural resources. This project builds on this educational foundation by establishing a graduate and undergraduate student exchange program between UPR-RP and leading research and educational institutions in Manaus, Brazil (INPA and UFAM). Such exchanges will result in an integrated research project that transcends national boundaries and will foster in these young scientists an integrative perspective to tackle biodiversity

problems. CATEC will continue to increase the numbers of trained Hispanic minority professionals engaged in research, teaching and management, empowering a more diverse generation of scientists by a) **contributing to the training of global scientists in a traditionally underrepresented minority-serving institution** and b) **fostering long-lasting international partnerships for research and human resource capacity building in aquatic biodiversity studies.**

## OVERVIEW AND MAIN AIMS

Starting with early naturalists such as Humboldt, Bates and Wallace, biodiversity of the Amazon basin has fascinated scientists and the lay public alike. While major groups of plants and animals have exceptionally high alpha and beta diversities in this region, it is freshwater fishes that show the highest species density and richness with an estimated 50% of all freshwater species occurring in only 0.3% of the world's freshwater habitat (from Reis et al., 2003 and AAA Atlas of Populations and Environment). A number of mechanistic hypotheses have been proposed to explain the biodiversity of the Amazon basin. One of these hypotheses is the now classic Pleistocene refugium (Haffer, 1969, Ab'Saber, 1977) and the related museum (Fjelds , 1994, Nores, 1999) hypotheses have recently been co-opted to explain diversity patterns in South American characid fishes (Hubert & Renno, 2006), one of the major groups of primary freshwater fishes of South America. The authors hypothesize that climatically driven marine incursions in the lower and central Amazon basin in the Pliocene has led to the elimination and subsequent colonization of the area by characid faunas (Hubert & Renno, 2006) from the refugial Brazilian and Guyana Shields and the western Amazon basin. The implication of this hypothesis is that large sections of the Amazon basin have only recently been colonized and that their faunas are a conglomeration of faunas existing in refugial areas. Even more importantly, the underlying implication of this process is that as an ecosystem, the central and eastern Amazon rainforest is ecologically young, is highly dynamic, and subject to radical environmental changes throughout its recent history. The validity of this hypothesis is predicated on the critical first step of deciphering the major processes that have led to the diversification of the Amazonian aquatic vertebrates. If this hypothesis is found to be valid, these data will furnish a historical perspective on climate change and together with ecological niche modeling will provide a plausible scenario for past refugia. These results can, in turn, be used in forward looking models of future climate change, and its impact on the aquatic fauna. To successfully complete the objectives outlined in this proposal, we propose three specific goals:

**Specific goal 1: Foster long-lasting international partnerships for research and human resource capacity building in aquatic biodiversity studies.** The project will bring together participants from 4 countries and members of the **Amazonian Ichthyology Research Network** (RIIA – Red de Investigaciones Ictiol gicas de la Amazon a – [www.riiaamazonia.org](http://www.riiaamazonia.org)) whose goal is to coordinate and facilitate cross national research on Amazonian fishes. RIIA members actively study taxonomy, systematics, phylogeography and population genetics of South American fishes. The network also engages in education workshops and by providing training opportunities for participants of all levels, from undergraduate student to senior research personnel. This proposal is designed to work within the conceptual framework of RIIA by promoting multinational collaborations to address solve some of our most urgent biodiversity issues.

**Specific goal 2: Identify key processes responsible for the diversification of aquatic fauna in the Amazon basin.** Our *working hypothesis* is that aquatic refugia have played a key role in the biological evolution of aquatic vertebrates in the Amazon basin and that their existence may be inferred from phylogenetic and population genetic data. We will investigate common patterns within and across river basins, and test the aquatic refugium hypothesis proposed as a major factor driving diversification of South American biota in contrast to the more orthodox hydrogeological hypothesis. As a result of this study we also anticipate the generation of new hypotheses of freshwater fish diversification in South America suitable for future research.

**Specific goal 3: Contribute to the training of global scientists in a traditionally underrepresented minority-serving institution.** Undergraduate and graduate students will be actively engaged in research, and mentored in the scientific problem solving to foster their potential to pursue a competitive and gratifying career in science. They will participate in all stages of the project development, from field research to laboratory data collection, data analysis and publication preparation. The international character of this supplement will also provide training opportunities to a new generation of scientists that will understand how international science collaborations work in a globally connected society.

This project is innovative in that, it tests a commonly invoked but hotly debated historical process that has only very recently been proposed to be of importance for aquatic vertebrates. It uses

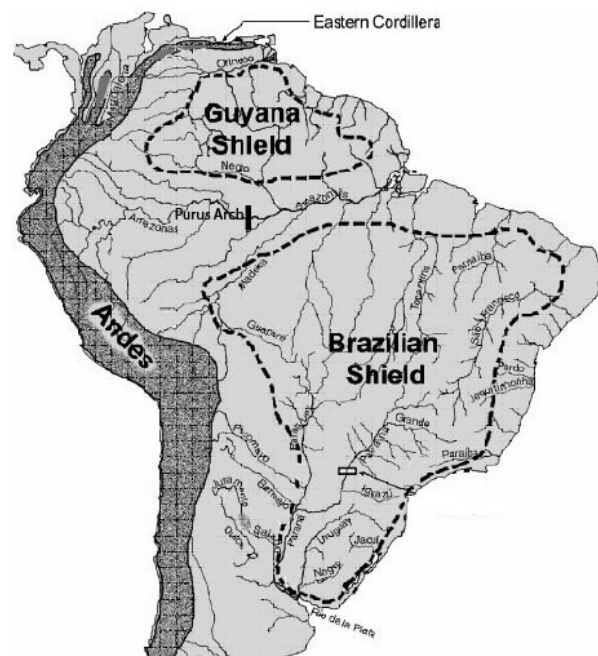
phylogenetic and population genetic approach which provide a powerful and statistically rigorous approach to hypotheses testing, allowing me to test both spatial as well as temporal concordance patterns obtained from different aquatic groups, and the concordance of these patterns to hypothesized causal climatic and geological processes. Recent theoretical advances also allow us to assign not only probabilities to the proposed evolutionary scenarios, but also to infer confidence intervals associated with these probabilities. These theoretical and computational advances will allow me to propose viable alternative hypotheses in the case that my current working hypothesis is rejected. The successful completion of this study therefore has a potentially important impact for our knowledge of how Neotropical aquatic biodiversity is formed, and for the training of next generation of researchers that will be directly impacted by how well we understand these processes, and what actions we take to preserve this biodiversity and the processes that generate it.

## BACKGROUND AND HYPOTHESES TO BE TESTED

The rise of the Andes and extensive Miocene marine incursions driven by orogenic events had drastic effects on the evolution of hydrographical and geomorphological patterns (Lundberg et al., 1998) and, therefore, the distribution of freshwater fishes in South America (Lundberg, 1998). Until approximately 8 mya, much of South America was drained by a single major drainage that ran along the much-smaller Andes northwards to drain into the Caribbean Sea. Included in this drainage were the Magdalena (until ~12 mya) and Maracaibo (until ~8 mya) basins that currently have trans-Andean (they drain western Andean slopes). Also included in this drainage was the western Amazon basin including the western Brazilian Shield, and the western Orinoco basin including the western Guyana Shield, while the eastern Amazonas and eastern Orinoco regions were isolated as separate Atlantic versant basins. It has been suggested that much of this paleodrainage formed a shallow epicontinental sea (Räsänen et al., 1995), or more likely in its southern portion by an extensive freshwater lake (Paxton et al., 1996, Marshall & Lundberg, 1996, Hoorn, 1996) draining north into the Caribbean. This area initially became flooded starting around 14 mya (Haq et al., 1987). The Purus paleoarch has been proposed as the western boundary of this so called Lake Pebas, and the area occupied by it was the present day western Amazon basin. An additional major marine transgression lasting approximately 800,000 years occurred at the Miocene/early Pleistocene boundary at 5 mya (Haq et al., 1987). Andean orogenic events also affected southern South American drainages, including the proposed capture of the Bolivian basin by the Amazon basin to the exclusion of the Paraguay basin (Lundberg et al., 1998).

