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Non-invasive ancient DNA protocol for fluid-preserved specimens and phylogenetic systematics of the genus *Orestias* (Teleostei: Cyprinodontidae)

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Abstract

Specimens stored in museum collections represent a crucial source of morphological and genetic information, notably for taxonomically problematic groups and extinct taxa. Although fluid-preserved specimens of groups such as teleosts may constitute an almost infinite source of DNA, few ancient DNA protocols have been applied to such material. In this study, we describe a non-invasive Guanidine-based (GuSCN) ancient DNA extraction protocol adapted to fluid-preserved specimens that we use to re-assess the systematics of the genus *Orestias* (Cyprinodontidae: Teleostei). The latter regroups pupfishes endemic to the inter-Andean basin that have been considered as a 'species flock', and for which the morphology-based taxonomic delimitations have been hotly debated. We extracted DNA from the type specimens of *Orestias* kept at the Muséum National d'Histoire Naturelle of Paris, France, including the extinct species *O. cuvieri*. We then built the first molecular (control region [CR] and rhodopsin [RH]) phylogeny including historical and recently collected representatives of all the *Orestias* complexes as recognized by Parenti (1984a): *agassizii*, *cuvieri*, *gilsoni* and *mulleri*. Our ancient DNA extraction protocol was validated after PCR amplification through an approach based on fragment-by-fragment chimera detection. After optimization, we were able to amplify < 200 bp fragments from both mitochondrial and nuclear DNA (CR and RH, respectively) from probably formalin-fixed type specimens bathed entirely in the extraction fluid. Most of the individuals exhibited few modifications of their external structures after GuSCN bath. Our approach combining type material and 'fresh' specimens allowed us to taxonomically delineate four clades recovered from the well-resolved CR tree into four redefined complexes: *agassizii* (*sensu stricto*, i.e. excluding *luteus*-like species), *luteus*, *cuvieri* and *gilsoni*. The *mulleri* complex is polyphyletic. Our phylogenetic analyses based on both mitochondrial and nuclear DNA revealed a main, deep dichotomy within the genus *Orestias*, separating the *agassizii* complex from a clade grouped under shallow dichotomies as (*luteus*, (*cuvieri*, *gilsoni*)). This 'deep and shallow' diversification pattern could fit within a scenario of ancient divergence between the *agassizii* complex and the rest of *Orestias*, followed by a recent diversification or adaptive radiation within each complex during the Pleistocene, in- and outside the Lake Titicaca. We could not recover the reciprocal monophyly of any of the 15 species or morphotypes that were considered in our analyses, possibly due to incomplete lineage sorting and/or hybridization events. As a consequence, our results starkly question the delineation of a series of diagnostic characters listed in the literature for *Orestias*. Although not included in our phylogenetic analysis, the syntype of *O. jussiei* could not be assigned to the *agassizii* complex as newly defined. The CR sequence of the extinct *O. cuvieri* was recovered within the *cuvieri* clade (same haplotype as one representative of *O. pentlandii*), so the mtDNA of the former species might still be represented in the wild.

Key words: Ancient DNA, ethanol-fixed specimen, formalin-fixed specimen, inter-Andean basin**,** museum collections, phylogeny, pupfishes, species flock, species complex

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Introduction

Specimens stored in museum collections represent a crucial source of morphological and genetic information to tackle evolutionary and taxonomic issues. The added value of museum specimens becomes even more important when considering morphological groups with 'fuzzy' species delimitations (e.g. species complexes), and extinct or very elusive taxa (Chakraborty *et al.* 2006; Stuart *et al.* 2006; Wandeler *et al.* 2007).

Although 'ancient DNA' extraction from museum specimens nowadays is done routinely (Ramakrishnan & Hadly 2009; Shapiro & Hofreiter 2012; Wandeler *et al.* 2007), its level of success heavily depends on the chemical treatments the specimens have been subjected to. The situation is especially complex for fluid-preserved collections, due to the variety of preservatives, methods of fixation and storage conditions that have been used during the last two centuries. It also happens that a specimen or series of specimens are subjected to different preservatives and storage conditions during their time in the collections. Unfortunately, information on the specific chemical treatments applied to collections is rarely available in museums' records (Chakraborty *et al.* 2006; Koshiba *et al.* 1993; Schander & Halanych 2003). The resulting challenge for ancient DNA investigators is to find a balance between the investment of time and resources (i.e., the number of museum specimens to include in the study) and the quality of obtained data (i.e., amplifiable DNA fragments sufficiently informative and taxonomically representative).

Fluid-preserved teleostean collections mostly fall in one of the following categories: ethanol-fixed and -preserved, formalin-fixed and ethanol-preserved, and formalin-fixed and -preserved (Koshiba *et al.* 1993; Raja *et al.* 2011). The use of formalin as a preservative for museum specimens has been a common practice since the late 1800s (De Bruyn *et al.* 2011; Schander & Halanych 2003). DNA extraction and PCR amplification from specimens that were fixed or preserved in formalin may be problematic because of cross-linking between proteins and DNA following DNA fragmentation and nucleotide alteration, often resulting in low amounts and poor quality genomic fragments of small size, varying between 100 and 500 bp (Koshiba *et al.* 1993; Pääbo *et al.* 2004; Schander & Halanych 2003; Wirgin *et al.* 1997). The deleterious effects of formalin on the genome are expected to be higher with a greater time of specimen fixation, making the extraction of DNA unlikely from specimens that were fixed in formalin for long periods of time (Chakraborty *et al.* 2006; Koshiba *et al.* 1993; Raja *et al.* 2011; Zhang 2010).

Many ancient DNA protocols adapted to a variety of museum material (e.g. formalin-fixed and paraffin-embedded tissues, molluscs, and teleostean scales) have been published (e.g. Chase *et al.* 1998; Goelz *et al.* 1985; Impraim *et al.* 1987; Rohland & Hofreiter 2007; Wisely *et al.* 2004; Yue & Orban 2001). However, only few protocols for DNA extraction from fluid-preserved teleostean specimens are available, particularly in the case of formalin-preserved specimens (Chakraborty *et al.* 2006; De Bruyn *et al.* 2011; Raja *et al.* 2011; Shedlock *et al.* 1997; Wirgin *et al.* 1997; Zhang 2010). In addition, most of the methods developed for ancient DNA extraction can be highly invasive, damaging to the specimens or leading to the total destruction of samples, sometimes discouraging museum curators from authorizing such investigations on their collections (Bolnick *et al.* 2012; Hofreiter 2012; Rohland *et al.* 2004; Wisely *et al.* 2004). In the context of fluid-preserved teleostean specimens, non-invasive methods for DNA extraction remain a challenge.

The genus *Orestias* Valenciennes (Cyprinodontidae: Teleostei) comprises pupfishes endemic to the high-latitude lakes and tributary streams from the inter-Andean basin of South America. Its main area of distribution covers the Lake Titicaca and most of the Andean Altiplano, from southern Peru to Bolivia and northeastern Chile. It represents one of the three native teleostean genera endemic to the area (Parenti 1984a; Vila *et al.* 2007; Villwock 1986). *Orestias* pupfishes are externally characterized by the absence of pelvic fins, a reduced and irregular body squamation pattern and a unique head pore pattern (Parenti 1984a; Villwock 1986). Given their great ecological and phenotypic diversity, particularly within the species assemblage from Lake Titicaca, they have been considered as a 'species flock' (Kosswig & Villwock 1964; Villwock 1962; but see Parenti 1894b). The first main revision of *Orestias* was proposed by Tchernavin (1944). The author recognized 20 species structured into four groups, distinguished by overall body shape and/or squamation pattern (Table 1). A later revision from the lesser Lake Titicaca delineated 15 species divided into eight morphological groups on the basis of morphometric and meristic characters (Lauzanne 1982). The most exhaustive taxonomic revision was based on morphometric, osteological and meristic characters and included the complete series of type material (Parenti 1984a). The author delineated 43 species grouped into four monophyletic complexes: *cuvieri* Valenciennes, *mulleri* Valenciennes and *gilsoni* Tchernavin, all endemic to Lake Titicaca and nearby water bodies, and *agassizii* Valenciennes (see Eschmeyer &

TABLE 1. Classification of the species of *Orestias* in the three main taxonomic revisions of the genus. Species described by Valenciennes [in Cuvier and Valenciennes] (1846)[a], Castelnau (1855)[b], Cope (1876)[c], Garman (1895)[d], Eigenmann & Allen (1942)[e], Tchernavin (1944)[f], Arratia (1982)[g], Lauzanne (1982)[h] and Parenti (1984a)[i]. Numbers in parentheses correspond to species groups.

