Molecular taxonomy of *Plagioscion* Heckel (Perciformes, Sciaenidae) and evidence from mtDNA RFLP markers for an invasive species in the Paraná river, Southern Brazil [1]

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ABSTRACT. Mitochondrial RFLP markers were developed to examine whether *Plagioscion squamosissimus* (Heckel, 1840) is invasive in natural environments of the congener *P. ternetzi* in the Paraná river, in southern Brazil. Specimens of *P. squamosissimus* and of the putative *P. ternetzi* (Boulenger, 1895) were obtained from the Negro river (Manaus, Amazonas, Brazil) and from Paraná river, respectively. Fragments of the cytochrome b gene (900bp) were amplified by PCR and four restriction enzymes (Eco RI, Mbo I, Bam HI and Alu I) yielded the mitochondrial markers. An additional RFLP analysis with a cytochrome b gene sequence of *Plagioncion* sp. from GeneBank was carried out to validate the prior analysis. No genetic differentiation was found among either sample. While molecular variation in the cytochrome b analysis was no substantial among individuals, the combined analysis was important for demonstrating that there is no evidence for differentiation of the putative sample *P. ternetzi* from that of *P. squamosissimus*. The ecological implications of the introduced occurrence of *P. squamosissimus*, as well as the role of molecular taxonomic approaches for biodiversity studies are discussed.
KEY WORDS. *Plagioscion squamosissimus*; Cytochrome b; genetic identity; biological invasions.

RESUMO. **Taxonomia molecular de *Plagioscion* Heckel (Perciformes, Sciaenidae) e evidências de marcadores moleculares RFLPs de mtDNA para uma espécie invasora no rio Paraná, Sul do Brasil.** Marcadores RFLPs mitocondriais foram desenvolvidos para verificar se *Plagioscion squamosissimus* (Heckel, 1840) é invasora nos ambientes naturais da espécie congênere *P. ternetzi* no rio Paraná, no sul do Brasil. Exemplares de *Plagioscion squamosissimus* e supostamente de *P. ternetzi* (Boulenger, 1895) foram obtidos, respectivamente, do rio Negro (Manaus, AM, Brasil) e rio Paraná (Foz do Iguaçu, PR, Brasil). Foram amplificados, via PCR, fragmentos de cerca de 900pb do Citocromo b e foram utilizadas quatro enzimas de restrição (Eco RI, Mbo I, Bam HI e Alu I) para os fins de geração dos marcadores moleculares. Foi desenvolvida, a partir de uma seqüência de Citocromo b de *Plagioscion* sp. (genebank), uma análise de RFLP adicional, objetivando validar a primeira análise acima mencionada. Considerando a inexistência de significativa variação observada no Citocromo b dos indivíduos analisados, a análise combinada com todas as enzimas foi importante para demonstrar que não existe diferenciação molecular para o nível específico entre a suposta amostra de *P. ternetzi* e aquela de *Plagioscion squamosissimus*. São discutidas as implicações ecológicas da introdução de *P. squamosissimus*, bem como a aplicação da taxonomia molecular para estudos de biodiversidade.
PALAVRAS-CHAVE. *Plagioscion squamosissimus*; Citocromo b; identidade genética; invasões biológicas.

Molecular phylogenetics and systematics have shown great strides in recent years, due to the development of new and diverse methods for analysis of molecular DNA markers. These methods allow assessing the genetic variability of the biota carrying to a superestimation on the global biodiversity besides the relationships among taxa (Grechko 2002). Molecular taxonomic approaches may be defined as DNA-based methods that permit an exact and rapid method of distinguishing specimens based on their interspecific variations. Molecular taxonomy has many benefits such as (1) data can be obtained from a single specimen, (2) morphologically indistinguishable taxa can be separated, (3) all stages and morphs of taxa are accessible and (4) a single technique is applicable to all taxa, such as RFLP markers (Blaxter & Floyd 2003).