With the uplift of major Andean mountain ranges in Northern South America, and the uplift of the Merida Andes beginning the last 10-8 mya, the Magdalena and Maracaibo basins became isolated from the western proto-Amazonas River. The Orinoco River became isolated from the western Amazonas basin by the uplift of the Vaupes arch shortly after 8 mya. Shortly thereafter, Lake Pebas breached its western border, the Purus arch, and commenced to drain eastward, in the process capturing Guyana and Brazilian Shield drainages, and establishing the modern day Amazon River (Hoorn et al., 1995, Campbell Jr. et al., 2006). These events would have resulted in the transformation of a lacustrine habitat in the western Amazon basin into a riverine habitat, and occupation of western Amazon by riverine fish fauna. The second transgression 5 mya would have resulted in the reoccupation of the eastern, central and eastern portions of the western Amazon basin.

Pleistocene climatic changes have also been implicated in marine transgressions that have occurred within the last 0.8 mya (Hooghiemstra & Ran, 1994). The Pleistocene has seen large



oscillations in sea level relative to present, most resulting in lower sea levels than present, but other in higher sea levels than present (Siddall et al., 2003, Crowley et al., 1986); particularly large oscillations occurred in the last 1 million years (Billuops, 2004). A major high sea level stands occurred around 50,000 and 400,000 years ago (Liu & Herbert, 2004, Billuops, 2004). The observed differences in  $^{18}\text{O}/^{16}\text{O}$  ratios from present day ratios are indicative of around 100 m rise in sea level. Such a rise is sufficient to flood all of the eastern and central Amazon basin, and the eastern portions of the western Amazon basin (Nores, 1999).

Published data indicates that major areas of endemism for lowland freshwater fishes correspond, by and large, to the most ancient landforms in the continent: the Brazilian and Guyana Shields (2004, Hrbek & Larson, 1999, Hubert & Renno, 2006, Montoya-Burgos, 2003, Lovejoy & Collette, 2001) (Fig. 1), and in cases of molecular phylogenetic analyses, Amazonian areas outside the Guyana and Brazilian Shield occupy phylogenetically nested positions (Hrbek & Larson, 1999, Montoya-Burgos, 2003). This pattern has also been observed in Neotropical passerine birds (Aleixo, 2002).

These geologic, climatic and biogeographic data allow for the formulation of several null historical biogeographic hypotheses that are suitable for falsification with phylogenetic and population genetic data. Major hypotheses are:

- 1) Lineages in the lowland areas of the eastern portions of the western Amazon basin, and central and eastern Amazon basin should have nested phylogenetic positions within clades whose basal members are found on the Guyana and/or Brazilian Shields and/or in the western Amazon basin. Having colonized these lowland areas in the Pleistocene following the last marine incursion, members of these derived clades should show a signal of a demographic expansion – the **Pleistocene refugium/museum hypothesis**.
- 2) Lineages in the lowland areas of the central and eastern Amazon basin and including eastern sections of the western Amazon basin should have nested phylogenetic positions within clades whose basal members are found on the Guyana and/or Brazilian Shields and/or in the western Amazon basin. Having colonized these lowland areas in the early Pliocene following a marine incursion, members of these derived clades are not expected to show a signal of a demographic expansion – the **Pliocene refugium/museum hypothesis**.
- 3) Lineages in the lowland areas of the western Amazon basin should have nested phylogenetic positions within clades whose basal members are found on the Guyana or Brazilian Shields and/or the central and eastern Amazon basin. Because colonization of the western Amazon basin would have occurred shortly after the breach of the Purus Arch in the late Miocene, members of these derived clades are not expected to show a signal of a demographic expansion – the **hydrogeographic/Purus Arch hypothesis**.

All three hypotheses are mutually exclusive. Although there is some uncertainty about the duration of Lake Pebas and its final breach through the Purus Arch, as well as the extent and duration of some of the marine incursions into the eastern and central Amazon basin during the Pleistocene, these uncertainties do not preclude the testing of the major hypotheses of this study. **The effects of Miocene events will have left a demographic signature in the western Amazon basin (nested clades), while the genetic signature Pliocene events will be observable in the eastern part of western, eastern and central Amazon basin (nested clades), and Pleistocene events will be observable in the eastern part of western, eastern and central Amazon basin (nested species showing a signal of a demographic expansion).**

#### TARGET TAXON



**Species of the family Serrasalminidae:** Serrasalminids are the famous piranhas and pacus. Piranhas are highly carnivorous while pacus are largely frugivorous; the carnivorous piranhas are phylogenetically nested within the vegetarian pacus (Ortí et al., 1996, Ortí et al., 2008). Serrasalminids are economically important, and especially the pacu species of the genera *Colossoma*, *Piaractus* and *Mylossoma* are heavily exploited (Goulding, 1980, Goulding & Carvalho, 1982, Reinert & Winter, 2002). Piranhas are also exploited, but on a local scale and in

tourist fishery. *Colossoma* are known to undergo localized movements for spawning (Santos et al., 2007, Araújo-Lima & Goulding, 1998, Loubens & Panfili, 1997). Serrasalminids have also received much scientific attention. Classical systematic studies include those of Machado-Allison (Machado-Allison, 1983, Machado-Allison, 1985). Several molecular phylogenetic hypotheses using different taxonomic and gene sampling schemes have been proposed (Ortí et al., 1996, Ortí et al., 2008, Freeman et al., 2007, Hubert et al., 2007a), providing an excellent basis for future studies. Piranhas have also been subject to studies of ontogenetic shape variation and developmental integration (e.g. Fink & Zelditch, 1995, Fink & Zelditch, 1996, Zelditch et al., 1995, Zelditch et al., 2000), and the study of piranha shape variation has resulted in major advances in the field of geometric morphometrics (see the textbook of Zelditch et al., 2004).

## MATERIALS AND METHODS

**Study Area, Field Collections, and Tissue acquisition:** Extensive tissue collections have been made over the years for the target clade, from a large fraction of their area of distribution in South America. Population level sampling has been completed for the species of interest; however, there are several species that will be included in higher level phylogenetic analyses that have yet to be sampled. Therefore, field work is a vital part of this proposal. Areas of interest that need to be sampled are the Trombetas, Tapajos and Xingu Rivers both upstream and downstream of their major waterfalls. The current and past sampling design has been designed to specifically test the hypotheses outlined earlier in this proposal. The localities were further chosen at a regular geographic spacing to avoid the pitfalls irregular or incomplete sampling design. Specimens collected at sampling localities will be photographed, and representative specimens will be deposited as vouchers. Specimens will be collected using appropriate fishing gear (gill nets, hook & line, cast nets) with the help of locally hired fishermen. Sampling will be conducted by a CREST-CATEC faculty (Tomas Hrbek) and graduate student, and a Brazilian partner (Izeni Farias) and graduate student.