Tchernavin (1944)	Lauzanne (1982)	Parenti (1984a)
		cuvieri complex
cuvieri (1) [a]		*cuvieri*
pentlandii (1) [a]	*pentlandii* (6)	*pentlandii*
	ispi (4) [h]	*ispi*
	forgeti (5) [h]	*forgeti*
		mulleri complex
		gracilis [i]
mulleri (4) [a]	*mulleri* (2)	*mulleri*
crawfordi (4) [f]	*crawfordi* (8)	*crawfordi*
tutini (4) [f]		*tutini*
incae (4) [d]		
		gilsoni complex
gilsoni (3) [f]	*gilsoni* (2)	*gilsoni*
taquiri (3) [f]		*taquiri*
mooni (4) [f]	*mooni* (3)	*mooni*
uruni (3) [f]		*uruni*
minutus (3) [f]		*minutus*
minimus (3) [f]	*minimus* (2)	*minimus*
	tchernavini (2) [h]	*tchernavini*
		tomcooni [i]
		imarpe [i]
		robustus [i]
		agassii complex
agassii (four variants, 2) [a]	*agassii* (7)	*agassii*
		empyraeus [e]
		frontosus [c]
polonorum (2) [f]	*polonorum* (7)	*polonorum*
		elegans [d]
jussiei (one subspecies, 2) [a]	*jussiei* (1)	*jussiei*
		puni [f]
		parinacotensis [g]
		laucaensis [g]
		tschudii [b]
		gymnotus [i]
		hardini [i]
		ctenolepis [i]
		ascotanensis [i]
		richersoni [i]
		multiporis [i]
		mundus [i]
luteus (2) [a]	*luteus* (1)	*ututo* [i]
		silustani [e]
		luteus
albus (2) [a]	*albus* (1)	*rotundipinnis* [i]
	olivaceus (8)	*farfani* [i]
		albus
		olivaceus
Hybrids		Hybrids
cuvieri x *pentlandii*		*cuvieri* x *pentlandii*
affinis [d] = *olivaceus* x *agassii*		
		Nomen nudum
		rospigliossi
		pentlandii var. *fuscus*

Fong 2010 for the correct use of the epithet name), the most speciose (24 species) and widely distributed complex, found in lake Titicaca but also in other hydrological systems within the inter-Andean basin. Since then, three additional species within this complex have been described from Chile (Vila 2006; Vila & Pinto 1986; Vila *et al.* 2011). Taxonomic delimitations within *Orestias* by means of classical taxonomic characters (i.e., morphometrics and meristic counts) have been hotly debated (Lauzanne 1992; Loubens 1989; Lüssen *et al.* 2003; Müller 1993; Villwock 1986; Villwock & Sienknecht 1995; Villwock & Sienknecht 1996), for four main reasons: i) the great morphological variation within the genus, ii) the difficulty in segregating among the different developmental stages (Villwock & Sienknecht 1995; Villwock & Sienknecht 1996), iii) the definition of diagnostic characters from small series of specimens that appear non-reliable when considering larger series (Villwock 1986; Villwock & Sienknecht 1996), and iv) the bias in the calculation of body indexes from warped fluid-preserved specimens (Villwock 1986). In addition, the occurrence of hybridization within and between complexes (Tchernavin 1944; Villwock & Sienknecht 1995; Aspiazu 2002; Esquer Garrigos *et al*. submitted), might blur species boundaries, further complicating the establishment of reliable diagnostic morphological characters. More recently, the phylogenetic systematics of *Orestias* was partially revised through a phylogenetic analysis based on mitochondrial DNA (mtDNA) (Lüssen *et al.* 2003). The authors failed to recover the monophyly of the *agassizii* complex, and showed that the latter should exclude *O. luteus* Valenciennes in order to be phylogenetically valid. Besides, the reciprocal monophyly of several species within the *agassizii* complex was not recovered.

In this context, we aim to evaluate the taxonomic boundaries within the genus *Orestias* in a taxonomically exhaustive (i.e. including representatives from all the complexes), molecular phylogenetic framework. For this, we first apply a modified, non-invasive ancient DNA extraction protocol (Rohland *et al.* 2004) to fluid-preserved specimens representing the type series of *Orestias* kept at the Muséum National d'Histoire Naturelle, Paris, France (MNHN), including the extinct species *O. cuvieri* (Harrison & Stiassny 2004; CREO extinctions database http://creo.amnh.org/pdi.html). This protocol using guanidinium-thiocyanate (GuSCN) was designed for non-destructive DNA extraction from 'hard' material (bones, teeth and skin) of mammalian specimens, but has never been tested on 'soft', fluid-preserved collections. We then build a molecular phylogeny encompassing all the species complexes of *Orestias*, through the combination of both historical (type material) and freshly collected representatives of each complex.

Material and methods

Sample collection. Twenty-eight specimens and 11 species, representing the four traditionally delimited complexes of *Orestias* (*cuvieri*, *mulleri*, *gilsoni*, *agassizii*) were collected from the lake Titicaca and wetlands ('bofedales') from Sur Lipez (Bolivia) between 2008 and 2010 (Table 2). Complexes and species have been identified according to external characters and identification keys given in Parenti (1984a) and Lauzanne (1982), and also by comparison with the teleostean collection of the Limnology Department of the Universidad Mayor de San Andres (UMSA), La Paz, Bolivia. Most of the specimens belonging to the g*ilsoni* and *mulleri* complexes could not be identified to the species level. Such individuals were grouped into 'morphotypes' according to their level of similarity in their external aspect (e.g. body shape, coloration and squamation), and were —whenever possible— related to a species or group of species within the complex to which they belong. Collected specimens were preserved in 99 % ethanol solution and deposited in the collections of the UMSA. A small tissue sample was taken from the dorsal muscle of each specimen for genetic analysis.

Sampling of historical fluid-preserved type specimens stored at MNHN included sixteen individuals representing eleven species as described by Valenciennes (1846) Lauzanne (1981) and Castelnau (1855) (Table 3). No information concerning the specific chemical treatment used for fixation was available. However, the external appearance of some specimens (e.g. with very soft and flaccid bodies; see Table 3) suggested they had been dried out several times and re-hydrated with over-concentrated preservative (Villwock 1986). All the fluid-preserved specimens used in our study were photographed before and after the DNA extraction procedure to visualize potential changes in color, scale loss, degradation or loss of fin rays, resulting from the processing of the individuals. In addition, a 'damage scale' was established to assess dehydration and distortion of body surfaces (1 = no damage, 2 = slight distortion and dehydration of body surface, and 3 = strong dehydration and distortion of body surface; see Table 3).

TABLE 2. List of the specimens used in this study, specifying sample locality and Genbank accession numbers. *Species complexes as defined by Parenti (1984a). Species described by Valenciennes [in Cuvier and Valenciennes] (1846)[a], Castelnau (1855)[b] and Lauzanne (1981)[c]. Numbers in parentheses correspond to the new MNHN catalogue numbers assigned to the specimens that were exposed to GuSCN bath.

Species / Taxon	Sample code	Complex*	Sample locality	Genbank acc. numb.	
				CR	RH
O. agassizii	P234	*agassizii*	Lake Poopo, Bolivia	**KC408006**	**KC408044**
O. agassizii	T113	*agassizii*	Huatajata, Lake Titicaca, Bolivia	**KC408007**	**KC408045**
O. cf polonorum (sensu Lauzanne*)*	H260	*agassizii*	HichuKhota lake, Bolivia	**KC408008**	**KC408046**
O. cf agassizii	KH290	*agassizii*	Khotia, Bolivia	**KC408009**	**KC408047**
O. cf agassizii	K300	*agassizii*	Khara Khota, Bolivia	**KC408010**	**KC408048**
O. cf agassizii	TP323	*agassizii*	Puno Bay, Peru	**KC408011**	**KC408049**
O. cf agassizii	BE1182	*agassizii*	Bofedal Celeste, Bolivia	**KC408012**	**KC408050**
O. cf silustani	1682	*agassizii*	Moho Bay, Peru	**KC408013**	**KC408051**
O. luteus	GENT21	*agassizii*	Huatajata, Lake Titicaca, Bolivia	**KC408014**	**KC408052**
O. luteus	GENT24	*agassizii*	Huatajata, Lake Titicaca, Bolivia	**KC408015**	**KC408053**
O. luteus	T077	*agassizii*	Huatajata, Lake Titicaca, Bolivia	**KC408016**	**KC408054**
O. luteus	U175	*agassizii*	Lake UruUru, Bolivia	**KC408017**	**KC408055**
O. luteus	U217	*agassizii*	Lake UruUru, Bolivia	**KC408018**	**KC408056**
O. albus	1351	*agassizii*	Puno Bay, Peru	**KC408019**	**KC408057**
O. 'gilsoni' morphotype A	1609	*gilsoni*	Huatajata, Lake Titicaca, Bolivia	**KC408026**	**KC408062**
O. 'gilsoni' morphotype A	1612	*gilsoni*	Huatajata, Lake Titicaca, Bolivia	**KC408027**	**KC408063**
O. 'gilsoni' morphotype B	GENT38	*gilsoni*	Huatajata, Lake Titicaca, Bolivia	**KC408024**	**KC408064**
O. 'gilsoni' morphotype B	GENT41	*gilsoni*	Huatajata, Lake Titicaca, Bolivia	**KC408025**	**KC408065**
O. cf crawfordi	1613	*gilsoni*	Huatajata, Lake Titicaca, Bolivia	**KC408028**	**KC408066**
O. ispi	T046	*cuvieri*	Huatajata, Lake Titicaca, Bolivia	**KC408029**	**KC408067**
O. ispi	T047	*cuvieri*	Huatajata, Lake Titicaca, Bolivia	**KC408030**	**KC408068**

.....continued on the next page

TABLE 2. (Continued)