Plagioscion Gill, 1861 (Sciaenidae) is a neotropical freshwater genus of fish comprising seven species (*sensu* Froese &

Pauly 2006, Soares & Casatti 2000, Aguilera & Aguilera 2000). *Plagioscion squamosissimus* (Heckel, 1840) is widely distributed throughout the Orinoco, Amazon and Parnaíba basins, and was also introduced into reservoirs of northeastern Brazil during the 1970s. Currently, it is among the dominant species in the Itaipu reservoir (Benedito-Cecilio & Agostinho 2000). In that reservoir, the species varies with respect to the size at maturation and location and timing of the spawning season (Carnelós & Benedito-Cecilio 2002). *Plagioscion squamosissimus* is an opportunistic fish predator (fishes comprise 80% of its diet) (Torloni *et al.* 1993, Braga 1998, Loubens 2003).

Human impacts on the ecosystems continue to grow (McNeely 1996). One critical element in this increased impact is the movement of organisms from one region to another through trade, transport, and tourism. Many of these introductions of organisms into new ecosystems are beneficial to people and detrimental to the ecosystem receiving them. Thus, an invasive species may be defined as a species whose establishment and spread threatens ecosystems, habitats or species with economic or environmental harm (Colautti & MacIsaac 2004). Since *P. squamosissimus* may be considered as an invasive species into the Paraná river, here we wished to confirm its occurrence in natural environments of the endemic *Plagioscion ternetzi* (Boulenger, 1895), downstream to the Itaipu powerplant, based on MT (molecular taxonomy) analysis.

MATERIAL AND METHODS

Ten specimens of *P. squamosissimus* were obtained from the Negro river (Manaus, Amazonas, Brazil; Figs $1a_1/a_2$) and thirteen specimens of the putative *P. ternetzi* were obtained from the Paraná river, downstream from the Itaipu power plant (Foz do Iguaçu, Paraná, Brazil; Figs $1b_1/b_2$). Of these, 5/6 individuals (see results for details) of *P. squamosissimus* from Amazon basin and 6 individuals of the putative *P. ternetzi* were analysed. Genomic DNA was isolated from ethanol-preserved muscle tissue by the Salting-out methodology (Miller & Poleski 1998).

The mitochondrial cytochrome b gene was amplified by polymerase chain reaction (PCR) in 25µl reactions containing 4µl dNTPs (5mM), 2.5µl reaction buffer (10X), 2µl $MgCl_2$ (25mM), 2µl of each primer (10µM), 1µl Taq DNA polymerase (1U/µl), 2µl of template DNA (50ng/µl) and 9.5µl of H_2O. PCR conditions were as follows: 30 cycles of 94ºC (1min), 40ºC (45secs.), 72ºC (90secs), 18 cycles 94ºC (1min), 53ºC (30 secs), 72ºC (1min). The following primers set were used for PCR: Cytblscie 5' CGAAACTAATGACTTGAAAAACCACCGTTG 3' and Cytbhscie 5' AAATAGGAARTATCAYTCTGGTTTRAT 3'. Both, PCR conditions and the primers used followed Santos *et al.* (2003).

RFLP markers were yielded by using 4 restriction enzymes (Tab. I). Experiments used 1 µl of the PCR-based fragments plus 1 U of each enzyme at 37ºC for three hours following the manufacturer's suggestions. The products were resolved by electrophoresis in 1% agarose gels run with TBE buffer (0.89 m Tris, 0.89 m boric acid and 0.08 m EDTA, pH 8.3). Electrophoresis was conducted at $3V/cm^{-1}$. Gels were stained with ethidium bromide and the image was captured by using the digital gel documentation system Vilber Lourmat IP.010.SD.

An additional analysis used a Cytochrome b sequence from *Plagioscion* sp. (genebank accession nº AY374296) to verify the consistency of the mtDNA markers. Thus, the number of restriction site repeats within each sequence was verified and compared, given each enzyme used.

Table I. Summary of the restriction enzimes used with their sequence restriction sites (arows).

Enzime	Sequence restriction site
Alu I	AG ↓ CT
Bam HI	G ↓ GATCC
Eco RI	G ↓ AATTC
Mbo I	↓ GATC

RESULTS

Fragments (approximately 900bp) were obtained from the mitochondrial cytochrome b gene in all samples (Fig. 2a). The Eco RI and Mbo I restriction profiles presented a single band of 900bp for both Paraná and Negro river samples (Figs 2b, d). The restriction profile for Bam HI was an identical 725bp band in both samples (Fig. 2e). Alu I restriction profile was the most revealling and had three bands, corresponding to 400, 275 and 225bp (Fig. 2c).

