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DOTTORATO DI RICERCA IN SCIENZE DELLA TERRA E DELL'AMBIENTE

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Phylogeography and diversification of the extremophile fish
genera *Danakilia* (Actinopterygii: Cichlidae) and *Aphanius*
(Actinopterygii: Cyprinodontidae) of the Danakil Depression

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*“The desert is beautiful,” the little prince added.
And that was true. I have always loved the desert.
One sits down on a desert sand dune, sees nothing, hears nothing.
Yet through the silence something throbs, and gleams...
“What makes the desert beautiful,” said the little prince,
“is that somewhere it hides a well...”*

Antoine de Sainte-Exupéry (1900-1944)



Lake Abaeded, Danakil Depression of Eritrea

To Anna Rita, because also common men sometimes have a great woman behind,
to my loving parents, who have always allowed me to do what was right and good to me,
I dedicate this endeavour.

ACKNOWLEDGMENTS

I wish to thank all the people who gave me support and friendship during this research. My brotherly friend Giuseppe De Marchi, with whom we dreamed of this study long before it started and with whom I share a thirty-two years old passion for the African wilderness. My supervisor Mauro Fasola, who since I was a younger student than now always encouraged and supported me with unconditioned confidence. Melanie Stiassny, who let me learn from her expertise, funded one of my expeditions, then offered me one of the greatest opportunities I had in my life and an unexpected tartlet on a lonely birthday in New York. My favoured geneticists Adriana Bellati and Liz Alter, who patiently taught a dunce how to deal with things a lot smaller than the smaller fish, while nicely tolerating my clumsiness. Carmelo Fruciano, who patiently explained me how to manage geometric morphometrics with much more confidence than before. Anton Lamboj, who taught me how to fish with a 6 meter-long seine net in a puddle, how to keep fish alive in a plastic bag for days and how not to lose patience while counting numberless scales, and spines, and rays under a stereoscope. My Eritrean and Ethiopian old and new fellows Futsum Hagos, Yohannes Mebrathu, Misikire Tessema and Berhan Asmamaw, peacefully reunited in the passion for nature. My colleagues at the Museo di Storia Naturale di Milano, which endured the weight of my absence for three years. The Municipality of Milano that endorsed my training goal and continued to pay me the salary during my study leave. Many more people than those listed here contributed to this work in many ways: my heartfelt thanks to all and everyone!

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CHAPTER 1

INTRODUCTION



Danakilia franchettii in a desert spring in the Lake Afrera basin.

WHY DESERT FISHES?

Deserts as extreme environments for fish

What makes a desert is not a particular temperature but rather a lack of precipitation. Deserts are defined by their arid conditions, which leads to the fact that most of the area occupied by desert is barren and monotonous. For this reason, many people view desert as a wasteland and a place devoted of life. In contrast, biologists have long seen deserts as laboratories of nature, where natural selection manifests at its most extreme. Deserts are characterized by their aridity, yet each desert is different in its abiotic qualities. Deserts are so widely spaced on the planet and have arisen for so many different reasons that the variation among deserts is probably greater than for any other biome. For example, North American continental deserts are far hotter in the summer and wetter than African and Middle Eastern deserts (Ward, 2016).

Until fairly recently, a desert was considered a place that received less than 250 mm of rainfall. However, if this amount of rain is distributed evenly over the entire year, it can be enough to maintain a grassland. Conversely, when rainfall is concentrated in 1 or 2 months over the year, plants can use only a certain amount of rain at a time. Torrential rain, though abundant, usually runs off the ground before it can be used by desert organisms because most of it is not absorbed by the soil, leaving the ground nearly as dry as it was without rain (Ward, 2016).