Species / Taxon	Sample code	Complex*	Sample locality	Genbank acc. numb. CR	RH
O. ispi	T054	*cuvieri*	Huatajata lake, Bolivia	**KC408031**	**KC408069**
O. pentlandii	1323	*cuvieri*	Saracocha lake, Peru	**KC408032**	**KC408070**
O. pentlandii	1324	*cuvieri*	Saracocha lake, Peru	**KC408033**	**KC408071**
O. 'mulleri' morphotype A	T093	*mulleri*	Huatajata, Lake Titicaca, Bolivia	**KC408020**	**KC408058**
O. 'mulleri' morphotype A	T094	*mulleri*	Huatajata, Lake Titicaca, Bolivia	**KC408021**	**KC408059**
O. 'mulleri' morphotype B	1408	*mulleri*	Capachica, Lake Titicaca, Peru	**KC408022**	**KC408060**
O. 'mulleri' morphotype B	1412	*mulleri*	Capachica, Lake Titicaca, Peru	**KC408023**	**KC408061**
Syntype of *O. agassizii* [a]	MNHN 1905-0180	*agassizii*	Lake Poopo, Bolivia	**KC408034**	**KC408072**
Syntype of *O. agassizii* [a]	MNHN 1905-0181	*agassizii*	Lake Titicaca, Peru	**KC408035**	**KC408073**
Syntype of *O. agassizii* [a]	MNHN A-9602 (2012-0123)	*agassizii*	Chambira river, Peru	**KC408036**	-
Syntype of *O. tschudii* [b]	MNHN A-9604	*agassizii*	Lake Titicaca, Peru	**KC408037**	-
Syntype of *O. humboldti* (syn. *O. cuvieri*) [a]	MNHN A-9595 (2012-0128)	*cuvieri*	Lake Titicaca, Peru	**KC408038**	-
Syntype of *O. pentlandii* [a]	MNHN 0000-4415	*cuvieri*	Lake Titicaca, Peru	**KC408039**	-
Syntype of *O. pentlandii* [a]	MNHN A-9594	*cuvieri*	Lake Titicaca, Peru	**KC408040**	**KC408074**
Paratype of *O. ispi* [c]	1981-0606 (2012-0127#1)	*cuvieri*	Lake Titicaca, Tiquina, Peru	**KC408041**	-
Paratype of *O. ispi* [c]	1981-0606 (2012-0127#22)	*cuvieri*	Lake Titicaca, Tiquina, Peru	**KC408042**	-
Paratype of *O. forgeti* [c]	1981-0604 (2012-0126#38)	*cuvieri*	Lake Titicaca, Tiquina, Peru	**KC408043**	-

TABLE 3. Success of DNA extraction and PCR amplification in historical *Orestias* specimens (including non-type and types). Species described by Valenciennes [in Cuvier and Valenciennes] (1846)[a], Castelnau (1855)[b] and Lauzanne (1981)[c]. Numbers in parentheses correspond to the new MNHN catalogue numbers assigned to the specimens that were exposed to GuSCN bath. Tissue type: ES - entire specimen, PF - pectoral fin, PFM - pectoral fin including a small fragment of muscle, CP - caudal peduncle, M - muscle. See Material and methods for damage scale.

Specimen	MNHN CG number	Tissue Type	External body appearance before DNA extraction	Sample size	Incubation time	DNA (ng/µl)	External body appearance after DNA extraction (damage scale)	PCR amplification							
								Control region					Rhodopsin		
								1	2	3	4	consensus	1	2	consensus
O. mulleri	1981-1428 # 13	ES	rigid	67.2 mm	20 min	120-120	1	+							
	1981-1428 # 4	ES	rigid	62.1 mm	50 min	83.3-99.7	2	+							
	1981-1428 # 7	ES	rigid	63.5 mm	50 min	19.8-30.5	2	+							
	1981-1428 # 17	ES	rigid	57.1 mm	120 min	1.11-3.73	3	+							
	1981-1428 # 29	ES	rigid	63.9 mm	120 min	12.9-51.6	3	+							
	1981-1428 # 3	CP	rigid	< 15 mm	7 days	0.28	NA	+							
	1981-1428 # 3	PF	rigid	< 15 mm	7 days	0.11	NA	+							
	1981-1428 # 31	CP	rigid	< 15 mm	7 days	0.29	NA	+							
	1981-1428 # 31	PFM	rigid	< 15 mm	7 days	0.2	NA	+							
	1981-1428 # 10	ES	rigid	62.2 mm	10 min	22.4-32.3	1	+							
O. ispi	1622-10-11 # 3	ES	rigid	49.2 mm	10 min	0.19	3	-							
Syntype of *O. agassizii* [a]	1905-0180	ES	rigid	58.1 mm	25 min	63.6-66.6	2	+	+	+	+	validated	+	+	validated
Syntype of *O. agassizii* [a]	1905-0181	ES	rigid	57.1 mm	25 min	81.4-84.2	1	+	+	+	+	validated	+	+	validated
Syntype of *O. agassizii* [a]	A-9602 (2012-0123)	ES	soft and flaccid	60.2 mm	25 min	0.12-0.13	3	+	+	R	+	validated	-	-	NA
Syntype of *O. tschudii* [b]	A-9604	M	soft and flaccid	< 15 mm	7 days	0.24	NA	+	+	+	+	validated	-	-	NA
Syntype of *O. jussiei* [a]	A-9599 (2012-0125)	ES	soft and flaccid	83.1 mm	25 min	0.44 - 0.58	2	R	R	-	+	validated	-	-	NA
Holotype of *O. luteus* [a]	A-9608	M	soft and flaccid	< 15 mm	7 days	0.28-0.30	NA	-	-	-	-	NA	-	-	NA
Syntype of *O. albus* [a]	A-9607	M	soft and flaccid	< 15 mm	7 days	<0.1-0.15	NA	R	R	R	R	chimeric	-	-	NA
Syntype of *O. albus* [a]	A-9607	M	soft and flaccid	< 15 mm	7 days	0.35-0.35	NA	+	R	+	+	chimeric	-	-	NA
Paratype of *O. tchernavini* [h]	1981-0771 (2012-0129#22)	ES	rigid	56.1 mm	25 min	0.17	1	+	R	R	R	chimeric	-	-	NA
Syntype of *O. humboldti* (syn. *O. cuvieri*) [a]	A-9595 (2012-0128)	ES	soft and flaccid	59.3 mm	25 min	<0.1-0.20	3	R	R	R	R	validated	-	-	NA
Syntype of *O. pentlandii* [a]	0000-4415	M	soft and flaccid	< 15 mm	7 days	0.22-0.25	NA	+	+	+	+	validated	-	-	NA
Syntype of *O. pentlandii* [a]	A-9594	M	soft and flaccid	< 15 mm	7 days	0.24	NA	+	+	+	+	validated	+	+	validated
Paratype of *O. ispi* [c]	1981-0606 (2012-0127#1)	ES	rigid	76.9 mm	25 min	0.34-0.4	1	+	R	+	+	validated	-	-	NA
Paratype of *O. ispi* [c]	1981-0606 (2012-0127#2)	ES	rigid	69.1 mm	25 min	0.17-0.85	1	R	R	R	R	validated	-	-	NA
Paratype of *O. forgeti* [c]	1981-0604 (2012-0126#38)	ES	rigid	86.1 mm	25 min	0.78-0.99	1	+	+	R	+	validated	-	-	NA
Syntype of *O. mulleri* [a]	A-9605 (2012-0130)	ES	soft and flaccid	65.5 mm	25 min	0.10-0.13	3	R	-	+	R	chimeric	-	-	NA

[+] Sequence obtained from the 1st PCR amplification; [R] Sequence obtained from PCR re-amplification; No PCR amplification.

DNA extraction and PCR amplification from fresh samples. Genomic DNA was extracted using ABI Prism TM 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA) following manufacturer's recommendations. We amplified (i) 398 bp of the control region (CR; mitochondrial DNA) using the primer pair L-Smel—H-Smel (Falk *et al.* 2003), and (ii) 689 bp of Rhodopsin (RH; nuclear DNA) using a specific internal primer pair (OrRhF 5'-TTGTCAACCCAGCAGCCTAT-3'–OrRhR 5'-GCCGATGACCATGAGAATG-3') designed from a preliminary alignment of sequences obtained with the primer pair Rh193F –Rh1039R (Chen *et al.* 2003). Transmembrane domains in our amplified sequences, covering domains I–VI, were localized using as a reference the Rhodopsin transcript of *Oryzias latipes* deposited in the Ensembl database (http://www.ensembl.org/index.html; access code: ENSORLT00000013289). PCR amplification were performed in a 25 µl final volume with ~100 ng of template DNA, 0.1 mg/ml BSA, 1X PCR direct loading buffer with $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM each of forward and reverse primers, and 1 U *Taq* DNA Polymerase (Q-BIOgene, Illkirch, France). PCR cycling conditions for CR were as follows: initial denaturation at 94°C for 3 min, followed by 35 denaturation – annealing – extension cycles respectively at 94°C for 30 s, 50 °C for 30 s and 72°C for 30 s, and final extension step at 72°C for 15 min. PCR cycling conditions for RH included an initial denaturation at 94°C for 3 min, followed by 35 denaturation – annealing – extension cycles respectively at 94°C for 45 s, 61°C for 1 min and 72°C for 1 min 15 s, and a final extension step at 72°C for 15 min.