The additional RFLP analysis involving the 1085bp of the Cytochrome b sequence collected from genebank revealed the high consistency for the mtDNA markers. Specific RFLP markers were observed for the species sampled in the confluence between Amazonas and Tamshiyacu rivers in Peru (Sloss *et al.* 2004) (Figs 3a, b, c, d).

DISCUSSION

DNA/PCR-based analyses have been considered one of the most important methodological revolutions for biodiversity analysis (Whelan 2001)

PCR results showed that the primer set performed well, by amplifying a single band corresponding to a fragment of the cytochrome b gene, (Fig 2a) for all specimens analyzed (also see Santos *et al.* (2003) for *Macrodon ancylodon* Bloch & Schneider, 1801). Also, it suggests that very similar flanking complementary sequences such as 5'GCTTTGATTACTGAACTTTTTGGTGGCAAC 3' and 5' TTTATCCTTRATAGTYAGACCAAARTA 3' could be conserved in the sciaenid cytochrome b gene. The 900bp for the cytochrome b gene of *Plagioscion* specimens here studied are very similar with that obtained from genebank (*Plagioscion* sp.), suggesting an average size of 900-1000bp for this mitochondrial gene, similar to those from several representatives of the main fish groups (Tab. II). Such molecular genetic pattern seems to be also

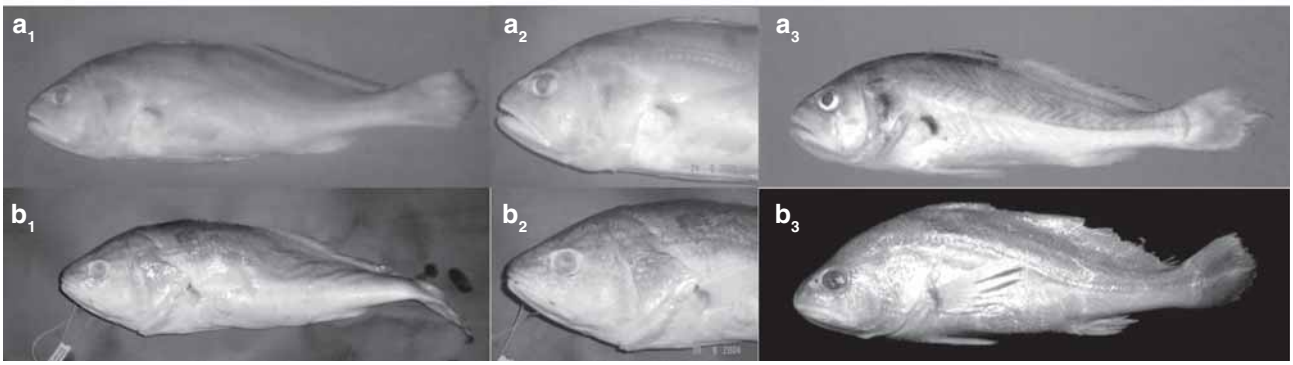

Figure 1. Samples (a_1/a_2) *Plagioscion squamosissimus* from the Rio Negro (Manaus, Amazonas, Brazil); (a_3) *Plagioscion squamosissimus* (photo by Ana L. Casatti); (b_1/b_2) Putative sample of *Plagioscion ternetzi* from the Paraná river (Foz do Iguaçu, Paraná, Brazil); (b_3) *Plagioscion ternetzi* (photo by Dra. Lilian Casatti).

