The utilization of ancient DNA to assess fish biodiversity: example of Mormyridae

Sébastien Lavoué
Geneticist

Jean-François Agnèse
Geneticist

Introduction

The zoological and botanical collections of the world's Natural History Museums are an incredible heritage. By their richness, they are supports for important systematic studies. These collections have become even more precious because many species of animals and plants can no longer be harvested either because certain areas cannot be easily accessed (high costs, geopolitical problems) or because of the rarity or disappearance of the species in its natural habitat (LEVEQUE, 1994).

The goal of these collections is to allow the description and the classification of all living and fossil organisms. Classically, the use of these collections was apparent only with morphological, morphometrical or anatomical approaches. Only recently has the molecular exploitation of these collections been envisaged (HIGUCHI *et al.*, 1984; THOMAS *et al.*, 1989), giving them a new dimension. In fact, the *in vitro* chained amplification technique or PCR (MULLIS

and FALOONA, 1987; SAIKI *et al.*, 1988) allows the study of a DNA sequence from a very small quantity of genetic material. The strength of this technique suggests the ability to study rare and/or damaged DNA like that found in fossils or of tissues preserved in museums (ancient DNA, *s.l.*).

After having reviewed the advantages and difficulties of utilizing the DNA of collection specimens during the comparative biology study, and more particularly of those fixed in formaldehyde, we present our results obtained from specimens of the Mormyridae family preserved at the Musée royal d'Afrique centrale (Mrac) in Tervuren and at the Muséum national d'histoire naturelle (MNHN) in Paris.

DNA studies from formaldehyde-fixed tissues

Why use DNA from formaldehyde-fixed tissues ?

The use of DNA from collection specimens may be an answer to the sampling problems encountered in many comparative biology studies, particularly those using molecular biology techniques (LECOINTRE, 1993; WHEELER, 1992). For example, GAUTHIER *et al.* (1988), showed the importance of fossils on the phylogeny of Amniotes and were therefore obliged to modify the phylogeny proposed by GARDINER (1982).

The study of the genetic structures of populations and of the processes of speciation could benefit from a spatial-temporal dimension thanks to the use of ancient individuals whose capture dates and locations are noted in collection registers. Several works (THOMAS *et al.*, 1990; WAYNE *et al.*, 1991; HARDY *et al.*, 1994) have shown the interest of such an approach.

Likewise, the introduction of individuals from exogenous populations of a species into an area could lead to a genetic modification of a local population by introgression. The molecular analysis of specimens captured prior to the introduction and preserved in collections would allow the determination of the degree of introgression. This is even more important considering that the introduction of new species or of geographically distinct populations is quite common, especially in the teleosts (WELCOMME, 1988).

Generally speaking, all types of population genetics studies can benefit from the study of this DNA.

Fixation conditions influence DNA extraction and amplification

Studies of molecular biology exploit two types of samples from which ancient DNA can be extracted (*s.l.*), fossils and animals that have been subjected to a preservation treatment after their deaths and stored in zoological collections. HERMANN and HUMMEL (1993) reviewed the entire body of work on fossil DNA. For animals from zoological collections, there are basically two types of preservation techniques: either by drying (naturalized) or preserving in a liquid medium (generally 70% ethanol) after having been fixed in formaldehyde (diluted to 4-10%, buffered or not, from a few hours to several days). Formaldehyde is the most commonly used fixative because of its ease of use (available in large quantities and not expensive).

The preservation technique chosen is based partially on the lifestyle of the animal. Therefore, terrestrial vertebrates and invertebrates are almost always dried, while aquatic vertebrates especially the teleosts, are preserved in liquid, size permitting. Therefore, the use of ichthyological collections in molecular biology must first pass through the development of techniques capable of rendering the DNA of these formalin-fixed specimens usable.