**Gene Sampling and Sequencing Protocols:** Total genomic DNA for all molecular analyses will be extracted using Qiagen DNEasy kits. Data from multiple loci increase phylogenetic resolution, and more closely approximate the history of species rather than genes (Hare, 2001, Avise, 2004). As sequencing nuclear genes becomes more feasible, this strategy is increasingly more common in phylogenetic and phylogeographic studies (e.g. Sota & Sasabe, 2006, Li et al., 2007, Hrbek et al., 2008, Hrbek et al., 2007b, Weisrock et al., 2005, Townsend et al., 2008). We will follow this strategy. MtDNA sequences will be collected following the general protocol outlined by Santos et al. (2007) and Willis et al. (2007) for the mitochondrial control region or CR (500-1000 bp), and Ivanova et al. (2007) for COI genes. The mtDNA PCR primers are highly conserved, but will be optimized for each taxon as necessary. Nuclear gene fragments corresponding to the exon 2 and introns 1 and 2 (ca. 900 bp) of the s7 ribosomal protein gene (following protocols in Chow & Hazama, 1998, Lavoué et al., 2003, Johnson et al., 2004); intron 2 (ca. 850 bp) of the single-copy nuclear gene RAG-1 (following protocols in Grande et al., 2004, Hrbek et al., 2007b), plus the single copy genes Gylt, Prt and myh6 (following protocols in Li et al., 2007). The last three nuclear genes are as or slightly more variable than RAG-1 gene (Li et al., 2007 and unpublished data from *Fundulus* phylogeny). Primer regions for all nuclear genes are highly conserved, and have been successfully tested in representatives of all clades that will be analyzed in this proposal. All forward primers have M13 sequence tails and all reverse primers have SP6 sequence tails added to them to standardize cycle sequencing conditions and facilitate sequencing throughput. PCR products will be purified with ExoSap. Sequencing reactions will follow standard Perkin Elmer Big Dye sequencing protocol for double-stranded cycle sequencing reactions. Sequences will be determined on an ABI 3130xl automatic DNA sequencer. Nuclear sequences may result indicate heterozygous alleles. In cases when only one site is variable, determination of the two constituent alleles is unambiguous. However, in other cases, inference of allelic states will be necessary either statistically using the program PHASE (Stephens & Scheet, 2005, Stephens et al., 2001) if sufficient statistical power for haplotype estimation exists, or haplotypes will be inferred experimentally by SSCP (Sunnucks et al., 2000, Ortí et al., 1997) or bacterial vector cloning, and sequenced individually. Haplotype inference will not be necessary for species level phylogenetic analyses, but will be necessary for individual species analyses. *For species selected for phylogenetic studies, for each sampling locality* two random individuals will be selected, and sequenced for the two mitochondrial and five nuclear genes. *For species selected for population level studies, from each sampling locality* 20 individuals will be sequenced for the two mitochondrial and five nuclear genes. Sequencing of the COI barcode region will be reduced to a random subset of 5 individuals that differ in



their CR region haplotype. Individuals that have the same haplotype at the faster evolving, physically linked CR region, are unlikely to vary at the more slowly evolving COI region (see Morando et al., 2004 for an example). A strategy that samples a large number of individuals at fewer loci is most likely to accurately recover phylogenetically shallow species trees (Maddison & Knowles, 2006).

**Microsatellite Development and Genotyping Protocols:** Recent molecular advances have resulted in efficient protocols for microsatellite development. In the last four years, my group and that of my main collaborator in Brazil (I. Farias) have jointly developed and published seven microsatellite marker panels (Farias et al., 2006, Farias et al., 2003, Amado et al., 2008, Santos et al., 2009, Gravena et al., 2009, Fantin et al., 2007, da Silva et al., 2008). Majority of these panels contain 15 or more highly polymorphic loci. These panels include microsatellite makers for the target species *Colossoma macropomum*, and *Piaractus brachypomus*. An additional eight microsatellite markers have been developed for *Piaractus mesopotamicus* (Calcagnotto et al., 2001) and successfully tested in my laboratory. These markers have been successfully tested for variability in *Pygocentrus nattereri*, a target species of this proposal. Our aim is to develop and optimize a minimum of 15 microsatellite loci per target species. Once developed, 20 individuals per locality per species will be screened for variation at 15 markers. Genotypes will be determined on a Perkin Elmer ABI 3130xl.

**Data analysis:** Base calling and data quality assessment will be done in PHRED (Ewing & Green, 1998, Ewing et al., 1998). Orthologous sequences will be grouped by clade, aligned using Clustal X (Thompson et al., 1997) and adjusted by hand if necessary. For nuclear genes, both alleles will be analyzed. Microsatellite data will be scored using binning algorithms constructed in the ABI Genotyper software, manually checked for accuracy, and further checked in the software MicroChecker (Van Oosterhout et al., 2004). The polished data will be used for testing: 1) phylogenetic relationships among species within clade; 2) single species hypotheses; and 3) hypotheses of concordance among clade and among single species hypotheses.

**Single clade analyses:** Phylogenetic relationships among species of each clade will be inferred, the resulting phylogenetic hypotheses will be assessed for robustness, and robust phylogenetic hypotheses will be used for testing of the main hypotheses in this proposal. Prior to analyses, the species status of sampled individuals will be inferred using taxonomic keys and by referencing to catalogued museum specimens, and when necessary expert advice will be sought (see letters from expert taxonomists). Species that will not be sampled in the Amazon basin, will be obtained from colleagues experts on the particular groups (Ortí – University of Nebraska). Two randomly chosen individuals per species per locality will be analyzed. Generated sequence data partitions (mtDNA and individual nuDNA genes) will be assessed for significant incompatibility using the ILD test (Farris et al., 1994). Although combined vs. separate analyses are of considerable debate, it has been shown that combining different datasets, even if they show statistical incongruence, is the only way to resolve conflicts among datasets, and to investigate homoplasy. A combined data analysis should, therefore, favor the emergence of congruent phylogenetic signal, allowing different data partitions to contribute to overall phylogenetic resolution (Cunningham, 1997, Cognato & Vogler, 2001). Combined analysis is also likely to reflect the phylogenetic history of the species as a whole. Species-level phylogenies will be assessed using standard methods as follows. The Akaike Information Criterion (AIC) in ModelTest (Posada & Buckley, 2004) will be used to identify the model of molecular evolution that best explains variation in DNA sequences within each clade. Both maximum likelihood (Felsenstein, 1981) and Bayesian inference (Huelsenbeck et al., 2001) estimates of phylogenetic hypotheses under the (AIC) selected model. Robustness of phylogenetic inferences will be assessed using non-parametric bootstrapping (Felsenstein, 1985) or via posterior probabilities (Ronquist & Huelsenbeck, 2003) as applicable. Where phylogenetic inference is plagued by introgression or incomplete lineage sorting, several methods have recently been developed which allow phylogenetic inference using samples from many individuals and multiple loci (Sota & Sasabe, 2006, Maddison & Knowles, 2006, Joly & Bruneau, 2006). Accurate rooting of the inferred topology is critical for evaluation of hypotheses in a phylogenetic framework. Therefore in addition to traditional outgroup rooting methodology, we plan to examine root inference using Bayesian methods (Huelsenbeck et al., 2002).