DNA extraction and PCR amplification from historical samples. DNA extraction procedures on historical specimens were conducted in a dedicated ancient DNA box equipped with autonomous ventilation system and UV-irradiation. We adapted the Guanidine thiocyanate (GuSCN)-based, non-invasive ancient DNA protocol of Rohland *et al*. (2004) to fluid-preserved historical specimens, including types. We used seven non-type specimens representing *O. mulleri* and a single individual of *O. ispi* Lauzanne collected by Lauzanne (1981) to optimize the protocol according to three criteria: quantity and quality of extracted DNA, level of damage inflicted to specimens after the extraction bath, and PCR amplification success of a short control fragment of the control region. We varied the initial quantity of tissue used for extraction, from entire specimen to small samples of pectoral fins and caudal peduncle. Samples were gently washed in 1X TE buffer (pH=8) at room temperature during 1 h and immediately after, incubated with soft, flat rotation at 40 °C in 2 ml–50 ml (depending on sample size) of a GuSCN buffer (4M GuSCN, 50 mM Tris pH 8.0, 25 mM NaCl, 1.3% Triton X-100, 2.5 mM PTB, 20 mM EDTA). Negative extraction controls were included for each extraction. Incubation times for the entire specimens varied between 10 and 120 minutes, in 15–50 ml sterile centrifuge tubes with conical bottom (Corning, NY). Small tissue samples were incubated during seven days (Rohland *et al.* 2004). DNA was purified using two successive washes of a chloroform-isoamyl alcohol solution (CIA 96:4), precipitated overnight at 4 °C in isopropanol (2/3 volume), and eluted in 50 µl $_{MQ}H_2O$. The final concentration of extracted DNA was estimated with a Qubit® Fluorometer using the Quant-iTTM dsDNA HS Assay Kit (Invitrogen, Villebon-sur-Yvette, France).

Our first test based on non-type, fluid-preserved historical specimens allowed us to determine the optimal incubation time for entire specimens to 20–25 min (Table 3). We thus proceeded to the DNA extraction of entire type specimens (for type series including more than one representative with total length < 9.5 cm) following the procedure described above. In the case of large type specimens and/or unique holotypes, a sample of muscle from the pleural cavity was dissected for extraction. Extraction baths for entire specimens were generally divided in two volumes after the incubation period, and processed separately. After DNA extraction, specimens were washed in a series of increasingly concentrated baths of ethanol (50%, 60% and 70%) to remove possible traces of GuSCN, before being transferred and stored in ethanol 95%.

PCR amplification of short (< 190 bp) and overlapping CR and RH fragments was done with specific primer pairs (Fig. 1; Table 4) designed from nucleotide sequence alignments obtained from fresh samples, using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/) and OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx). In total, we amplified 398 bp bp for CR and 168 for RH (covering trans-membrane domains I–II) (Fig.1). PCR amplification was performed in a final volume of 25 µl under cycling conditions described above, but containing 1–5 µl of template DNA and 2 U *Taq* DNA Polymerase (Q-BIOgene). Negative and positive controls were included for each series of PCR amplifications. PCR amplifications were done in a separate room to avoid contamination. When PCR products were weak or non-visible, we proceeded to re-amplifications, with the following modifications: [mix] 0.8 µl of PCR product as template, 0.1 µM each of forward and reverse primers, 1 U *Taq* DNA Polymerase (Q-BIOgene), [cycling conditions] 30 denaturation–annealing–extension cycles, and annealing temperatures lowered by 2°C. We assessed congruency among PCR products by

sequencing as many products as possible, obtained from different DNA aliquots, both first round PCRs and/or re-amplifications.

TABLE 4. List of the specific primer pairs designed to amplify the short fragments of the control region (CR) and the rhodopsin (RH) from fluid-preserved specimens of *Orestias*. See also Figure 1.

Locus	Fragment	bp	Primer sequence (5'–3')	Ta (°C)
CR	1	139	L-Smel (Falk *et al*. 2003)	51
			aCR-R1: GTMGGCTYACAYTWCTYTAAATG	
	2	194	aCR-F2: CTAGGRTTCTAAATTAAACYRTTCTTTG	54
			aCR-R2: TAGTAGGGRCATTATMYTKTGATGG	
	3	172	aCR-F3: CCATCAMARKATAATGYCCCTACTA	54
			aCR-R3: CAACCGATGCGATGTTCTTAC	
	4	171	aCR-F4: GCTAAAAACTCATARGTCGAGTTATAC	51
			H-Smel (Falk *et al*. 2003)	
RH	1	184	aRH-F1: TTGTCAACCCAGCAGCCTAT	56
			aRH-R1: GTGAATCCTCCGAACACCAT	
	2	164	aRH-F2: TCTCATTCTTGTCGGCTTCC	51
			aRH-R2: CGTTCATGGAGGTGTACATCG	

Ta: annealing temperature.

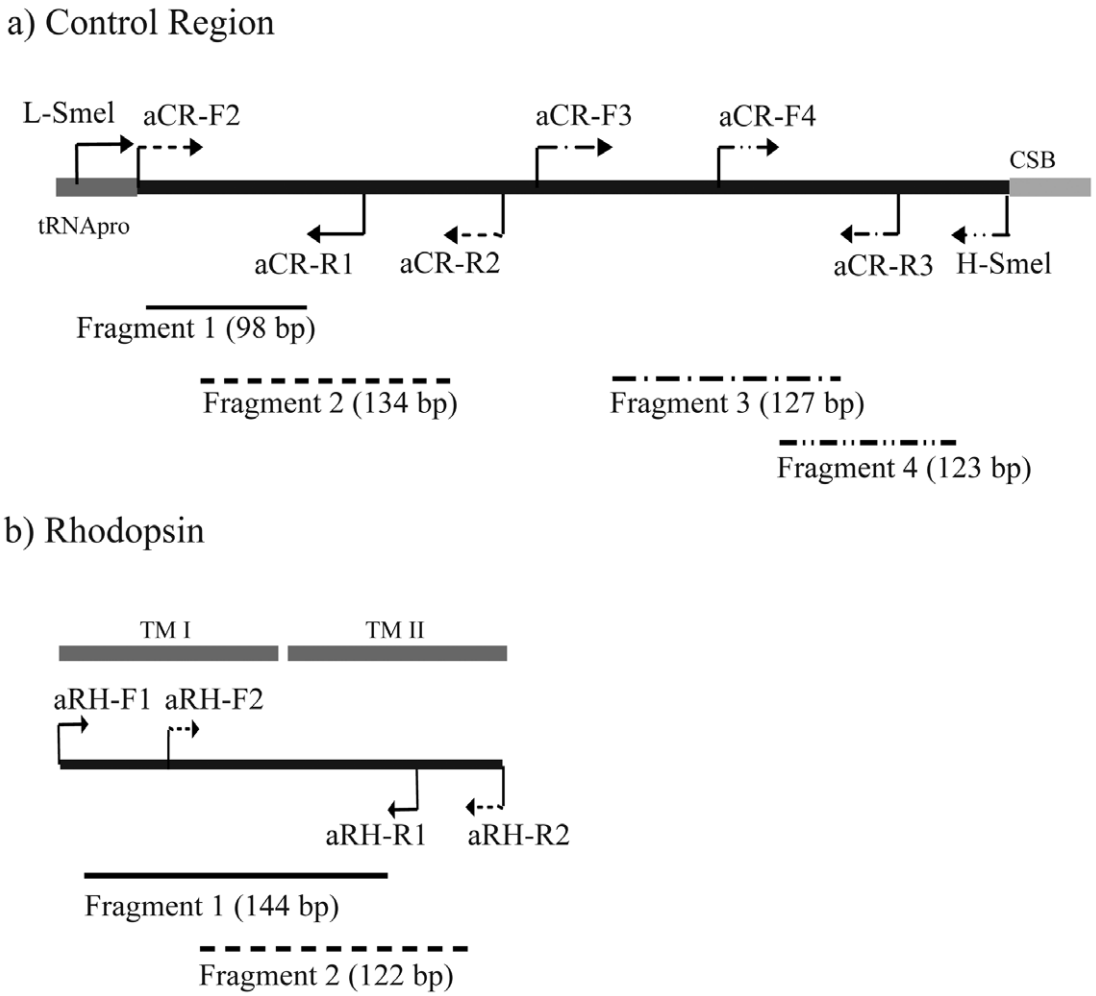

FIGURE 1. Schematic diagram of amplified PCR fragments of control region (a) and rhodopsin (b) from historical specimens, indicating primer location. See Table 4 for primer acronyms.

DNA sequencing and alignment. PCR products were sequenced in both directions on ABI 3730xl DNA Analyzer 96-capillary sequencers (Applied Biosystems) at GENOSCOPE (Consortium National de Recherche en Génomique, Evry, France) and at Eurofins (MWG Operon, Ebersberg, Germany). Nucleotide sequences were edited and aligned by eye using BioEdit 5.0.6 (Hall 1999). Sequences were deposited in Genbank under accession numbers: KC408006 to KC408073 (Table 2).

Validation of the nucleotide sequences obtained from type specimens. In order to identify putative assembly chimeras (composite nucleotide sequences with fragments originating from different organisms) in the nucleotide sequences obtained from type specimens, we ran a pairwise distance analysis on a fragment-by-fragment basis, assessing the match of ancient DNA sequences with the four clades that were recovered in the phylogenetic analysis (see below) using the nucleotide sequence alignment derived from our fresh samples. We used the mean number of pairwise character differences adjusted for missing data (allowing to consider gaps) in PAUP 4.0b10 (Swofford 2003). We returned the set of lowest distances (from 0.0 to 0.03) between each ancient DNA fragment and the series of nucleotide sequences representing the 'fresh' morpho-species. We considered that we obtained a chimeric sequence when at least one of the four fragments had a taxonomic match (criterion of lowest pairwise distance) attributed to a different clade from that of the other fragments.