There is a certain number of studies where part of the sampling has been taken from dry tissues found in museums, such as skin,

feathers or bones (HOUDE and BRAUN, 1988; COOPER *et al.*, 1992; HIGUCHI *et al.*, 1984; THOMAS *et al.*, 1989, 1990; WAYNE *et al.*, 1991; PAABO, 1989; PAABO *et al.*, 1988; etc). The difficulty of extracting and amplifying the DNA from formalin-treated samples is reflected in the lack of work on the subject. If we exclude the successes observed in medicine on tissues of human origin preserved after having been fixed for a very short time (a few hours), carefully washed and included in paraffin (TSAI and O'LEARY, 1993; FITZGERALD *et al.*, 1993; SHIBATA *et al.*, 1991), in 1997 and in spite of certain methodological studies (CRISCUOLO, 1992, on reptiles; DE GIORGI *et al.*, 1994, on nematodes; VACHOT and MONNEROT, 1996, on amphibians), no molecular phylogeny whose sampling includes formalin-fixed specimens has been published. In teleosts, to our knowledge, only the work of SHIOZAWA *et al.* (1992) on a subspecies of trout (Salmonidae) has been published. However, the results obtained must be confirmed.

The quality (DNA fragmentation and successful amplification by PCR) and the quantity of DNA extracted are subject to several factors related to the fixation (GOEBEL and SIMMONS, 1993; VACHOT and MONNEROT, 1996). These factors are the formalin concentration, the pH and the temperature of the fixing solution (KOSHIBA *et al.*, 1993), the duration of the fixation (HAMASAKI *et al.*, 1993; KARLSEN *et al.*, 1994), the age of the collections (VACHOT and MONNEROT, 1996). These different parameters act upon the size of fragments recovered (and thus on the size of fragments that can be amplified) but also on the quantity of DNA extracted (GOEBEL and SIMMONS, 1993; VACHOT and MONNEROT, 1996). Also, the action of the formalin causes the creation of covalent bonds between certain proteins and the DNA, especially with certain histones (BRUTLAG *et al.*, 1969; JACKSON, 1978; KOSHIBA *et al.*, 1993), and the creation of bonds within the DNA molecule. The fragmentation of the DNA molecule is an irreversible process, whereas the creation of bonds between proteins and the DNA within the DNA molecule can be a reversible phenomenon. The fixative can also induce modifications in the sequence (PAABO, 1985; DE GIORGI *et al.*, 1994). If the mechanisms of the formation of these bonds are still unknown and under discussion, the

fragmentation of the DNA is caused by the acidification of the medium, induced by the oxidation of the formalin into formic acid, that acidification being greater still if the formalin is not buffered. It should be noted that even a non-oxidized solution of formalin is acidic. These structural modifications of the DNA limit the size of the amplified fragment to a maximum of a few hundred pairs of bases.

Faced with these problems, some authors have proposed new methods of preservation. GOEBEL and SIMMONS (1993) reviewed the different techniques of preservation, and proposed alternative methods to preserve both the morphology of the animal and its DNA. Their works view the problem from the angle of preserving fragments of maximum size. However, no amplification by PCR was made with the fragments to test these methods. Also, they proposed no particular extraction protocol. VACHOT and MONNEROT (1996), proposed both new preservation techniques and an extraction protocol applicable to amphibian specimens already stored in collections.

Because of the poor quality and the small quantity of DNA extracted and of the extreme sensitivity of PCR, the tissue sampling, the extraction and the amplification are all more susceptible to contamination than in the case of work involving fresh DNA. When working with formalin-fixed DNA, appropriate anti-contamination measures must be taken (HUMMEL and HERMANN, 1993). The measures recommended for avoiding all contamination are not specific to formalin-fixed DNA, but are necessary whenever extracting any type of ancient DNA. A dedicated room is necessary, reserved for pre-PCR manipulations of ancient DNA and equipped with a laminar flow hood with a UV sterilization unit. All equipment used there in must be specific to the room. Technicians should not have been at work in any other labs before entering. Containers and solutions must have been autoclaved and then UV treated for 15 minutes. Also, control samples must be used at every step. The extraction controls allow the determination of the extent of contamination at the extraction phase. They undergo the same treatments as the sample to be extracted but contain no tissue. The first control is processed as the first sample and the second control is

processed as the last sample. The first control reveals contamination from the environment, the second reveals contamination from the other samples. The other controls during the amplification are not specific to the DNA. Because of these contamination problems, the use of one or two probes reserved for the group being studied to amplify the desired fragment is indispensable.