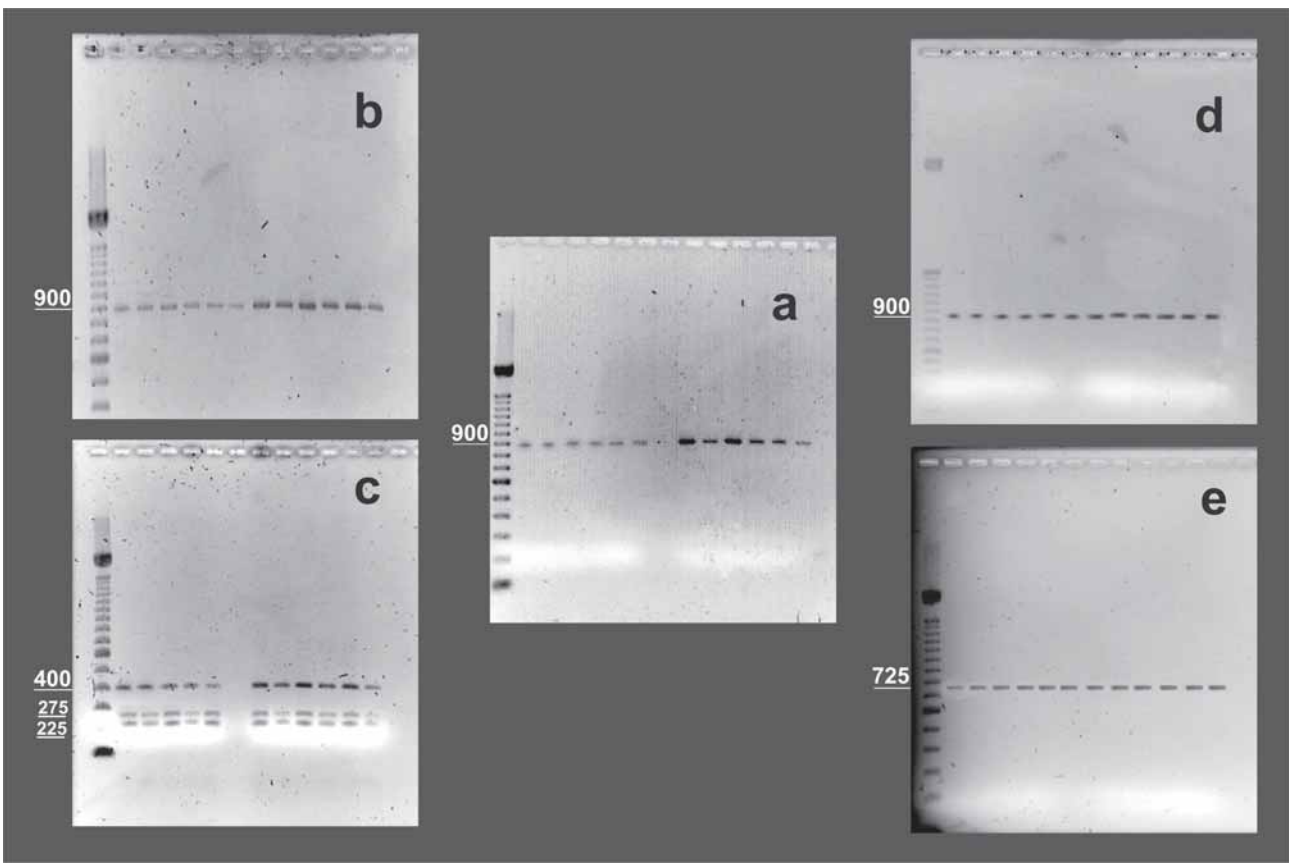

Figure 2. PCR and RFLP profiles. In (a) PCR products of the cytochrome b gene of 900bp; (b) and (d) the single band (900bp) yielded with EcoRI and MboI enzymes, respectively; (c) RFLP profiles using the AluI enzyme; (e) the single band (725bp) obtained with BamH1 enzyme. The first 5/6 specimens (left to right) are of *P. squamosissimus*. The other individuals are putatively *P. ternetzi*.

a common feature among vertebrates considering that similar sizes are also detected in members of mammals, such as bats and rodents (Bradley & Baker 2001).