The hypotheses tested in this proposal require an estimate of divergence time of diversification events. These estimates will be estimated under Bayesian (Kishino et al., 2001) and maximum likelihood (Sanderson, 1997, Sanderson, 2002) implemented in the programs divtime and r8s. Both methods allow the estimation of 95% confidence interval around the divergence estimate. The topology used in the divergence time estimates will be the best maximum likelihood phylogenetic hypothesis. Numerous independently derived calibration points exist that can be used in combination and for cross referencing.

Major geological calibration points are summarized in Lundberg et al. (1998). Additional calibration points have been proposed for characid phylogenies (Freeman et al., 2007) which may be used in my study. Although cladogenic events were not dated, the study of (Calcagnotto et al., 2005) is amenable to divergence time calibration using fossil and geological data. Thus calibrated, the characiform phylogeny will provide additional divergence time estimates. Phylogenetic dating is an active but controversial area of research, and there are inherent inaccuracies in dating phylogenies. Nevertheless, the objective of this study is to distinguish between Miocene and Pleistocene divergences. Major marine/freshwater transgressions occurred in the western Amazon from 14-8 mya (Haq et al., 1987, Räsänen et al., 1995), and at the Miocene/early Pleistocene boundary at 5 mya (Haq et al., 1987), while Pleistocene transgressions have occurred within the last 0.8 mya (Hooghiemstra & Ran, 1994, Liu & Herbert, 2004, Billuops, 2004) providing a widely different expectation of divergence rates.

These tests are designed to explicitly address the hypotheses of this proposal. Last colonized region will have phylogenetically nested faunas, and nested species or clades will show divergence from their sister species/clades compatible with one of the three geological Epochs. Therefore through a combination of the distribution of nested species/clades, and phylogenetic dating of these species/clades, this test will distinguish between the **Pleistocene refugium/museum**, **Pliocene refugium/museum** and **hydrogeographic/Purus Arch hypotheses**.

**Single species analyses:** Geographically wide-ranging species selected for single species comparisons are *Colossoma macropomum*, *Piaractus brachipomus*, and *Pygocentrus nattereri*. These taxa will be assessed for the existence of cryptic population structure. The boundaries of independently-evolving populations will be determined using the consensus among both phylogenetic and non-tree-based methods. The consensus approach is robust to methodological differences and permits inference even in cases of limited introgression and incomplete lineage sorting among groups. A probabilistic assessment of population boundaries with no *a priori* information on population structure includes contingency tests of genetic differentiation among populations (Waples & Gaggiotti, 2006) and Bayesian clustering methods such as STRUCTURE (Pritchard et al., 2000) and BAPS (Corrander et al., 2003). For a genealogical-based inference of population structure and associated processes I will employ contingency tests of associations of haplotypes and geography at different clade hierarchies (Templeton et al., 1995, Zhang et al., 2005). Although this Nested Clade Analysis has been criticized recently (Knowles & Maddison, 2002, Knowles, 2004, Petit, 2008, Panchal & Beaumont, 2007) it is valuable as a hypothesis generating approach (Templeton, 2004, Templeton, 2008, Garrick et al., 2008) and can be made more reliable by the incorporation of additional loci (Knowles & Maddison, 2002, Knowles, 2004, Strasburg et al., 2007). Finally, while designed for delimiting species boundaries, model fitting analyses of difference in evolutionary dynamics that determine the fate of genetic polymorphisms within populations/species (coalescent processes) versus between populations/species (divergence-extinction processes) (Pons et al., 2006) will be used to infer population boundaries. The permeability of these boundaries will be tested in the Maximum likelihood and Bayesian flavors of MIGRATE (Beerli, 2006). An analysis in IMA (Hey & Nielsen, 2007), in addition to providing an estimate of gene flow between the inferred populations, will also provide an estimate of the time of divergence between the two inferred populations. Whether the inferred rates of gene flow provide a statistically better explanation than no gene flow will be assessed via hLTR and AIC tests.

In each population, but especially within those that have been identified as having expanded into a particular region or have colonized this region, I will test the hypothesis of demographic growth. Several statistics have been proposed, including comparisons of diversity statistics (e.g. Fu's  $F_s$  (Fu, 1997)) and mismatch distributions (Harpending et al., 1998), and Bayesian estimates of  $g$ , the population growth parameter inferred in the program LAMARC (Kuhner et al., 1998). A formal statistical comparison of demographic hypotheses under no population growth, and exponential growth will be carried out in the program BEAST (Drummond & Rambaut, 2007). The ML estimates and graphic visualization of the results may also be obtained in the program GENIE (Pybus & Rambaut, 2002). The program BEAST also allows a Bayesian approach (Drummond et al., 2005) to inferences of demographic population size changes. This Bayesian approach provides an estimate of the magnitude of the change, the time when growth commenced, and the duration of the growth period. The estimate of the time when growth began also provides a cross-validation of the results of IMA analyses if demographic expansion followed shortly after the two populations separated. For microsatellite data, the program Mdiv will be used for the signal of demographic growth.

These tests are designed to explicitly address the hypotheses of this proposal. A Pleistocene colonization of the western, central and eastern Amazon basin, the **Pleistocene refugium/museum hypothesis**, is expected to manifest itself with a signal of population differentiation and demographic expansion in these areas of the Amazon basin. Conversely, neither the **Pliocene refugium/museum hypothesis** nor the **hydrogeographic/Purus Arch hypothesis**, are expected to show this pattern.

#### PRELIMINARY DATA AND PROGRESS TO DATE

The clade proposed to be studied, the serrasalmids (piranhas and pacus), have received much scientific attention. Phylogenetic hypotheses are available and there have been several recent taxonomic treatments (Jégu, 2004). Species limits and distributions are also well known. Further, a robust, multi-gene molecular phylogeny for characid fishes has also been published (Calcagnotto et al., 2005). These studies provide a solid foundation for further studies; however, they do not provide the data or the resolution necessary to address the hypotheses of this proposal. Preliminary data specific to this project is also available.

**Serrasalminae:** Starting nearly two years ago, our group and that of Izeni Farias have commenced studying the phylogeography and population genetics of *Colossoma macropomum* and *Piaractus brachipomus*. We have amassed over 400 samples collected from approximately 15 localities for *C. macropomum*, and about ½ that amount for *P. brachipomus*. I also have sampling various other serrasalminid species from these 15 Amazonian localities. One of our students, Concy Santos, has published a small mtDNA study (Santos et al., 2007), and has developed and published a panel 15 microsatellite primers (Santos et al., 2009) for *C. macropomum*. These markers are together with a panel published by (Calcagnotto et al., 2001) provide the minimum 15 markers set for *P. brachipomus* and *Pygocentrus nattereri*. All markers for phylogenetic analyses have successfully amplified in our laboratory and the laboratory of G. Ortí (Li et al., 2007).