The intermittent regime of most water basins and watercourses present in deserts and the chemical and physical characteristics of their waters, seem apparently inhospitable to fish life. Facts however contradict this commonplace. Besides, an ever-changing climate and geology represent opportunities for the dispersal of fishes that can also promote speciation. Periods of wet climate created connections between waterbodies that allowed dispersal. Conversely, periods of dry climate, by breaking interconnection between waterbodies, triggered isolation and created vicariance or barriers to dispersal. Likewise, tectonic movements may enhance or reduce the availability of barriers. Under the pressure of climate change and vicariance a fish is compelled to survive in very different habitats. For example, in the shift resulting from desertification, a fish of relatively cold freshwaters can face the challenge of living in warm brackish or even hypersaline waters. Conversely, other species persist in rivers and streams that flow into or across deserts, where adaptation to variable stream flow is a prerequisite to survival. Moreover, the ever-changing environment poses always new evolutionary challenges to the physiology of the fish at some or all life stages. Thus, it is not strange to find that most desert species were likely preadapted to cope with harsh environments, i.e. they had already evolved adaptations to tolerate extremes of temperature and salinity and/or different water regimes (Hillyard *et al.*, 2015).

What is a desert fish?

A recent literature review showed that about 460 species of ray-finned fishes (Actinopterygii) belonging to 52 families live in these environments, with Cyprinidae (43%) and Cyprinodontidae (10%) accounting for the bigger number of species (Hillyard *et al.*, 2015, for a complete list of families). The analysis of Hillyard *et al.* (2015) also suggests that each desert retains a distinctive species assemblage reflecting the faunal composition of the surrounding regions, in this supporting the idea that fish life in deserts made its appearance not as the expression of an isolated evolutionary event, but through isolation and adaptation of local species whose presence predated the development of present-day conditions (Hillyard *et al.*, 2015). Fossil remains in fact reveal the widespread distribution of fish in what are now deserts; at the same time, drainage patterns are modified by tectonic activity and continue to affect current fish distributions. Distribution can vary between sympatric species (and upper taxa) suggesting a differential utilization of stochastic events: for example, a species may have taken advantage of a dispersal event, while another did not. This implies that interpreting the presence of extant desert fishes requires an understanding of their evolutionary history and ecology in the light of the species pattern of dispersal and vicariance and the correlations with geomorphological and climatic changes interesting their range. For example, looking back at the Sahara's hydrological history reveals that this area, now considered a hyper-arid environment, was characterized by interconnected streams and lakes as recently as 10,000 years ago; at the same time, the availability of aquifers that still feed oases where fish persist is another important feature to keep in mind and today the largest known aquifer underlies the Sahara Desert.

Ecological and physiological challenges to fishes in deserts

A review of the major factors influencing the life of fishes in desert is discussed in Deacon and Minckley (1974). These include:

- current and turbulence: flash-flooding seldom annihilates fishes from a habitat despite its impressive violence, but it may affect species with its tremendous molar action that may damage fish and fill pools with debris;
- suspended solids and sedimentation: high concentrations of suspended solids in rivers or lakes situated in areas of finely divided soils drained from denuded basins, windswept lakes or highly degrading streams may physically suffocate fishes by accumulation in the branchial chamber and on the gill filaments; the increased turbidity can shade aquatic habitats inhibiting the development of algae;
- drought and desiccation: fish populations displaced by floods from rivers to closed-basin

lakes, dry-pans or dry channels may be left on dry flats or injected into salinities of lethal concentrations;

- direct effects of light: extreme light exposure may affect both adults and larvae causing sunburns, inhibition of hatching of eggs and of growth of larvae;
- temperature: constant high temperature in springs and of widely fluctuating temperatures in other desert waters may introduce stresses (e.g. thermal, diminished oxygen concentration) to which only a few fish species have successfully adapted; seasonal changes, preceded by acclimation, appear less stressing than diurnal;
- hydrogen-ion Concentration (pH): is of little consequence to fishes except at its upper and lower ranges (>9.5 or <4.0) and most desert waters are in the alkaline range (pH 7.2-9.0);
- salinity: desert habitats are characterized by high content of dissolved solids, a natural consequence of surface water standing in a desiccating environment; for this reason, some of the most striking adaptations to hypersaline conditions are among desert fishes;
- dissolved oxygen (O_2): dissolved oxygen supply is probably the most influencing single factor influencing the life of desert fishes and low levels cause losses of weight, impaired swimming abilities, malformed or weakened larvae;
- dissolved carbon dioxide (CO_2): carbon dioxide in desert waters is rarely sufficiently high to create problems for fishes (carbonates tends to buffer any direct effects of the gas), but the ability of a fish to utilize oxygen seems to decrease as concentration of CO_2 increases;
- dissolved hydrogen sulfide (H_2S): this gas is normally present within the bottoms of desert aquatic habitats, but in daytime it may be counteracted by the oxidizing effects of intense sunlight coupled with high primary productivity; its toxicity seems based on sulfide ion (S^{2-}), which creates a tissue anoxia resulting from inactivation of carriers for O_2 .