Difficulties to obtain preserved tissues

Besides the technical difficulties posed by the use of this type of material in molecular biology, there is also the problem of the availability and accessibility of material from collections which are rare and limited in quantity. THOMAS (1994) list five criteria to help make the decision to destroy a sample from a museum collection for use in molecular studies: 1) Scientific value and feasibility of the study; 2) Qualifications of the researcher and/or laboratory to undertake this research; 3) Availability of samples from living populations (GRAVES and BRAUN, 1992); 4) Volume of material already taken from the collection in relation to the request; and 5) Efforts of museum personnel to satisfy the request. Within the framework of the fourth criterion, it seems obvious in the case of certain rare or reference specimens not to take material for molecular studies, nor to destroy a specimen for anatomical studies. In the case of fishes, collections are often very rich in numbers of individuals; for example, there are almost a million specimens of African fishes at Musée Royal de l'Afrique Centrale at Tervuren. Therefore it is often possible to obtain samples for the majority of species. It is also important to sample in such a way that the specimen is the least damaged to allow future morphological/anatomical studies. A small sampling of dorsal muscle does minimal damage. Lastly, THOMAS (1994) suggests returning the remaining DNA sample to the museum, which for but is not always reasonable, because not all museums are equipped to store DNA samples.

Example: Phylogenetic relationships in family Mormyridae

The Mormyridae (Teleostei; Osteoglossomorpha) are African fishes found only in fresh water. They are found in virtually every freshwater environment from the Sahara to Northern South Africa (including the Nile basin). The Mormyridae family has 17 genera and about 200 species (NELSON, 1994; GOSSE, 1984). They possess the ability to emit (and to receive) weak electrical signals due to the presence of muscular electrical organs in the caudal peduncle (HOPKINS, 1986). Because of this particularity they have been the major subjects of physiological and electrophysiological studies (MOLLER, 1995). To understand the formation and evolution of their electrical organs, there must first be well defined hypotheses on their phylogenetic relationships. From an osteological analysis, TAVERNE (1992) was the first to propose phylogenetic hypotheses for this family. AGNESE and BIGORNE (1992) and VAN DER BANK and KRAMER (1996) studied four and five genera respectively by enzymatic polymorphism. ALVES GOMES and HOPKINS (1997) studied one genus, *Brienomyrus*.

Within this study, we sequenced a fragment of mitochondrial DNA cytochrome *b* from two specimens coming from the Musée Royal de l'Afrique Centrale at Tervuren (MRAC) collection. We also sequenced four genera available fresh in order to be able to discuss the validity of the sequences coming from formalin-fixed specimens based on their phylogenetic positions.

Material and methods

The origin of the fixed specimens used in this study are *Boulengeromyrus knoepffleri* from Makokou, Ivindo, Gabon in 1975, MRAC (75-24-P1-2); *Paramormyrops gabonensis* from Makokou, Ivindo, Gabon in 1975, MRAC (75-24-P7-13); *Myomyrus pharao* from Kisangani, Zaire, Zaire in 1980, MRAC