**General preliminary data for the Amazon basin:** Over the last several years, my colleagues at UFAM and INPA and I have generated a large amount of data on the distribution of genetic diversity of aquatic vertebrates in the Amazon basin. Published studies include fishes (Toffoli et al., 2008, Santos et al., 2007, Hrbek et al., 2007a, Hrbek et al., 2005, Willis et al., 2007, Farias & Hrbek, 2008), and also Amazonian manatees (Cantanhede et al., 2005), the freshwater dolphins (Gravena et al., 2008), and two species of crocodylians (Farias et al., 2004, de Thoisy et al., 2006, Vasconcelos et al., 2008, Vasconcelos et al., 2006, Hrbek et al., 2008). Several Masters and Ph.D. theses have also been produced (Cantanhede, 2008, d'Assunção, 2006, Amado, 2008, Meliciano, 2008, de Souza, 2008). Nearly all phylogeographic and population level studies with the exception of cichlids genera *Symphysodon* and *Cichla* and the stingray genus *Potamotrygon* show genetic homogeneity over large geographic distances (thousands of kilometers), and large effective population sizes. *Symphysodon* from its natural distribution in the eastern portion of the western Amazon basin show a strong signature of a demographic expansion (Farias & Hrbek, 2008), and fishes from the central and eastern Amazon basin are an admixed hybrid group (unpub. microsatellite data and some evidence in Farias & Hrbek, 2008). A nearly identical pattern is observed in *Potamotrygon* (Toffoli, 2006). A recently submitted (Farias et al., 2009) analysis of mitochondrial DNA sequence variation in *Colossoma macropomum* clearly indicates a nearly two order magnitude increase in effective population size in the Amazon basin. Exponential growth is a significantly better explanation than no growth. This increase commenced approximately 350,000 years ago, and it supports the hypothesis of a demographic expansion associated with a colonization event in the Pleistocene. I have subsequently carried out Bayesian demographic analyses of population size change in BEAST for other taxa including the Amazonian manatee, two species of crocodiles, and four fish species that have appropriate sampling to address this question. In all instances demographic growth of an order of magnitude or larger was observed, and this growth began between 300,000 and 500,000 years before present. When I specifically tested the alternative hypotheses of demographic growth vs. no demographic growth, I rejected no demographic growth in all cases. These preliminary data lead me to conclude that Pleistocene colonization of Amazon basin lowlands is likely to have occurred. These findings are unexpected as previous hypotheses (Hubert et al., 2007a, Hubert & Renno, 2006) proposed a 5 mya colonization event in the Amazon basin lowlands following the retreat of the Miocene/Pliocene incursion. Hubert et al. (2007b) suggest a Pleistocene demographic expansion (~800,000 ybp) in the Bolivian basin, however, they correlated this event with changes in forest cover (marine incursions into the Bolivian basin have not occurred since the mid Miocene (Hoorn, 1996)).

## SYNERGY ACTIVITIES AND INTERNATIONAL COLLABORATIONS

The international component of the CREST proposal is of the benefits to both parties in the proposed collaboration and is a logical augmentation of the existing CREST's activities. Tomas Hrbek, senior personnel in the NSF-CREST proposal at the University of Puerto Rico – Rio Piedras is an active member of the Molecular Ecology, Evolution and Genetics group. He has long maintained informal collaborations with researchers and the graduate programs at the Federal University of Amazonas (UFAM) and the National Institute of Amazonian Research, and has contributed to the graduate programs at both institutions through giving courses and training graduate students. The CREST partnership supplement will allow for the formalization of these activities, and will establish the CREST-CATEC center as a premier center of its kind in the Caribbean and a partner to institutions with similar visions throughout Latin America. It will further provide a mechanism for the exchange of graduate students and faculty between the CREST-CATEC center and the Biology-UPR graduate program, and the GCBEV-INPA and DB-UFAM graduate program.

### Specific benefits to CREST-CATEC members at the University of Puerto Rico

1. Providing international research experience to graduate students
2. Exposing of students to a foreign research environment
3. Exposing of students to novel ideas and paradigms
4. Training of global scientists in a traditionally underrepresented minority-serving institution
5. Fostering long-lasting international partnerships for research and human resource capacity building in aquatic biodiversity studies

### Specific benefits to the Genetics, Conservation and Evolutionary Biology graduate program of the National Institute of Amazonian Research

1. Providing research opportunities for graduate students from partner institutions at the University of Puerto Rico
2. Internationalization of the GCBEV graduate program
3. Exposing of students to novel ideas, paradigms and techniques
4. Formalizing of a link between the GCBEV-INPA and Biology-UPR graduate programs

### Specific benefits to the Biological Diversity graduate program at the Federal University of Amazonas

1. Providing research opportunities for graduate students from partner institutions at the University of Puerto Rico
2. Internationalization of the DB graduate program
3. Exposing of students to novel ideas, paradigms and techniques
4. Formalizing of a link between the DB-UFAM and Biology-UPR graduate programs

### Specific benefits to Amazonian Ichthyology Research Network (RIIA)

1. Fortifying a network of scientists working to solve problems of common interest
2. Providing a mechanisms to train future scientists and members of this network
3. Providing opportunities of network members to visit the laboratories of other network members, and to collaboratively work on problems of common interest.

## POTENTIAL PITFALLS

**Low statistical support for phylogenetic hypotheses** – Number of the currently available phylogenetic hypotheses have low statistical support, potentially due to a rapid radiation of a relatively large number of lineages within a relatively short amount of time. This and the goal of obtaining complete family level phylogenetic hypotheses is one of the reasons for proposing to reconstruct phylogenetic relationships of the studied families. However, the issues of low statistical support for phylogenetic hypotheses will only play a role if this affects hypotheses addressed in this proposal. If this will remain the case, I will test if addition of more sequence data is likely to increase phylogenetic support (Philippe et al., 1994). If addition of more data does not raise statistical support, the interpretations of results will need to be treated with caution, or even discarded. **Dating of divergence events** – Estimation of divergence times at nodes of interests is not trivial. However, both Bayesian (Thorne & Kishino, 2002, Drummond et al., 2006) and Maximum-likelihood (Sanderson, 2002) methodologies provide statistically well supported and robust estimates, that have narrow and well supported confidence intervals. Furthermore, the order of

magnitude difference in divergence times expected between Pliocene and Pleistocene events is expected to be sufficiently large to not produce overlapping confidence intervals.

## MANAGEMENT PLAN

The size of this project and its international component requires a careful coordination of research activities. Initial coordination effort will be done via online conferencing (from UPR's CREST-CATEC facilities), and a schedule of activities will be maintained on secure webpages developed for this project. The purpose of these coordination activities will be to finalize: **(1) Educational plans** and opportunities for student exchanges among participants, and participation of students in field research. A mechanism to recruit promising candidates for student exchanges will be developed, under the auspice of the NSF-CREST program at UPR-RP. **(2) And formalize a mechanism of exchange** between graduate students and researchers at UPR-RP and Brazilian partner institutions. **(3) Collecting schedules and sampling design** for each region, based on the individual schedules of the participants and appropriate field season. Collecting trips have already been planned; however, the actual itineraries and participant involvement will need to be determined, as well as standardized sample collection and information management. **(4) Project workflow in the laboratory** will also need to be optimized. This is tightly lined with field schedule, and the availability of samples. **(5) Data management** via a centralized web site and electronic data repository, using UPR's *HPCf* bioinformatic resources will be presented to participants, and optimized for flexibility and ease of use.

A graduate student will participate in field work in Brazil, and a graduate student plus undergraduate students will participate in data collection, analysis and preparation of results for publication at UPR.

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## JUSTIFICATION FOR SUPPLEMENTAL FUNDING

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**Overview:** The University of Puerto Rico, Rio Piedras Campus (UPR-RP) proposes to submit a CREST Partnership Supplement to our currently funded CREST grant. The CREST grant supports the Center for Applied Tropical Ecology (CATEC) which serves as a catalyst for systemic change in educational, research development and structural institutional transformation in a Hispanic minority serving institution. CATEC's mission is to promote a) training of human resources at the graduate, undergraduate and post-doctoral levels, b) state-of-the-art research in conservation biology and environmental issues, c) infrastructure improvement, and d) production of relevant results for policy and conservation management.