Mitochondrial DNA has been extensively studied in fish and most vertebrate groups (Moyses & Almeida-Toledo 2002, Jérôme *et al.* 2003, Palo & Merilä 2003, Reedle *et al.* 2003,

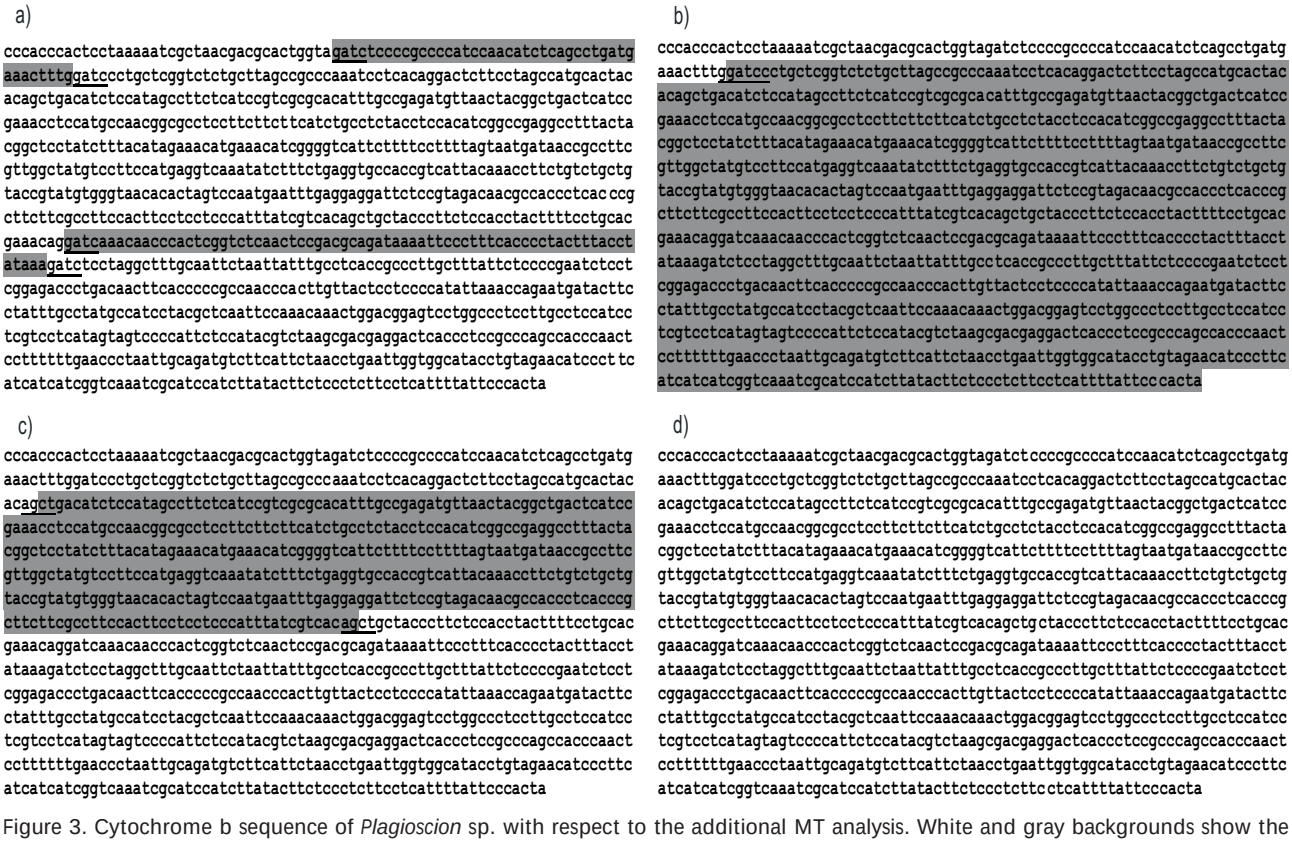

Figure 3. Cytochrome b sequence of *Plagioscion* sp. with respect to the additional MT analysis. White and gray backgrounds show the fragments yielded by the enzymes. (a), (b), (c) and (d) are the different RFLP profiles detected with MboI, BmaHI, AluI and EcoRI enzymes, respectively. The underlined tetra/hexanucleotide segments denote the restriction sites of the enzymes.