(82-25-P32-45) ; *Genyomyrus donnyi* Kisangani, Zaire, Zaire in 1980 MRAC (83-31P-39-40) ; *Stomatorhinus walkeri* from Route Loubomo, Loukénéné, Gabon MRAC (91-79-P-99-113) ; *Ivindomyrus opdenboschi* from Ma'an, Ntem, Cameroon MRAC (93-82-P-2) ; *Myomyrus macrops* from Epula, Epula, Zaire in 1986 MRAC (91-79-P405-416) ; *Mormyrops zanclirostris* from Loa-Loa, Ivindo, Gabon in 1964 MNHN (1987-897) ; *Isichthys henryi* from Marela, mongo, Guinea in 1986 MNHN (1986-525) ; *Stomatorhinus corneti* from Ybiegn, Nyabarélé, Gabon in 1964 MNHN (1987-910). The origin of the fresh specimens used in this study are *Marcusenius senegalensis* from Batamani, Niger, Mali in 1994 ; *Petrocephalus bovei* from Batamani, Niger, Mali in 1994 ; *Gnathonemus petersii* from Aquarium import ; *Mormyrops zanclirostris* Makokou, Ivindo, Gabon in 1997 and *Heterotis niloticus*, Bia, Côte d'ivoire in 1996.

For each individual, 1 cm^3 of muscle was taken from the dorsal position, and placed in 70°C ethanol. For the specimens coming from collections, no information about preservation techniques could be supplied. The extraction method used on samples from fresh fish was that of WINNPENNINCKX *et al.* (1993). The extraction method used for collection specimens was similar to that developed by VACHOT and MONNEROT (1996) for amphibians. The muscle sample was washed four times with TE pH=8 (SAMBROOK *et al.*, 1989) and left to incubate in the extraction solution (proteinase K 0.8 mg/ml, SDS 2%, EDTA 10 mM, Tris HCl pH=8 100 mM, 50 mM DTT, 100 mM NaCl (VACHOT and MONNEROT, (1996)) for 72 hours at 50°C with slight agitation. Every 12 hours the concentration was increased by 0.7 mg/ml of proteinase K. After digestion, the DNA is extracted by a classical phenol-chloroform method. The DNA was precipitated with two parts by volume of 100 % ethanol in the presence of 0.2 M NaCl for one night at -20°C. After centrifuging, the pellet was rinsed twice with 70 % ethanol. The extract obtained was collected in a volume of 20 µl of twice-distilled water where 10 µl were used to estimate the quantity and the quality of the DNA extract (migration on 1% agarose gel, visualization using ethidium bromide under ultraviolet light).

Two primers were used to amplify a 495 bp fragment from the 3' terminal part of the cytochrome *b*. One of these primers, L15930 was described by KOCHER *et al.* (1989). The other primer L'195 (5'-GAA-ACC-GGM-TCA-AAC-AAC-CC-3') was developed specifically for this study. It was first tested on DNA extracts from fresh fish tissues, Mormyridae, Perciformes and Siluriformes, to ensure its specificity. Amplifications were made using a « crocodile II » thermocycler (Appligene). They were performed in a volume of 50 µl (34.2 µl of water, 2.5 µl of DMSO, 5 µl of MIX 5 (2.15 mM of each dNTP), 1 µM of each probe, 1 µl of genomic DNA, 5 µl of buffer 10X and 2 U of Taq polymerase (Hitaq, Prolabo)). The first cycle was 1mn at 94°C, 1 mn at 54°C and 2 mn at 72°C. Finally a last cycle of 4 mn at 72°C was performed. Five µl of amplified product were set to migrate on 1% agarose gel in the presence of a length marker. The amplified fragments of satisfactory size were then cloned using a pCR-Script(+) cloning kit (Stratagene). The sequencing was performed using the method of SANGER *et al.* (1977) with the help of a T7 Sequencing™ Kit (Pharmacia Biotech). Sequence recording was performed using the MUST program (PHILLIPE, 1993). Analysis using the Distance Neighbor Joining method (SAITOU and NEI, 1987) was also performed with this program. Parsimony analysis was carried out using the PAUP 3.1.1. program (SWOFFORD, 1993). Tree robustness was estimated by the bootstrap method (FELSENSTEIN, 1985) using PAUP with 100 replicates and branch length.