The unifying research theme is biodiversity conservation under the scenario of climate change. The questions addressed were chosen for their potential to be applied at the scale of the Caribbean Basin, or to the Tropics in general, and to serve as foundations for projects at the regional or international level. The Molecular Ecology, Evolution and Genetics (MEEG) area address conservation issues using molecular markers to understand the phylogeny, the spatial distribution of genetic diversity, and species interactions in the Puerto Rico and the Caribbean. The Species and Population Management (SPM) area focuses on impact of interactions between exotic and native species, and understanding patterns of regional diversification and distribution of endangered species and species of economic importance. The Ecosystem Processes (EP) area addresses the question of how species, both terrestrial and marine, respond to climate variability, by comparing the effect of paleoclimatic change with the impact of temporal and spatial variation in climate as well as historical anthropogenic changes in land use on ecosystem structure and function. All projects foster inter- and trans disciplinary synergies and interactions among research fellows within each thrust area, with other thrust areas, the University of Puerto Rico, and the local, national and international scientific communities.

The goal of the current supplement is to expand and enhance our STEM educational capacities through the fostering of long-lasting international partnerships for research and human resource capacity building in aquatic biodiversity studies and our ability to understand the genetic basis of adaptive responses of organisms to climate change. To accomplish this goal, we propose to study the genetic signatures of past climate change in a group of fishes in the Amazon basin by establishing collaborations with the Amazonian Ichthyology Research Network (RIIA ? Red de Investigaciones Ictiol gicas de la Amazon a . The objective of this study is to identify historical and ongoing processes that are responsible for generating and maintaining aquatic biodiversity in the Amazon basin. The central hypothesis to be tested is that the central and eastern Amazon basin is ecologically young, and that it has only recently been colonized from refugia located on the Brazilian and Guyana Shields, and in the western Amazon basin. This is a test of the refugium theory applied to aquatic fauna, and it tests the important of past climatic events on shaping of current demographic patters. It also provides information on location of aquatic refugia, and data for predicting the effects of future climate change on Amazonian aquatic fauna.

The intellectual merits of this supplement are fourfold: 1) develops an understanding on how past and present climate change affect biodiversity in the Neotropics, one of the topmost world biodiversity hotspots, 2) develops an understanding the genetic basis of response to changing environmental conditions, 3) develops an understanding of how fast organisms can track environmental changes via adaptive responses and 4) provides higher visibility for applied ecology and conservation research as a science career option to Hispanic students by providing competitive and attractive research training opportunities.

The broader impacts of the proposed activities are based on CATEC's leadership as a Hispanic science community where faculty and students lead significant synergic and integrative activities in research, education and knowledge-transfer in applied ecology and conservation of natural

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## JUSTIFICATION FOR SUPPLEMENTAL FUNDING

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resources. CATEC will continue to increase the numbers of trained Hispanic minority professionals engaged in research, teaching and management, empowering a more diverse generation of scientists by a) contributing to the training of global scientists in a traditionally underrepresented minority-serving institution and b) fostering long-lasting international partnerships for research and human resource capacity building in aquatic biodiversity studies.

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# HRD PROPOSAL BUDGET

YEAR 1

ORGANIZATION <b>University of Puerto Rico-Rio Piedras</b>				FOR NSF USE ONLY					
				PROPOSAL NO.	DURATION (months)				
PRINCIPAL INVESTIGATOR / PROJECT DIRECTOR <b>Elvira Cuevas</b>				AWARD NO.	Proposed	Granted			
				A. SENIOR PERSONNEL: PI/PD, Co-PI's, Faculty and Other Senior Associates (List each separately with title, A.7. show number in brackets)				NSF Funded Person-months	
				CAL	ACAD	SUM			
1. <b>Elvira Cuevas - PI</b>				0.00	0.00	0.00	\$ 0	\$ 0	\$ 0
2. <b>Tomas Hrbek - Co-PI</b>				0.00	0.00	1.00	7,030	0	7,030
3.									
4.									
5.									
6. ( 0 ) OTHERS (LIST INDIVIDUALLY ON BUDGET JUSTIFICATION PAGE)				0.00	0.00	0.00	0	0	0
7. ( 2 ) TOTAL SENIOR PERSONNEL (1 - 6)				0.00	0.00	1.00	7,030	0	7,030
B. OTHER PERSONNEL (SHOW NUMBERS IN BRACKETS)									
1. ( 0 ) POST DOCTORAL SCHOLARS				0.00	0.00	0.00	0	0	0
2. ( 0 ) OTHER PROFESSIONALS (TECHNICIAN, PROGRAMMER, ETC.)				0.00	0.00	0.00	0	0	0
3. ( 1 ) GRADUATE STUDENTS							13,200	0	13,200
4. ( 0 ) UNDERGRADUATE STUDENTS							0	0	0
5. ( 0 ) SECRETARIAL - CLERICAL (IF CHARGED DIRECTLY)							0	0	0
6. ( 0 ) OTHER							0	0	0
TOTAL SALARIES AND WAGES (A + B)							20,230	0	20,230
C. FRINGE BENEFITS (IF CHARGED AS DIRECT COSTS)							949	0	949
TOTAL SALARIES, WAGES AND FRINGE BENEFITS (A + B + C)							21,179	0	21,179
D. EQUIPMENT (LIST ITEM AND DOLLAR AMOUNT FOR EACH ITEM EXCEEDING \$5,000.)									
<b>General - Purpose Spectrophotometer</b>				\$	8,000				
TOTAL EQUIPMENT							8,000	0	8,000
E. TRAVEL							3,900	0	3,900
1. DOMESTIC (INCL. CANADA AND U.S. POSSESSIONS)							3,900	0	3,900
2. FOREIGN							7,100	0	7,100
F. PARTICIPANT SUPPORT COSTS									
1. STIPENDS \$ _____				784					
2. TRAVEL _____				5,000					
3. SUBSISTENCE _____				2,024					
4. OTHER _____				0					
( 2 ) TOTAL PARTICIPANT COSTS							7,808	0	7,808
G. OTHER DIRECT COSTS									
1. MATERIALS AND SUPPLIES							24,326	0	24,326
2. PUBLICATION COSTS/DOCUMENTATION/DISSEMINATION							0	0	0
3. CONSULTANT SERVICES							0	0	0
4. COMPUTER SERVICES							0	0	0
5. SUBAWARDS							0	0	0
6. OTHER							0	0	0
TOTAL OTHER DIRECT COSTS							24,326	0	24,326
H. TOTAL DIRECT COSTS (A THROUGH G)							72,313	0	72,313
I. INDIRECT COSTS (F&A)(SPECIFY RATE AND BASE)									
<b>Modified direct costs rate (Rate: 49.0000, Base: 56504)</b>									
TOTAL INDIRECT COSTS (F&A)							27,687	0	27,687
J. TOTAL DIRECT AND INDIRECT COSTS (H + I)							100,000	0	100,000
K. RESIDUAL FUNDS							0	0	0
L. AMOUNT OF THIS REQUEST (J) OR (J MINUS K)							\$ 100,000	\$ 0	\$ 100,000
M. COST SHARING PROPOSED LEVEL \$				0	AGREED LEVEL IF DIFFERENT \$				
PI/PD NAME <b>Elvira Cuevas</b>				FOR NSF USE ONLY					
ORG. REP. NAME* <b>Escalona de Motta, Gladys</b>				INDIRECT COST RATE VERIFICATION					
				Date Checked	Date Of Rate Sheet		Initials - ORG		