Whenever chimeric sequences were identified, and if DNA templates were available to allow for additional PCR amplifications, we used the TOPO TA Cloning Kit for Sequencing (Invitrogen) to clone the problematic fragments. Although not detected as chimeric, we also cloned CR fragments obtained from the extinct species *O. cuvieri*. Four to 27 clones per fragment were selected and sequenced using universal M13 primers (Invitrogen).

Phylogenetic reconstruction. Markov Chain Monte Carlo (MCMC) Bayesian inferences were performed to reconstruct the phylogenetic relationships within *Orestias* for both CR and RH sequences, using BEAST 1.6.1 (Drummond & Rambaut 2007). Sequences from type specimens identified as chimeric (on the basis of pairwise distance analysis) or with a high proportion of missing data (e.g. *O. jussiei* Valenciennes; see below) were removed from the analysis.

The most likely evolutionary models were determined in jModelTest 0.1.1 (Posada 2008), using a dataset of the sequences from the 'fresh' specimen without their indels (CR). Model selection was based on the Bayesian Information Criterion (BIC), as performance analysis on simulated datasets has suggested that BIC showed a higher accuracy (Luo *et al.* 2010). The best fitting substitution models were HKY + I for CR and F81 for RH. Single-site gaps in CR alignment were recoded as a different character (base) before running the BEAST analysis. BEAST was run with the following settings: tree prior using the coalescent and assuming constant size (Kingman 1982), and 100,000,000 generations of MCMC steps. Trees and model parameters were sampled every 10,000 generations. Acceptable mixing and convergence to the stationary distribution of the MCMCs was assessed with Tracer 1.5 (Rambaut & Drummond 2007). Two independent runs were performed in order to ensure that posterior probabilities were stable. Log and tree files were concatenated in LogCombiner 1.6.1 (Drummond & Rambaut 2007) with 1,000 generations excluded as burn-in. Trees were summarized as maximum clade credibility trees using TreeAnnotator 1.6.1 (Drummond & Rambaut 2007), and were visualized and edited using Figtree 1.3.1 (Rambaut 2009).

Results

DNA extraction and PCR validation from fluid-preserved specimens

The amount of DNA extracted from entire specimens was significantly higher than for small tissue samples such as caudal peduncle muscle and pectoral fin (one-way ANOVA: F=4.43; P=0.045). However, the amount of extracted DNA was variable in the case of entire specimens, ranging from 0.11 to 120 ng/µl (mean=26.44; Table 3). No correlation was detected among sample size, incubation time and amount of extracted DNA, although this may be due to the overall small number of samples of processed specimens. In general, extracted DNA was highly degraded (< 500 bp; Fig. 2).

Observations of the external appearance of non-type historical specimens of *O. mulleri* showed that a sufficient DNA quantity could be obtained without inflicting significant damage to the external appearance of specimens (but see the case of *O. ispi* MNHN 1622-10-11#3; Table 3), with an incubation time between 20 and 25 min. Longer incubation times inflicted serious damages to the external appearance. More specifically, body surfaces were

highly dehydrated and distorted. The damage levels to the external appearance among type specimens were variable (Fig.3), and ranged from no evident damage (level 1, e.g. *O. forgeti* Lauzanne MNHN2012-0126#38 and *O. ispi* MNHN2012-0127#1 and #2) to very evident dehydration and distortion of the body, mostly for specimens with soft and flaccid bodies (level 3, e.g. *O. humboldti* Valenciennes MNHN 2012-0128). On the other hand, no evident change in colors, scale loss or degradation of fin rays was detected among all the treated historical series.

Irrespective of the extraction conditions (i.e., incubation time, tissue sample and DNA amount) and DNA fragment sizes, most specimens yielded a clear PCR amplification product of the expected size in our first test of amplifying the first fragment of CR (Table 3 and Fig. 2). PCR amplification success rate for the four fragments of CR in type specimens was higher (69 %) than for Rhodopsin (19 %). The CR nucleotide sequences were validated for eleven type specimens, whereas four were identified as chimeric. The consensus sequence obtained for the syntype of *O. jussiei* was considered validated as no conflict between amplified fragments was detected. However, given its level of missing data, this sequence was discarded from the phylogenetic reconstruction (but see Discussion). Within the *cuvieri* complex all the nucleotide sequences obtained from the type specimens were considered validated as no conflict between sequenced fragments was detected. Sequences of the type specimens for *O. tchernavini* Lauzanne (MNHN 1981-0771 // 2012-0129 #22), *O. mulleri* (MNHN A-9605 // 2012-0130), and *O. albus* Valenciennes (MNHN A-9607) were considered chimeric as we detected conflicts among taxonomic attributions of the different fragments constituting their nucleotide sequences (Tables 3 and 5). As a consequence, these specimens were discarded from the phylogenetic reconstruction. The RH sequences that we could obtain for three type specimens representing *agassizii* (*O. agassizii* MNHN 1905-0180 and MNHN 1905-0181) and *cuvieri* complexes (*O. pentlandii* Valenciennes MNHN A-9594) were validated, although taxonomic resolution was lower (Tables 3 and 5; see below). The PCR amplification of the DNA extracted from the holotype of *O. luteus* was not successful.

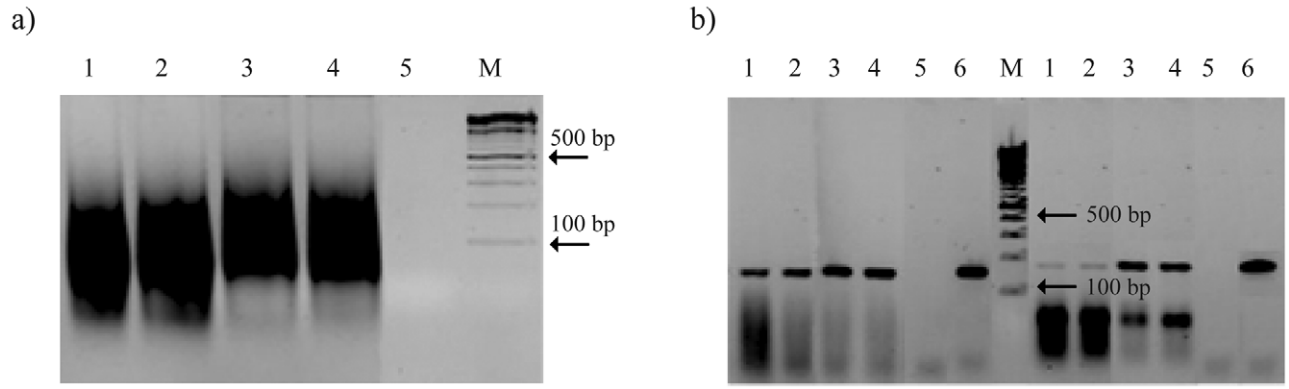

FIGURE 2. Gel electrophoresis displaying extracted DNA (a) and amplified PCR fragments (b) in two historical samples of *O. mulleri* (MNHN 1981-1428#4 and #13). In a, columns 1–2: extracted DNA for specimen #4 (aliquots a and b); columns 3–4: extracted DNA for specimen #13 (aliquot a and b); column 5: negative DNA extraction control; M: molecular weight marker (100 bp ladder). In b, PCR amplification products for control region and rhodopsin are left and right to the molecular weight marker, respectively. Columns 1–2: specimen #4 (aliquots a and b); columns 3–4: specimen #13 (aliquots a and b); column 5: negative PCR control; column 6: positive PCR control. M: molecular weight marker (100 bp ladder).

Phylogeny of the genus *Orestias*

The CR phylogenetic tree recovered four strongly supported clades, branching as follows: Clade A, (Clade B, (Clade C, Clade D))) (Fig.4). We could not recover the reciprocal monophyly of any of the 15 species or morphotypes included in our analysis (Fig. 4). Clade A included all the type specimens and recently collected representatives of *O. agassizii*, *O. cf agassizii* and *O. cf silustani* Allen. Clade B included representatives of *O. luteus*, *O. albus* and two morphotypes attributed to *mulleri* complex. Clade D included the two morphotypes attributed to the *gilsoni* complex and a representative of *O. cf crawfordi* Tchernavin. Clade C included the type specimens of *O. cuvieri*, *O. pentlandii*, *O. ispi* and *O. forgeti*, and all the recently collected representatives of *O. ispi* and *O. pentlandii*. The *mulleri* complex was polyphyletic, with its morphotype representatives spread between clades B and D.

The RH phylogenetic tree was less resolved than the CR tree. Two strongly supported sister-clades were

recovered, Clades 1 and 2 (Fig. 5), corresponding to the mitochondrial Clade A and Clades B-C-D (Fig. 4), respectively. The three type specimens representing *O. agassizii* and *O. forgeti* grouped within the former and latter clades, respectively. The two clades showed a slightly lower support when type specimens were included in the phylogenetic analysis (Fig. 5).