Results and discussion

Extractions and amplifications DNA from fresh and fixed tissues

During this study a fragment of the 3' part of cytochrome *b* of 589 bp was amplified and sequenced for four species of Mormyridae freshly available: *Mormyrops zanclirostris*, *Petrocephalus bovei*, *Marcusenius senegalensis*, *Gnathonemus petersii*, and an extra group, *Heterotis niloticus*.

We extracted total DNA from ten samples of muscular tissue collected from individuals fixed in formalin and preserved in the collections of the Mrac and the MNHN. From these extracts, the quality of DNA was visualized on 1.5% agarose gel under ultraviolet light with BET. In all cases the DNA was degraded. The size of fragments was between 100 and 2000 bp. There seemed to be no obvious relationship between the mean size of fragments obtained and the age (duration) of the specimens in the collection.

From these extracts we tried to amplify using PCR a fragment of 495 bp of the mitochondrial gene coding for cytochrome *b* in the 3' part. One of the two primers used was specific to Mormyidae, in order to avoid some of the threat of contamination. The second primer was universal (KOCHER *et al.*, 1989). The PCR program was slightly modified from that used for samples of fresh tissue (JACKSON *et al.*, 1991). The time of hybridation and elongation were both extended (1 mn 30 instead of 1 mn). Two positive amplifications were obtained from 10 extracts. These two specimens were of two different species, *Genyomyrus donnyi* and *Myomyrus pharao*, preserved in the MRAC collection since 1980.

The two positive amplifications come from these two specimens which were collected during the same mission. Their fixation conditions were similar, and no doubt conducive to the proper preservation of the DNA. Access to the fixation parameters, knowing which type of formalin was used (buffered or not), its concentration, its fixation period, would allow a selection of suitable specimens whenever possible. Unfortunately, this information is never available with specimens in the collections. It has been shown that the greater the size of the fragment to be amplified, the harder it is to amplify it. Classically, it has been recommended to work with fragments less than 500 bp. In this study, the size of the fragment was fairly large (495 bp), but we have not better results with short fragments.

Authenticity of sequences

After treating the amplified fragment, the two sequences were determined and compared to sequences belonging to different Mormyridae species.

All the controls were negative. For *Myomyrus pharao*, we amplified and sequenced the piece of cytochrome *b* twice from two different extractions. The two sequences were identical. The alignment of the sequences of *Genyomyrus donnyi* and *Myomyrus pharao* to those from fresh tissues posed no difficulties. No insertion or deletion were detected. The majority of substitutions were found in the third positions of the codons, which is in agreement with a degeneration of the genetic code. After translation of the sequences into amino acids, no stop codon was found.

In order to validate the sequences by their respective position within the Mormyridae, the sampling for the comparison was determined on several systematic levels: the extra-Mormyridae level (with *Heterotis niloticus*); the Petrocephalinae versus Mormyridae level (with *Petrocephalus bovei*) and the intra-Mormyrinae level (with *Mormyrops zanclirostris*, *Gnathonemus petersii* and *Marcusenius senegalensis*). Numerous morpho-anatomic and physiological characters derived support the validity of each of these levels. The Mormyridae and the Mormyrinae are considered to be monophyletic, these last are grouped as closely related to the Petrocephalinae (TAVERNE ,1972; VAN DER BANK and KRAMER, 1996 (electrophoretic data); ALVES GOMES and HOPKINS, 1997).
After having eliminated the transitions, we obtained by the research option « Branch and Bound » from PAUP 3.1.1., two trees of a minimal length of 107 steps (CI=0.832, RI=0.635). They differed only by the position of *Genyomyrus donnyi*: 1) either *Genyomyrus donnyi* is closely related to *Gnathonemus petersii* (grouping supported by 2 synapomorphies and a bootstrap value of 51%, figure 1); or *Genyomyrus donnyi* is closely related to *Marcusenius senegalensis* (grouping supported by only one synapomorphy and a bootstrap value of 41%). The group formed by *Genyomyrus donnyi*, *Gnathonemus petersii* and *Marcusenius senegalensis* is confirmed by a very high bootstrap value (94%). *Mormyrops zanclirostris* is closed to this group. *Mormyrus pharao* is in a basal position within the Mormyrinae. The Mormyrinae ensemble is supported by a bootstrap value of 83%. The Petrocephalinae (*Petrocephalus bovei*) are sister group of the Mormyrinae. By the Neighbor-Joining method, the topology of the tree is the same as that where *Genyomyrus donnyi* is closely related to *Gnathonemus petersii* (confirmed by a bootstrap value of 67%).