# HRD PROPOSAL BUDGET

Cumulative

ORGANIZATION				FOR NSF USE ONLY		
<b>University of Puerto Rico-Rio Piedras</b>				PROPOSAL NO.	DURATION (months)	
					Proposed	Granted
PRINCIPAL INVESTIGATOR / PROJECT DIRECTOR				AWARD NO.		
<b>Elvira Cuevas</b>						
A. SENIOR PERSONNEL: PI/PD, Co-PI's, Faculty and Other Senior Associates (List each separately with title, A.7. show number in brackets)	NSF Funded Person-months			Funds Requested From NSF	Non-Federal Matching Funds	Total Project Cost
	CAL	ACAD	SUM			
1. <b>Elvira Cuevas - PI</b>	0.00	0.00	0.00	\$ 0	\$ 0	\$ 0
2. <b>Tomas Hrbek - Co-PI</b>	0.00	0.00	1.00	7,030	0	7,030
3.						
4.						
5.						
6. ( 0 ) OTHERS (LIST INDIVIDUALLY ON BUDGET JUSTIFICATION PAGE)	0.00	0.00	0.00	0	0	0
7. ( 2 ) TOTAL SENIOR PERSONNEL (1 - 6)	0.00	0.00	1.00	7,030	0	7,030
<b>B. OTHER PERSONNEL (SHOW NUMBERS IN BRACKETS)</b>						
1. ( 0 ) POST DOCTORAL SCHOLARS	0.00	0.00	0.00	0	0	0
2. ( 0 ) OTHER PROFESSIONALS (TECHNICIAN, PROGRAMMER, ETC.)	0.00	0.00	0.00	0	0	0
3. ( 1 ) GRADUATE STUDENTS				13,200	0	13,200
4. ( 0 ) UNDERGRADUATE STUDENTS				0	0	0
5. ( 0 ) SECRETARIAL - CLERICAL (IF CHARGED DIRECTLY)				0	0	0
6. ( 0 ) OTHER				0	0	0
TOTAL SALARIES AND WAGES (A + B)				20,230	0	20,230
<b>C. FRINGE BENEFITS (IF CHARGED AS DIRECT COSTS)</b>						
TOTAL SALARIES, WAGES AND FRINGE BENEFITS (A + B + C)				949	0	949
<b>D. EQUIPMENT (LIST ITEM AND DOLLAR AMOUNT FOR EACH ITEM EXCEEDING \$5,000.)</b>						
			\$ 8,000			
TOTAL EQUIPMENT				8,000	0	8,000
<b>E. TRAVEL</b>						
1. DOMESTIC (INCL. CANADA AND U.S. POSSESSIONS)				3,900	0	3,900
2. FOREIGN				7,100	0	7,100
<b>F. PARTICIPANT SUPPORT COSTS</b>						
1. STIPENDS \$ _____			784			
2. TRAVEL _____			5,000			
3. SUBSISTENCE _____			2,024			
4. OTHER _____			0			
( 2 ) TOTAL PARTICIPANT COSTS				7,808	0	7,808
<b>G. OTHER DIRECT COSTS</b>						
1. MATERIALS AND SUPPLIES				24,326	0	24,326
2. PUBLICATION COSTS/DOCUMENTATION/DISSEMINATION				0	0	0
3. CONSULTANT SERVICES				0	0	0
4. COMPUTER SERVICES				0	0	0
5. SUBAWARDS				0	0	0
6. OTHER				0	0	0
TOTAL OTHER DIRECT COSTS				24,326	0	24,326
<b>H. TOTAL DIRECT COSTS (A THROUGH G)</b>						
				72,313	0	72,313
<b>I. INDIRECT COSTS (F&amp;A)(SPECIFY RATE AND BASE)</b>						
TOTAL INDIRECT COSTS (F&A)				27,687	0	27,687
<b>J. TOTAL DIRECT AND INDIRECT COSTS (H + I)</b>						
				100,000	0	100,000
<b>K. RESIDUAL FUNDS</b>						
				0	0	0
<b>L. AMOUNT OF THIS REQUEST (J) OR (J MINUS K)</b>						
				\$ 100,000	\$ 0	\$ 100,000
<b>M. COST SHARING PROPOSED LEVEL \$ 0</b>				<b>AGREED LEVEL IF DIFFERENT \$</b>		
PI/PD NAME <b>Elvira Cuevas</b>				FOR NSF USE ONLY		
				INDIRECT COST RATE VERIFICATION		
ORG. REP. NAME* <b>Escalona de Motta, Gladys</b>				Date Checked	Date Of Rate Sheet	Initials - ORG

## BUDGET JUSTIFICATION

Senior Personnel: One month of summer salary is requested for Tomas Hrbek to release him from teaching obligations during the summer and allow him to travel and concentrate on the administration of the project. Requested amount is \$7,030 over a two year period.

Graduate Students: Full time (12 month) funding for one graduate student at the University of Puerto Rico – Rio Piedras (UPR) is requested for the duration of the grant. The amount includes tuition and health insurance. These funds will go to support a new student that will be recruited to develop a doctoral dissertation on one or more aspects of this project. Requested amount is \$13,200 over a two year period.

Undergraduate Students: No funds are requested at this time, but several undergraduates will be involved through UPR's RISE and CREST programs that support undergraduate research experiences. REU supplements from NSF will be sought to increase undergraduate involvement as soon as promising students are identified through these programs.

Equipment: A Beckman Coulter A23615 Du 720 General-Purpose Spectrophotometer (DU 700 Series UV/Vis) is requested; price \$8,000.

Travel: Funds are requested to support a collecting trip to Brazil. The trip will involve CREST senior personnel (Tomas Hrbek) and one graduate student. Funding is requested for round trip air transportation from San Juan, Puerto Rico to Manaus, Brazil via Miami, FL (\$1,500/person), for accommodations and maintenance in Manaus for one week (\$90/day/person), and for travel to the Trombetas, Tapajos and Xingu Rivers (\$1,000/person), per diem (\$28/day/person) and hotel accommodations (\$30/day/person). An additional three days stay in Manaus during which time final preparations will be made and a research talk will be presented at Universidade Federal do Amazonas (UFAM) and the Instituto Nacional de Pesquisas da Amazonia (INPA) will be presented, is requested (\$70/day/person). The total foreign travel support requested is \$7100. Additional funds are requested to attend one annual meeting for the PI and a graduate student such as the SSE meetings or the ASIH meetings over a two year period. Requested amount is \$3,900.

Participant Support Costs: Funds are requested for two participants, a PI from our partner laboratory (Izeni Farias), and for a graduate student (Maria da Conceição Freitas Santos) to travel to San Juan, Puerto Rico from Manaus, Brazil via Miami, FL in the amount of \$1,500/person. While at the University of Puerto Rico, we request a per diem of \$28/day/person plus a housing allowance of \$200/person for the projected two week visit. Further support is for inland travel within Brazil (\$1,000/person), per diem (\$28/day/person) and hotel accommodations (\$30/day/person). The total requested participant support is \$7808.