Discussion

Non-invasive ancient DNA protocol applied to fluid-preserved historical specimens of *Orestias*

To our knowledge, our study reports on the first test of non-invasive Guanidine-based protocol (Rohland *et al.* 2004) adapted to fluid-preserved, historical teleostean collection specimen. In our case, i.e. for individuals measuring between 49.2 and 86.1 mm, we suggest that the protocol is 'optimal' (i.e., balance between extracted DNA quantity and damage level of the specimens) when extraction baths of entire specimens are set to 20–25 min. All the historical specimens showed more rigid bodies and appeared 'cleaner' (Rohland *et al.* 2004) after the extraction procedure. Nevertheless, no changes in color, scale loss, and degradation of fin rays were detected, leaving external structures nearly intact for future morphological studies. Our observations are congruent with the generally non-deleterious impact of the Guanidine-based protocol on modern samples of terrestrial arthropods fixed in EtOH 80% (Rowley *et al.* 2007). On the other hand, some *Orestias* type specimens with soft and flaccid bodies seemed more impacted by dehydration and showed severely distorted body surfaces, compared to specimens with originally more rigid bodies (e.g. *O. cuvieri vs. O. forgeti* in Fig.3 and Table 3). According to our observations, variation in the damage level might be attributed to (i) the initial conditions of fixation and storage (which are usually unknown), and (ii) the size of the specimens, since specimens with smaller and thinner bodies were more severely damaged as GuSCN buffer could penetrate the tissues more quickly (e.g. type specimens of *O. cuvieri* 2012-0128 in Fig.3, and specimen of *O. ispi* in Table 3).

FIGURE 3. Example of type specimens treated by incubation in GuSCN bath. Left/right sides: specimens before/after DNA extraction procedure. Damage scale (see Material and Methods): A and B = 1, C = 2 and D = 3. Scale bar represents 2 cm.

Our adaptation of Rolhand *et al.*'s (2004) protocol may prove useful to improve the success of non-invasive DNA extraction from fluid-preserved teleostean specimens. De Bruyn *et al.* (2011) applied an ancient DNA protocol based on silica spin columns (Yang *et al.* 1998) to 80 years old teleostean tissues. This method yielded amplifiable mtDNA fragments from alcohol-fixed specimens, but failed for formalin fixed tissues. The other traditional extraction methods that were also tested (phenol:chloroform, high-salt procedure, QIAmp DNA microkit) proved to be even less efficient. Zhang (2010) reported on a DNA extraction protocol from formalin-preserved teleosteans using a CTAB method with a hot alkali pre-treatment, yielding PCR amplification of fragments of the cytochrome c oxidase subunit 1 (COI). Nevertheless, this method was tested in samples less than 23 years old, and proved to be optimal for samples fixed within the year. Raja *et al.* (2011) reported on the successful DNA extraction from formalin-fixed teleostean tissues using a standard extraction protocol (Nishiguchi *et al.* 2002), but did not discuss the possibility of DNA amplification from the resulting extracts. Our extraction protocol may be relevant for both alcohol and formalin fluid-preserved teleostean specimens. Even if we did not have access to detailed information about the specific fixation conditions of the specimens (a common situation in museum collections; Chakraborty *et al.* 2006; Koshiba *et al.* 1993; Schander & Halanych 2003), the latter showed differences in body stiffness (i.e. flaccid *versus* rigid bodies) suggesting that they were probably subjected to different fixation procedures and storage conditions during their 'life' as vouchers (e.g. ethanol-fixed and -preserved, formalin-fixed and ethanol-preserved, or formalin-fixed and-preserved; see Villwock 1986).

Although the protocol we have set up here can be considered non-invasive, we insist on the fact that the estimation of the optimal incubation time in GuSCN buffer may have to be adjusted depending on (i) the length and width (volume) of the specimen, and (ii) their initial conservation state, e.g. specimens with flaccid body *versus* specimens with more rigid bodies. The protocol was originally designed to extract DNA from 'hard' material of mammalian voucher specimens (bones, teeth and samples of skin; Rohland *et al.* 2004), so we cannot anticipate the potential long-term effects on the conservation of extracted specimens, and the feasibility and advisability of successive DNA extractions from the same specimen. In addition, as GuSCN is a hazardous substance, it is important to take precautions before returning the treated specimens into collections (Bolnick *et al.* 2012), such as washing with diluted ethanol baths, and storing in separate jars to avoid potential diffusion of fluids from the treated specimens.

A total of 11 out of 15 type material nucleotide sequences were validated by our chimera-detection approach (Table 5). Although we consider that our protocol is validated following this procedure, we could not totally remove the incidence of contamination, one of the most difficult aspects when working with ancient or historical samples (Hebsgaard *et al.* 2005; Kelman & Kelman 1999; Yang & Watt 2005). The presence of four chimeric sequences in our alignment could be explained by cross-specimen contamination via organic micro-fragments and mucus through the steps of collective storage during field work and once in the collections (Wandeler *et al.* 2007), and/or the proneness of samples with low DNA quantity and quality, such as historical samples, to exogenous DNA contamination (Pääbo *et al.* 2004) and artifact formation (e.g. chimeras) during PCR amplification. In the latter case, chimeric DNA sequences are produced during "jumping PCR" events, from two or more template molecules owing to incomplete strand synthesis and blocking lesions (Fulton *et al.* 2012; Hebsgaard *et al.* 2005; Kelman & Kelman 1999; Pääbo *et al.* 1989).

In our case, the adaptation of Rohland *et al.*'s (2004) protocol led to the successful amplification of nDNA (RH) fragments. In comparison, Rohland *et al.* (2004) were unable to amplify nDNA from samples of skin, bone and tooth of historical specimens of mammals. The success rate of PCR amplification was clearly higher for mtDNA (CR) than for nDNA (11 *versus* three validated sequences, out of 15 DNA extracts; Table 5). This might be explained by the greater copy number of mtDNA *versus* nDNA (~500-fold; De Bruyn *et al.* 2011). In addition, and independent of DNA quantity, the presence/absence of PCR inhibitors and the level of DNA fragmentation (Fulton *et al.* 2012; Turci *et al.* 2010; Zhang 2010), parameters that were not assessed as part of this study, may have shaped the variation in PCR success rates that we observed among DNA extracts.

Phylogeny of the genus *Orestias*

Our approach combining type material and recently collected specimens allowed us to ascertain the taxonomic delineation of two of the four clades that we recovered from the well-resolved mtDNA tree (Fig. 4). Because Clade A included all the type specimens and recently collected representatives of *O. agassizii*, *O. cf agassizii* and *O. cf silustani*, we assign it to the *agassizii* complex as newly defined, which corresponds to the *agassizii* complex *sensu*

TABLE 5. Minimum pairwise distances calculated between type specimen nucleotide sequences and clade representatives from each fragment of control region (CR) and rhodopsin (RH). *Species complexes as defined by Parenti (1984a). Species described by Valenciennes [in Cuvier and Valenciennes] (1846)[a], Castelnau (1855)[b] and Lauzanne (1981)[c]. Numbers in parentheses correspond to the new MNHN catalogue numbers assigned to the specimens that were exposed to GuSCN bath.

Type specimen	MNHN CG number	Complex *	CR-clade assignment				RH-clade assignment	
			Fragment 1	Fragment 2	Fragment 3	Fragment 4	Fragment 1	Fragment 2
Syntype of *O. agassizii* [a]	1905-0180	*agassizii*	0.000 clade A	0.007 clade A	0.007 clade A	0.000 clade A	0.000 clade A	0.000 clade A
Syntype of *O. agassizii* [a]	1905-0181	*agassizii*	0.000 clade A	0.000 clade A	0.007 clade A	0.000 clade A	0.000 clade A	0.000 clade A
Syntype of *O. agassizii* [a]	A-9602 (2012-0123)	*agassizii*	0.020 clade A	0.007 clade A	0.007 clade A	0.00 clade A		
Syntype of *O. tschudii* [b]	A-9604	*agassizii*	0.000 clade A	0.000 clade A	0.007 clade A	0.000 clade A		
Syntype of *O. jussiei* [a]	A-9599 (2012-0125)	*agassizii*	0.030 clades B, C and D	0.030 clades B, C and D	NA	0.000 clades B, C and D		
Syntype of *O. albus* [a]	A-9607	*agassizii*	0.020 clade D	0.020 clade D and C	0.010 clade A	0.000 clade B		
Syntype of *O. albus* [a]	A-9607	*agassizii*	0.030 clades B and D	0.02 clade D	0.010 clade A	0.008 clade A		
Paratype of *O. tchernavini* [c]	1981-0771 (2012-0129#22)	*gilsoni*	0.010 clade B	0.010 clade C	0.000 clades B, C and D	0.000 clade B		
Syntype of *O. humboldti* (syn. *O. cuvieri*) [a]	A-9595 (2012-0128)	*cuvieri*	0.010 clade C	0.007 clade C	0.000 clade C	0.000 clade C		
Syntype of *O. pentlandii* [a]	0000-4415	*cuvieri*	0.000 clade C	0.000 clade C	0.000 clade C	0.000 clade C		
Syntype of *O. pentlandii* [a]	A-9594	*cuvieri*	0.010 clade C	0.007 clade C	0.000 clade C	0.000 clade C	0.000 clade BCD	0.000 clade BCD
Paratype of *O. ispi* [c]	1981-0606 (2012-0127#1)	*cuvieri*	0.010 clade C	0.007 clade C	0.007 clade C	0.000 clade C		
Paratype of *O. ispi* [c]	1981-0606 (2012-0127#22)	*cuvieri*	0.010 clade C	0.010 clade C	0.000 clade C	0.000 clade C		
Paratype of *O. forgeti* [c]	1981-0604 (2012-0126#38)	*cuvieri*	0.010 clade C	0.010 clade C	0.007 clade C	0.000 clade C		
Syntype of *O. mulleri* [a]	A-9605 (2012-0130)	*mulleri*	0.000 clade B	NA	0.000 clade A	0.000 clade A		

Parenti 1984 minus the *luteus* group. Clade C, including the type specimens of *O. cuvieri*, *O. pentlandii*, *O. ispi* and *O. forgeti*, and all the recently collected representatives of *O. ispi* and *O. pentlandii*, is equivalent to the *cuvieri* complex. Although clades B and D did not include any type material, their taxonomic delineation was also

possible. Clade B included all the representatives of *O. luteus* and *O. albus*, two species that are morphologically clearly distinct from the rest of *Orestias* (Lauzanne 1982), and belong with three other species not analyzed here to the *luteus* group defined by Parenti (1984a) as a monophyletic subdivision within the *agassizii* complex. We therefore delineate Clade B as representing the newly, provisionally defined *luteus* complex. We consider that the presence into this clade of two morphotypes attributed to the *mulleri* complex is evidence for the polyphyly of the *mulleri* complex as defined by Parenti (1984a), since *O. cf crawfordi*, also a representative of the *mulleri* complex, is included within another clade (clade D). Clade D includes the two morphotypes attributed to the *gilsoni* complex, and we consider provisionally that it corresponds to this complex.