Most desert species show physiological, morphological and behavioural adaptations evolved to cope with desert extreme conditions, but strangely enough, some desert species do not show any, a part from their broad ecological tolerance (Hillyard *et al.*, 2015).

These adaptations' most outstanding expressions are present in species living in temporary rivers and pools, common ephemeral aquatic habitats in the deserts of Africa, the Arabian Peninsula and South America. Ecological conditions can vary rapidly and dramatically in these ecosystems, and represent important stressors influencing the life of desert fishes. Species living in pools along dry riverbeds periodically becoming torrential will experience wide seasonal and daily fluctuations affecting water temperatures, ranging from 6 °C (South America) to 39 °C (Africa) extremes, variations of dissolved oxygen content in relation to the vegetation photosynthetic activity (with supersaturation during the

day and hypoxia or even anoxia during the night), the stochasticity of inundations despite the quite predictable seasonality of rainfall either in terms of water amount and duration of temporary pools, the isolation of pools precluding secluded fishes from moving to safe locations when their habitat desiccates or becomes inhospitable (Polačik and Podrabsky, 2015).

A major influence on desert river-dwelling fishes is the periodic occurrence of flash floods, when water regime can change from lentic conditions (low flow or stagnant) to lotic (torrential) in seconds (Deacon and Minckley, 1974; Naiman and Soltz, 1981). Flash floods, however, rarely remove fishes from a habitat in spite of their impressive strength but, in some instances, can decimate entire populations; in particular, fish populations living in broad and shallow braided rivers not confined in defined banks, normally do not experience the severe effects of flash flooding on their habitats (Deacon and Minckley, 1974). Temporary aquatic environments are common across almost all terrestrial ecosystems with seasonally changing patterns of precipitation and/or evaporation. Hypersaline waters (i.e. with salinity exceeding that of seawater: >35 g/l) are considered among the harshest aquatic environments where relatively few fish species can live. Those that do, usually estuarine, euryhaline species, can osmoregulate in salinities well over 100 g/l (Gonzales, 2012).

Simple vs complex environments

Isolated springs, lakes or waterholes along temporary rivers in desert areas are critical permanent or semi-permanent sources of water supporting both terrestrial and aquatic biodiversity that can be assimilated to islands of water in a sea of dry land, and the high diversity of isolated aquatic habitats has very likely played a large role in the evolution of desert fishes (Ward and Blaustein, 1994; Faulks *et al.*, 2010; Hillyard *et al.*, 2015). Nonetheless, these environments show various degrees of environmental stability, from desert springs being the most stable at seasonal and daily timescales, to ephemeral waterholes along dry riverbeds being subject to fluctuations of their physical (temperature, pH, turbidity) and chemical (conductivity, dissolved oxygen) parameters with dramatic daily and seasonal shifts of temperature, variation of dissolved oxygen following the circadian rhythms of photosynthetic activity and seasonal flash floods (Hillyard *et al.*, 2015; Polačik *et al.*, 2015). For these reasons, desert fishes in general can represent profitable “natural experiments” for the study of phenotypic variation in geographical space.