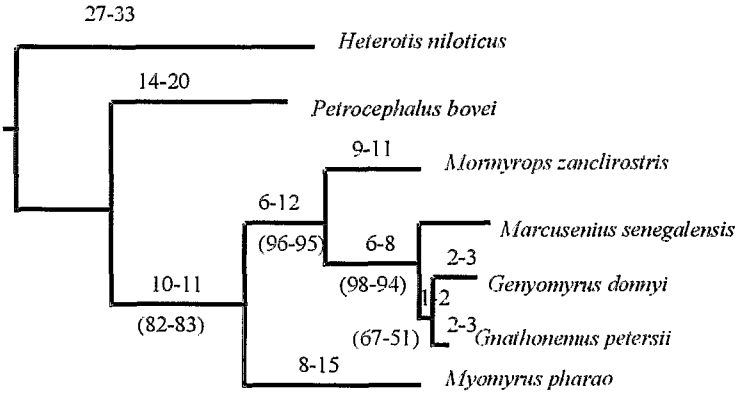

Figure 1
One of two most parsimonious tree obtained from a branch and bound search of PAUP (Swofford, 1993). Transitions are excluded from the analysis. Length = 107 steps. C.I.= 0.822, R.I.= 0.635. Branch lengths are proportional to the number of changes occurring along the branches under Acctran optimization. Numbers above branches are minimal and maximal branch lengths according to the optimization of homoplasies. Numbers below branches are bootstrap proportions obtained from 100 replicates using PAUP and NJ.

The position of *Genyomyrus donnyi*, close to *Gnathonemus senegalensis*, is congruent to that established by osteological data (TAVERNE, 1972). These two genera resemble each other a great deal, showing numerous derived character and therefore naturally very close.

The position of *Myomyrus pharao* is not in total agreement with the morpho-anatomical data. While it is a Mormyrinae, its grouping among them and despite the fact that the sampling was not sufficient, differs from that defined by TAVERNE in 1972 which grouped *Myomyrus*, *Isichthys* and *Mormyrops*. However, Taverne did not support this grouping by derived characters, he merely underlined the similarities between these three genera.

Even if we therefore cannot totally exclude contamination by a close species or the presence of a nuclear pseudo-gene whose divergence time is recent based on its weak argumentation, we still support the authenticity of the sequence. Even if the best proof of the

authenticity of the sequences obtained is not available, that of a comparison to the sequence of a fresh specimen, we are confident of this one. In fact, it is a cytochrome *b* sequence, whose systematic position in our sampling is not inconsistent with data acquired in morpho-anatomy.

Conclusion

To our knowledge, this is the first time that DNA sequences obtained from formaldehyde-fixed Teleost specimens have been used in a phylogenetic analysis. The extraction method used (VACHOT and MONNEROT, 1996, slightly modified) allows extraction of the DNA which is not always amplifiable. We believe that the fixation conditions play an important role in the results obtained. We confirm that the Petrocephalinae are sister group of the other Mormyrinae. Within the Mormyrinae and despite the fact that our sampling was incomplete, *Myomyrus pharao* is closely to the rest of the Mormyrinae. *Genyomyrus* is sister group to *Gnathonemus*.

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