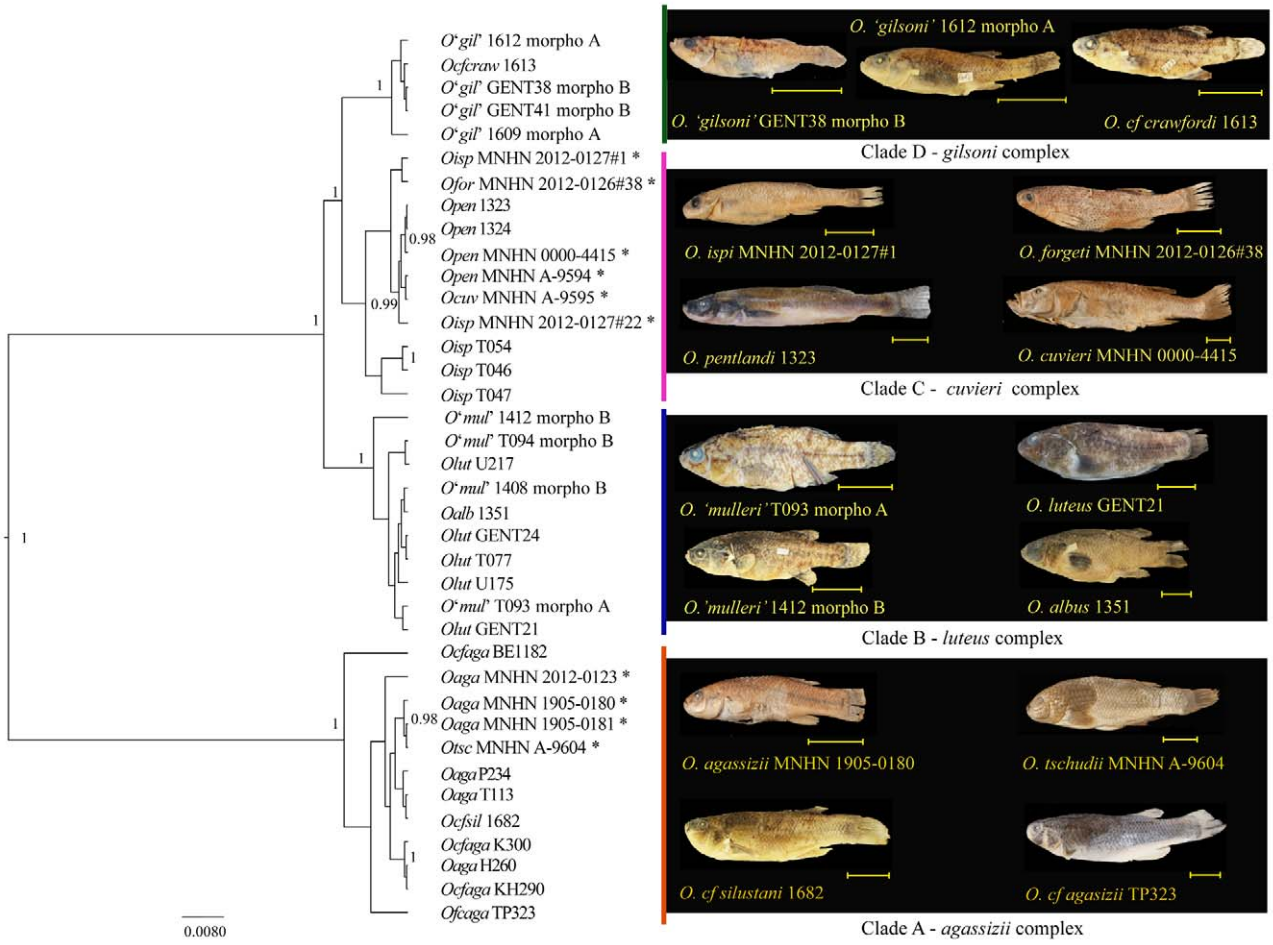

FIGURE 4. Molecular phylogeny of *Orestias* complexes including 'fresh' species and morphotype ("morpho") representatives, and type specimens. Maximum clade credibility tree for control region sequences. Values at branch nodes refer to highest posterior probability of occurrence for clades (> 0.95). Scale bar below tree indicates sequence divergence. Scale bar on pictures represents 2 cm. Type specimens are highlighted with an asterisk. See Table 2 for specimen acronyms. See Discussion for attribution of complex names.

Molecular phylogenetic studies on the genus *Orestias* that would contrast the traditional, morphological-based systematics of the genus are currently lacking or very partial (Lüssen *et al.* 2003; Parker & Kornfield 1995). Our study constitutes the first molecular phylogenetic reconstruction including representatives of all the complexes defined by Parenti (1984a). Our phylogenetic analyses based on both mt- and nDNA revealed a main, deep dichotomy within the genus *Orestias*, separating the *agassizii* complex from a cluster of three clades (Figs. 4 and 5), the latter grouping under shallow dichotomies in the mtDNA tree as complexes (*luteus*, (*cuvieri*, *gilsoni*)). The 'deep and shallow' diversification pattern observed in our mtDNA tree could fit with a scenario of ancient divergence, between the *agassizii* complex and the rest of *Orestias* followed by a recent diversification during the Pleistocene, in- and outside the Lake Titicaca (Lüssen *et al.* 2003; Parenti 1981; Parenti 1984a), possibly as an event of adaptive radiation within each complex (Lauzanne 1982; Lüssen *et al.* 2003; Maldonado *et al.* 2009; Villwock 1986).