Francesca *et al.* 2004, Bremer *et al.* 2005, Ferreira *et al.* 2005). Thus, the well-characterized cytochrome b gene is particularly useful in the analysis of the relationship between recently diverged taxa (Stepien & Kocher 1997) and is a powerful mtDNA marker for species levels (Meyer 1994) since two specimens with different molecular profiles may be considered as different species (see Parson *et al.* 2000 for details). Therefore, results herein that the two samples represent one species is supported by those studies, given the same molecular profile of cytochrome b in both samples (Figs 2b, c, d, e). Furthermore, RFLPs detected *Plagioscion squamosissimus* as an introduction into the Paraná river, downstream to Itaipu, within the distribution of the endemic congener, *Plagioscion ternetzi*. Cryptic invasive fish have been discovered also in others environments by the use of molecular techniques (Collins *et al.* 2002, Hickey *et al.* 2004).

Plagioscion squamosissimus is a top predator, with mostly fish comprising its diet (Braga 1998, Loubens 2003, Bennemann *et al.* 2006), and so its introduced occurrence may be an important threat to the native community, including the endemic congener *Plagioscion ternetzi*. Indeed, local fishermen have already noticed a decline in the abundance of *P. ternetzi* in their fish catch. The decline and ultimate extinction of native species as a consequence of biological invasions have been documented for neotropical ichthyofauna (Mooney & Cleland 2001, Latini & Petrere Jr 2004).

The additional RFLP analysis described herein was to test the conclusions of the MT analysis with a cytochrome b sequence from a *Plagioscion* species (Sloss *et al.* 2004). Specific molecular profiles were identified for *Plagioscion* sp., in comparison with those from *P. squamosissimus*. Thus, while a single band of 900bp identified *P. squamosissimus*, five bands (38bp, 43bp, 71bp, 423bp, 510bp) identified *Plagioscion* sp. (Figs 2d and 3a, respectively) by using MboI enzyme. Using BamHI enzyme, a single band of 725bp was found in *P. squamosissimus*, while 2 bands (81bp and 1004bp) were found in *Plagioscion* sp. (Figs 2e and 3b). Using AluI, the best resolution enzyme, three bands were found for both *P. squamosissimus* and *Plagioscion* sp., but of different molecular weights (400, 275 and 225bp; Fig. 2c and 150, 402 and 533bp; Fig. 3c, respectively). EcoRI enzyme did not differentiate the samples due to absence of its restriction site in both species (Figs 2b and 3d). Therefore, in comparing the geographical distributions among all *Plagioscion* species with that of *Plagioscion*

Table II. Survey of the size of cytochrome b gene for several fish species. The nomenclature used for taxonomic groups was based on Froese & Pauli (2006).

