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

YARELI ESQUER GARRIGOS[1,6], BERNARD HUGUENY[1], KELLIE KOERNER[1,2], CARLA IBAÑEZ[1,3], CELINE BONILLO[4], PATRICE PRUVOST[1], ROMAIN CAUSSE[1], CORINNE CRUAUD[5] & PHILIPPE GAUBERT[1]

[1]*UMR BOREA, Département Milieux et Peuplements Aquatiques MNHN - CNRS 7208 - IRD 207 - UPMC, Muséum National d'Histoire Naturelle, 43 rue Cuvier, 75231 Paris, France*
[2]*Current address: Charter School of the Dunes, 860 N Lake Street, Gary, IN 46403, USA*
[3]*Instituto de Ecología – Unidad de Limnología, Universidad Mayor de San Andrés, Campus Universitario Cota cota C.27 s/n, La Paz, Bolivia*
[4]*UMS Outils et méthodes de la systématique intégrative CNRS 2700, Département Systématique & Evolution, Muséum national d'Histoire naturelle, 57 rue Cuvier, 75005 Paris, France*
[5]*Genoscope. Centre National de Sequençage, 2 rue Gaston Crémieux, CP5706, 91057 Evry Cedex, France*
[6]*Corresponding author. E-mail: esquer@mnhn.fr*

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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