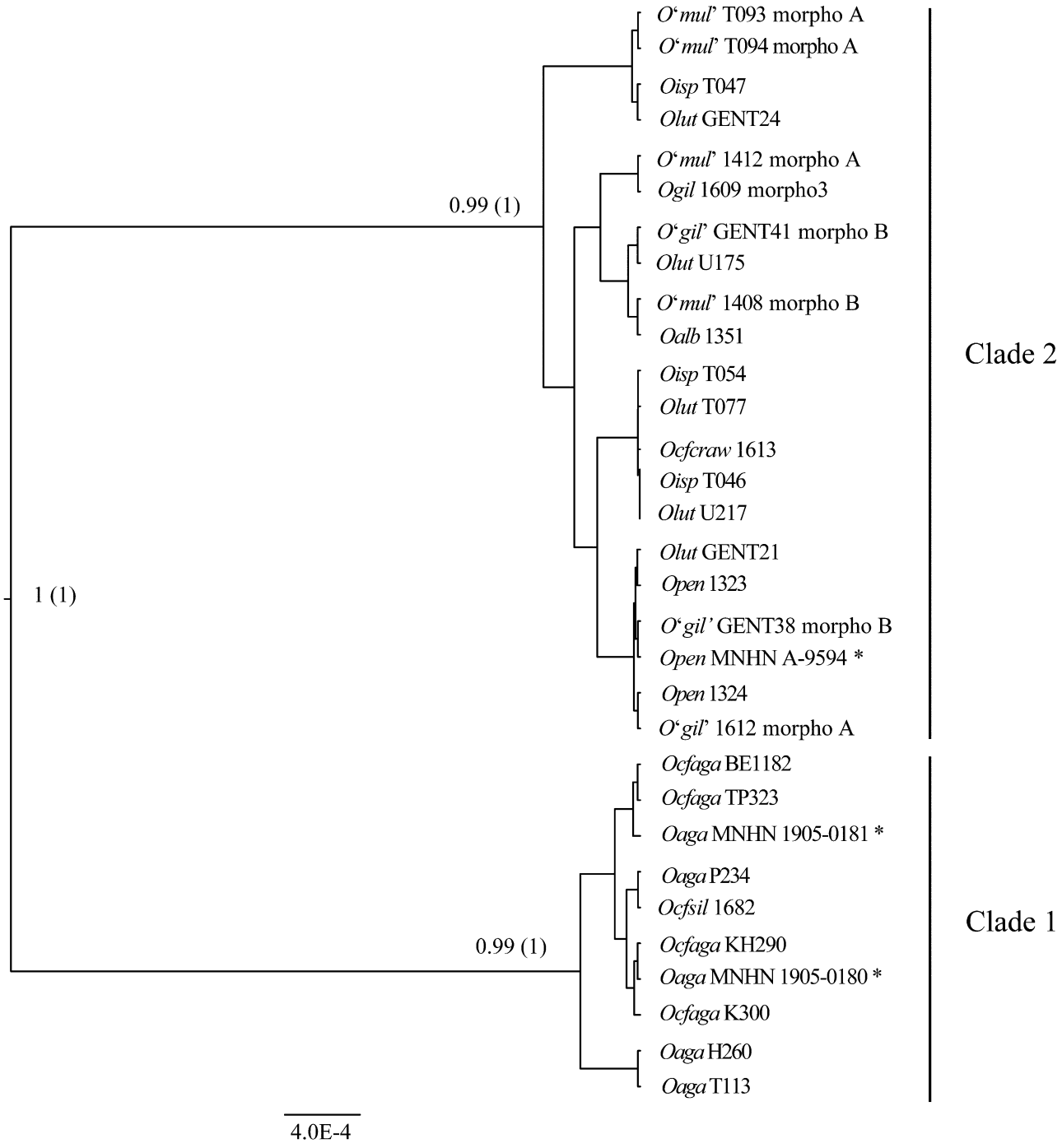

FIGURE 5. Molecular phylogeny of *Orestias* complexes including 'fresh' species and morphotype ("morpho") representatives, and type specimens. Maximum clade credibility tree for rhodopsin sequences. Values at branch nodes refer to highest posterior probability of occurrence for clades (> 0.95); within parenthesis are shown posterior values when removing type specimens from the analysis. Scale bar below tree indicates sequence divergence. Type specimens are highlighted with an asterisk. See Table 2 for specimen acronyms.

We propose, for the first time, a well-supported phylogenetic hypothesis and testing of the monophyly of complexes within the genus *Orestias*. Our results starkly question the use of a series of diagnostic characters established in the literature for complexes and groups of *Orestias* species. As a result, the synapomorphies proposed by Parenti (1984a) for the *agassizii* complex (i.e. including *luteus*-like species), consisting in a series of squamation characters (pattern and scale types) and the presence of a relatively deep caudal peduncle in adults, should be revised given the DNA-based exclusion from the complex of wide head and body morphotypes such as *O. luteus* and *O. albus* (also see Lüssen *et al.* 2003).

The *luteus* complex as defined by our analyses is temporarily left without any diagnostic characters, since it includes a combination of morphotypes (i) with wide head and body, and large and thick granulated scales that partially cover the body (*O. albus* and *O. luteus*), but also (ii) with smaller head and fully scaled body ("*mulleri*" morphotypes A and B). Interestingly, morphotype A shows granulated scales on the head, although these scales are thin and smaller than those present in *O. luteus* and *O. albus*. Parenti (1984a) delimited the *luteus* group from the rest of the species within the *agassizii* complex on the basis of their thick and granular scales present from the snout to the base of the dorsal fin (preopercular, opercular and dorsal region of the pectoral fins). However, granulated scales may also be present on the dorsal surface of the head of adults of *O. cuvieri* and *O. pentlandii* (Parenti 1984a). In addition, in *Orestias* as in other cyprinodontids (e.g. *Aphanius* Nardo), squamation pattern changes with age and growth (Parenti 1984a; Villwock & Sienknecht 1996). Since squamation pattern has been one of the primary characters used to distinguish among *Orestias* species (Parenti 1984a; Tchernavin 1944), its phylogenetic significance should be reconsidered (Villwock and Sienknecth 1996, Müller 1993, this study).

The *cuvieri* complex was fully recovered as a clade by our phylogenetic reconstruction. As a consequence, the five synapomorphies proposed by Parenti (1984a) still stand: i) elongate branchial apparatus with a narrow basihyal, ii) in adults, fifth ceratobranchials narrow and very close together or fused along midline, iii) interarcual cartilage long, thick rod, equal to or longer than first epibranchial bone, iv) interarcual cartilage equal or longer than first epibranchial bone, and v) increase in modal number of anal fin rays (16–17, range: 14 to 19). However, a detailed analysis of these osteological characters on large series of specimens is required to validate their diagnostic values.

The *gilsoni* complex as provisionally defined by our analyses included "*gilsoni*" morphotype B, which morphologically corresponds to a subset of species (*imarpe* Parenti, *robustus* Parenti, *uruni* Tchernavin, *tchernavini* Lauzanne) described by Parenti (1984a) as 'robust' and 'marbled', and morphotype A, which resembles *O. gilsoni*. Parenti (1984a) distinguished *O. gilsoni* from other species within the *gilsoni* complex as having a wide and dorsally flattened head and a narrow dorsal peduncle. The clade attributed to the *gilsoni* complex also included *O. cf crawfordi*, a deep-bodied, laterally compressed, fully scaled species belonging previously to the *mulleri* complex and closely related to *O. incae* Garman (see Parenti 1984a). The *gilsoni* complex was defined by Parenti (1984a) on a single synaphomorphic character state, namely "the procurrent caudal fin rays lying mostly interior to the body profile and closer to the vertebral column, giving the caudal fin a tapered external appearance". From our observations, this character proves very subjective to define and did not represent a reliable diagnostic character. As in the case of the newly defined *luteus* complex, *gilsoni* complex is temporally left without any diagnostic characters.

Eventually, the polyphyly of the *mulleri* complex (Parenti 1984a) as suggested by our analyses seriously questioned the validity of the single synapomorphy on which the complex was based (base of dorsal and anal fins projecting beyond the primary body profile and usually covered with small and thin scales; Parenti 1984a).

Importantly, we could not recover the reciprocal monophyly of any of the morphospecies (15) that were considered in our analyses, despite the fact that we used a fragment of the control region, one of the fastest-evolving mtDNA markers in vertebrates (Avise 2000). The evolutionary picture within the *Orestias* clades (or complexes) may correspond to that observed in recent adaptive radiations (0.001 to 2 Myr) within African Lake cichlids, where the occurrence of incomplete lineage sorting may prevent the recovery of monophyletic species lineages, despite clear-cut morphological, ecological and behavioral delineations among species (e.g. Bezault *et al.* 2011; Meyer *et al.* 1990; Moran & Kornfield 1993; Parker & Kornfield 1997). Moreover, cases of hybridization have been reported, notably between clearly differentiated taxa of *Orestias*. Within the *cuvieri* complex, natural hybrids between the large *O. cuvieri* and *O. pentlandii* were identified by Tchernavin (1944). Between *O. agassizii* and *O. luteus*, two morphologically and ecologically well-differentiated taxa (Maldonado *et al.* 2009), fertile hybrids have been reported based on crossbreeding experiments (Azpiazu 2002) and genetic data (Esquer Garrigos et al. submitted). Tchernavin (1944) and Villwock & Sienknecht (1995) also recorded hybrids *O. olivaceus* Garman x *O. agassizii* and *O. agassizii tschudii* Castelnau x *O. luteus*, respectively. Incidence of hybridization might blur species boundaries, further complicating the establishment of reliable and diagnostic, molecular and morphological characters. Furthermore, hybridization may in turn promote the morphological diversification and partly maintain the disruption of monophyly in *Orestias* morpho-species, notably if complexes are undergoing recent adaptive radiations where interspecific reproductive barriers are not completely efficient (e.g. Herder *et al.* 2006; Shaffer & Thomson 2007). In such a context, the systematics of the genus *Orestias* should be re-evaluated in depth through

more extensive and integrative approaches, including the analysis of co-dominant markers to better estimate gene flow and hybridization within and among complexes, the setting up of breeding experiments in controlled environments, and the search for morphological and osteological diagnostic features to reach a finer characterization of intra and inter-specific variation.

The cases of *O. jussiei* and *O. cuvieri*

Although not included in the phylogenetic analysis, the case of the syntype of *O. jussiei* (MNHN A-9599 // 2012-0125) can be discussed from a taxonomic point of view. Since all the CR fragments amplified from the specimen were assigned to different complexes than *agassizii* (to clades B, C and D at the same time, so without conflict among taxonomic/clade attribution), our results argue for the exclusion of *O. jussiei* from the *agassizii* complex as defined by our phylogenetic analysis. Whereas Parenti (1984a) included *O. jussiei* within the *agassizii* complex (but not within the *luteus* group), Lauzanne (1982) grouped the species with *O. luteus* and *O. albus*. The taxonomic identification of the type specimen of *jussiei* itself seems problematic, since its pronounced dorsal curvature has been suggested to be an artifact following poor fixation procedure (Lauzanne 1982) or a cranial deformation by *Metacercariae* brain infection (Heckmann 1992; Mueller 1972).

One of the most valuable aspects of our work consists in the sequencing of the CR haplotype of the extinct *O. cuvieri*, the most emblematic species within the genus *Orestias*. The nucleotide sequence of *O. cuvieri* was identical to that of the closely related species *O. pentlandii* (MNHN A-9594), and just slightly different (two base pairs) from haplotypes found in type specimens of *O. ispi*, highlighting the very close genetic proximity between morphologically and ecologically well differentiated species within the *cuvieri* complex. One of the probable causes of the extinction of *O. cuvieri* is competitive exclusion or predation through the introduction of non-native species such as trouts, salmonids and silversides between 1940 and 1950 (Lauzanne 1992; Parenti 1984a; Vila *et al.* 2007). However, given the genetic similarity among representatives of the *cuvieri* complex (especially between *O. cuvieri* and *O. pentlandii*), we may consider that the mitochondrial genome of the species is still represented in the wild, whether 'extinction' of the morphotype '*O. cuvieri*' occurred via competitive exclusion (genuine extinction) or hybridization and introgression with congeners ('extinction' by morphotype erosion).

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