In particular, cichlids' extraordinary ability to colonize freshwater habitats reaches an extent unmatched by any other vertebrate family (Liem, 1973). This is superbly exemplified by the booming adaptive radiations in the African Great Lakes (Tanganyika, Malawi and Victoria), each one containing hundreds of mostly endemic species. The African Great Lakes cichlid radiation are therefore universally recognized as a textbook example for the study of evolutionary patterns of

diversification. The surprising speed at which cichlids can explosively radiate is also renowned. In particular, some cichlid adaptive radiations developed in very short amounts of time, such as that of Lake Victoria (12,400-18,000 a depending on the study: Johnson *et al.*, 1996; Seehausen, 2002; Salzburger *et al.*, 2014) or the small adaptive radiation of the desert extremophile *Alcolapia* in Lakes Natron and Magadi (~10,000 a ago: Ford *et al.*, 2015 and references therein).

Far from the extreme ecological complexity of the African Great Lakes, smaller isolated aquatic systems are a more simplified and useful model, similar to some extent to the one offered by island ecosystems, thus allowing clearer insight into the main forces driving biological diversification of which ecomorphological differentiation is the main manifestation (Wellborn and Langerhans, 2015). In fact, the ecological opportunity provided by isolated or depauperate ecosystems and their unexploited ecological niches, where colonizers are released from interspecific competition, is one of the foremost forces driving phenotypic diversification and adaptive radiation (reviewed in Yoder *et al.*, 2010). Well known examples come from the Nicaraguan (Lake Nicaragua, Lake Managua and satellite crater lakes: Barluenga *et al.*, 2006; Elmer *et al.*, 2010; Franchini *et al.*, 2014) and Cameroonian lakes (Lake Barombi Mbo, Lake Bermin and Lake Ejagham: Schliewen *et al.*, 1994; Schliewen *et al.*, 2001; Schliewen & Klee, 2004; Martin *et al.*, 2014), the desert marshes of northern Mexico (Cuatro Ciénegas: Magalhaes *et al.*, 2015), and the smaller lakes of the East African Rift, such as the crater lakes of Uganda (Machado-Schiaffino *et al.*, 2015) and the East African soda lakes Natron (Tanzania) and Magadi (Kenya) (Ford *et al.*, 2015; Kavembe *et al.*, 2016).

The fish assemblages of the Danakil Depression of northeastern Africa

The Danakil Depression is one of the least explored and poorly studied desert regions of the World. Reasons for the little knowledge of the area are multiple: among these, punitive climatic conditions for most of the year, the past fierce opposition of the locals towards foreigners sometimes ending in the annihilation of entire European expeditions, the current presence of armed separatist groups, a 30-year guerrilla war for independence between Eritrea and Ethiopia (1961-1991) and their so far unsolved border disputes after the 1998-2000 conventional war.

Lack of information is especially evident on the subject of its biological diversity, which includes some valuable peculiarities, such as the healthiest population of the critically endangered African Wild Ass (*Equus africanus somalicus*) (Moehlman *et al.*, 2015). On the ichthyological side, our present knowledge of the fish fauna of the Danakil Depression is restricted to four species only: two belong to the genus *Danakilia* Thys van den Audenaerde, 1969 (Cichlidae, Pseudocrenilabrinae), endemic to the Danakil Depression, and two to the widely distributed Mediterranean and Near Eastern genus *Aphanius* Nardo, 1827 (Cyprinodontidae, Cyprinodontinae).

The discovery of the two cichlid species, *Danakilia franchettii* (Vinciguerra, 1931) and *D. dinicolai* Stiassny, De Marchi and Lamboj, 2010 (Fig. 1), happened well apart, both in time and in space. *D. franchettii* was first observed in 1928 in the hot mineral springs feeding Lake Afrera (=Lake Afdera or Giulietti or Egogi Bad) during the exploration of the Afar region from south to north carried out by the Nesbitt-Pastore-Rosina expedition of 1928 (Nesbitt, 1929, 1930a, 1930b, 1935), but the fish was collected only later, during the Italian scientific expedition in the Danakil of 1928-1929 led by baron Raimondo Franchetti (Franchetti, 1930, Vinciguerra, 1931).