Supplies: Supplies are requested each year molecular work and laboratory expenses, to support field efforts, shipping of material from the field, and for maintenance of laboratory stocks. Supplies are requested each year for high throughput volume molecular work, to support field efforts, shipping of materials, and museum curation. The supplies portion of this grant averages at \$25,000 per year, but is justified by the large number of samples that will need to be processed. We propose to sample three localities and at each locality I propose to sample at least 20 individuals from each species (with few exceptions, all localities have species of every clade, and some localities have more than one species per genus). Additional localities have already been collected. In total this represents 5100 specimens. *From each sampling locality* two individuals will be sequenced for the mtDNA control region, the mtDNA COI gene, and the 5 nuclear loci. This represents 2125 single stranded, one time only sequencing reactions. Sequencing costs at UPR are just under \$2 per sample. This includes price of BigDye chemistry, ABI matrix and buffers, alcohol and NaOAc for sample precipitation and 5¢ per sample for capillary wear. Single stranded sequencing without any failures or repeats will cost \$4,250. *From each sampling locality* these same 20 individuals will also be genotyped for 15 microsatellite markers for a total of 9000 genotyping reactions per species with three target species. Sample genotyping is around \$1 per sample largely due to the cost of ABI size standard, and ABI



matrix and buffers; genotyping estimates also include 5¢ per sample for capillary wear. Genotyping is estimated to cost \$9,000 (assuming 3-primer multiplexing – cost may be reduced further depending on multiplexing reliability and how primers are designed). All samples used in this study will need to be extracted, and high-quality archival extraction using Qiagen column kits (necessary since DNA extracts will be divided among labs) costs ~\$2 per sample. Total extraction cost is estimated at \$1,400. All samples also need to be PCR amplified. PCR amplification for sequencing reaction purification using PEG is ~30¢ per sample for a total cost of \$782 for reparation of samples for sequencing. PCR amplification for genotyping reaction is ~20¢ per sample for a total of \$5,400. These estimates also do not include any basic laboratory consumables such as gloves, pipette tips, PCR and sequencing plates, PCR plate covers, PCR tubes, agarose, buffers, chemicals, liquid media, DNA stains, etc. For the size of this project, costs are estimated at around \$4,500 per year. Furthermore these calculations do not assume any failure rate at any stage of this project, example that DNA may have to be reextracted, that PCR may need to be repeated, or that sequencing or genotyping reactions may need to be repeated. This can add additional 10% to the cost of this project. The total laboratory cost of the project is \$25,332.

Publication Costs: these funds are not requested in this proposal. Publication costs will be offset by the main CREST proposal.

Consultant Services: these funds are not requested in this proposal. However, I have requested that Dr. Guillermo Ortí provides consultation and progress evaluation as a professional courtesy.

Iquitos, February 25, 2009



Dr. Carmen García-Dávila  
Av. Quiñones, km 2.5  
Iquitos - Peru

**Att°: Dr Tomas Hrbek,**  
University of Puerto Rico  
Center for Applied Tropical Ecology

Dear Colleague,

I confirm my interest in collaborating in your NSF project entitled “**An Evaluation of Pleistocene Climate Change on the Demographic History of Amazonian Fishes**”, in the framework of the RIIA network (Red de Investigación sobre la Ictiofauna Amazonica, Amazonian Ichthyology Research Network), and in the strict respect of the institutional agreements between IIAP (Instituto de Investigaciones de la Amazonía Peruana) and IRD (Institut de Recherche pour le Développement).

The RIIA network ([www.riiaamazonia.org](http://www.riiaamazonia.org)) was officially created in June 2005 in Iquitos Peru. It mainly aims at understanding the evolution of the Amazonian fish diversity and providing tools for a better conservation and management of the ichthyological resources. This includes the sharing of local data and results for an integrative approach at the regional or continental scale. RIIA also seeks to promote the international formation and co-tutoring of students.

Dr. Carmen García- Dávila  
Jefe del Laboratorio de Biotecnología – LB  
Instituto de investigaciones de la Amazonía Peruana - IIAP  
Miembro integrante del RIIA



Ministério da  
Ciência e Tecnologia



February 23, 2008

To Whom it May Concern,

I am pleased to write this letter in support to Dr. Tomas Hrbek and the CREST-CATEC team at the University of Puerto Rico. The partnership formed between Dr. Tomas Hrbek and the graduate program at National Institute for Research in the Amazon (INPA) under my supervision, at which I was Head of Department, has been strong and firm. During the past four years, students and teachers of the INPA's Genetics Graduation Program have received his training. The partnership has provided substantial professional development to many of our students and teachers to improve the understanding of population genetics, phylogeography and evolutionary biology.

Besides, the partnership with at least four distinct research groups at National Institute for Research in the Amazon (evolutionary biology of Amazonian fish, birds, reptiles and mammals) has provided collaborative co-authoring.

I am particularly excited about this new project to be conducted by Dr. Hrbek that will test competing hypotheses that have resulted in differentiation of riverine faunas of the Amazon basin.

Because of the contribution this grant could bring both in terms of scientific knowledge and student development, as Researcher specializing in characids and former Head of the INPA Graduate Department, I strongly support the project of Dr. Hrbek and urge favorable consideration for its funding through the NSF-CREST supplement grant program.

Sincerely,

A handwritten signature in black ink, which appears to read 'Jorge I. R. Porto'. The signature is fluid and cursive, written over a light-colored rectangular background.

Dr, Jorge I. R. Porto,  
Department of Aquatic Biology  
National Institute for Research in the Amazon (INPA), Brazil.



**UNIVERSIDADE FEDERAL DO AMAZONAS**  
**INSTITUTO DE CIÊNCIAS BIOLÓGICAS**  
**DEPARTAMENTO DE BIOLOGIA**  
**LABORATÓRIO DE EVOLUÇÃO E GENÉTICA ANIMAL – LEGAL**  
Av. Gen. Rodrigo Octávio Jordão Ramos, 3000. Bloco E CEP 69077-000  
Tel +55 92 36474233  
Email: [izeni\\_farias@ufam.edu.br](mailto:izeni_farias@ufam.edu.br)



Manaus, 27 de Fevereiro de 2009

Dear Tomas,


I am excited to collaborate with you on the proposed research project entitled "An evaluation of Pleistocene climate change on the demographic history of Amazonia fishes ". I am very excited about this proposal since the primary focus of my laboratory is addressing how aquatic biodiversity is generated in the Amazon basin, and addressing these questions in a collaborative manner has the potential to really address some of the challenges in biodiversity research in the Amazon. Much new biological and geological information has come forth in the last few years, and we are finally in the position to critically test many of the hypotheses proposed for diversification in the Amazon basin.

I have a well equipped molecular lab, I manage the tissue collection of UFAM, and I have close collaborations with a number of colleagues at INPA, and thus an access to INPA's collections. I can provide you with these resources, and also provide you with a space where subsamples may be deposited and samples stored until export permits are obtained. I also have several excellent and excited students that I am sure will eagerly participate in field research, and will develop their theses projects through opportunities generated by this project. The successful funding of this project would allow the continued development of joint projects, collaborations at individual and institutional levels, and it would provide opportunities for the formation of many young scientists.

In short, I offer you logistic support in the field and the use of my laboratory. I also guarantee to help you to get any additional research permits you may need to carry out your work.

I wish you much success in obtaining the NSF-CAREER supplement grant.

Sincerely,

  
Profa Dra. Izeni Pires Farias  
Lab de Evolução e Genética Animal LEGAL  
Departamento de Biologia, ICB  
Universidade Federal do Amazonas - UFAM