Species	Taxonomic group	Gene size (bp)	Genebank accession	Reference
Myxine glutinosa (Linnaeus, 1758)	Agnatha/Myxiniformes	1158	Y15185	Rasmussen *et al.* (1998)
Chimaera monstrosa (Linnaeus, 1758)	Gnathostomata/Chimaeriformes	1144	AJ310140	Arnason *et al.* (2001)
Carcharinus plumbeus (Nardo, 1827)	Gnathostomata/Elasmobranchii	1146	L08032	Martin & Palumbi (1993)
Latimeria chalumnae (Smith, 1939)	Acanthodii/Sarcopterygii	1143	NC001804	Zardoya & Meyer (1997)
Lepidosiren paradoxa (Fitzinger, 1837)	Acanthodii/Sarcopterygii/Dipnoi	1140	AF302934	Brinkmann *et al.* (2004)
Acipenser baerii (Brandt, 1869)	Actinopterygii/Chondrostei	859	AF238656	Birstein *et al.* (2000)
Amia calva (Linnaeus, 1766)	Neopterygii/Amiiformes	1140	AB018999	Kumazawa *et al.* (1999)
Lepisosteus spatula (Lacepède, 1803)	Neopterygii/Semionotiformes	1141	AP004355	Inoue *et al.* (2003)
Arapaima gigas (Schinz, 1822)	Teleostei/Osteoglossomorpha	1141	AB035241	Kumazawa *et al.* (1999)
Elops hawaiensis (Regan, 1909)	Teleostei/Elopomorpha	1152	AB051070	Inoue *et al.* (2004)
Anguilla anguilla (Linnaeus, 1758)	Elopomorpha/Anguilliformes	1140	AP007233	Minegishi *et al.* (2005)
Sardinella aurita (Valenciennes 1847)	Teleostei/Clupeomorpha	1141	AF472584	Jerome *et al.* (2003)
Sardinops sagax (Girard 1854)	Teleostei/Clupeomorpha	1141	AF472585	Jerome *et al.* (2003)
Chanos chanos (Forsskål 1775)	Euteleostei/Anotophysi/Chanoidei	1140	AY504825	Lavoue & Sullivan (2004)
Gonorynchus greyi (Richardson 1845)	Euteleostei/Anotophysii/Gonorhyncoidei	1141	AB054134	Saitoh *et al.* (2003)
Phenacogrammus interruptus (Boulenger 1899)	Euteleostei/Otophysii/Characiformes	1141	AB018998	Kumazawa *et al.* (1999)
Chalceus macrolepidotus (Cuvier, 1817)	Otophysii/Characiformes	1141	AB054130	Saitoh *et al.* (2003)
Carassius auratus (Linnaeus, 1758)	Otophysii/Cypriniformes	1141	NC006580	Unpublished (GeneBank)
Apteronotus albifrons (Linnaeus, 1766)	Otophysii/Gymnotyformes	1141	AB054132	Saitoh *et al.* (2003)
Eigenmannia sp. (Valenciennes, 1842)	Otophysii/Gymnotyformes	1137	AB054131	Saitoh *et al.* (2003)
Hexanematichthys (Arius) platypogon (Günther, 1864)	Otophysii/Siluriformes	920	AJ580996	Unpublished (GeneBank)
Bagre marinus (Mitchill, 1815)	Otophysii/Siluriformes	920	AJ581355	Unpublished (GeneBank)
Corydoras rabauti (La Monte, 1941)	Otophysii/Siluriformes	1138	AB54128	Saitoh *et al.* (2003)
Salmo salar (Linnaeus, 1758)	Protachantopterygii/Salmoniformes	1141	NC001960	Hurst *et al.* (1999)
Alepocephalus tenebrosus (Gilbert, 1892)	Protachantopterygii/Argentiniformes	1141	AP004100	Ishiguro *et al.* (2003)
Esox americanus (Gmelin, 1789)	Protachantopterygii/Esociformes	1154	AY497436	Grande *et al.* (2004)
Ateleopus japonicus (Bleeker, 1854)	Sternopterygii/Ateleopodiformes	1141	AP002916	Miya *et al.* (2001)
Chauliodus sloani (Bloch & Schneider, 1801)	Sternopterygii/Stomiiformes	1137	NC002915	Kawaguchi *et al.* (2001)
Aulopus japonicus (Günther, 1877)	Cyclosquamata/Aloupiformes	1141	NC002674	Kawaguchi *et al.* (2001)
Aphredoderus sayanus (Gilliams, 1824)	Parachantopterygii/Percosiformes	1141	AP004403	Miya *et al.* (2003)
Porichthys myriaster (Hubbs & Schultz, 1939)	Parachantopterygii/Batrachoidiformes	1180	AP006739	Miya *et al.* (2005)
Mugil cephalus (Linnaeus, 1758)	Acanthopterygii/Mugiliformes	1138	NC003182	Miya *et al.* (2001)
Melanotaenia lacustris (Munro, 1964)	Acanthopterygii/Atheriniformes	1140	AP004419	Miya *et al.* (2003)
Mastacembelus favus (Hora, 1924)	Acanthopterygii/Synbranchiformes	1138	AP002946	Miya *et al.* (2001)
Oreochromis mossambicus (Peters, 1852)	Acanthopterygii/Perciformes	1135	NC007231	Unpublished (GeneBank)
Plagioscion sp. (Heckel, 1840)	Acanthopterygii/Perciformes	1085	AY374296	Sloss *et al.* (2004)

sp. (Sloss *et al.* 2004) we suggest that the present molecular characterization could be attributed to *Plagioscion montei* (Soares & Casatti 2000) since *P. montei* is the single species sampled for some river in Peru (also see Sloss *et al.* 2004).

Finally, the present approach suggests that molecular techniques may offer a very precise method of identifying biodiversity as well as recognizing invasive species. Therefore, we recommend the use of this tool in any study in which species richness need be tested, to test whether local samples represent actually native species, as well as to request the human responsibilities over the environmental impacts made.

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