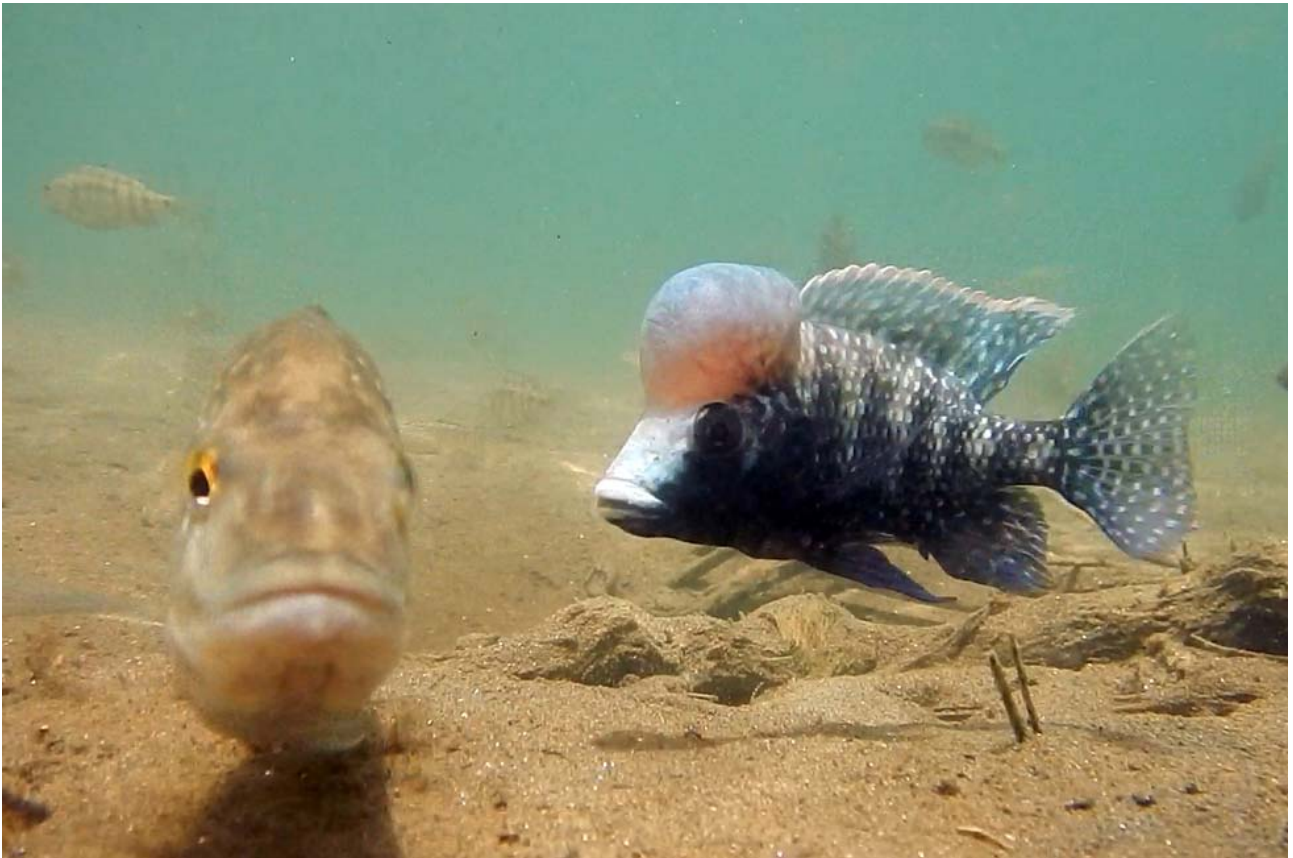


Fig. 1. *Danakilia dinicolai*

D. dinicolai, was collected only about seventy years later, between 1998 and 2001 during three joint Italian-Eritrean expeditions for the ecological investigation of Lake Abaeded, a small crater lake (10 ha) situated along the north-western margin of the Eritrean Danakil Depression (Stiassny *et al.*, 2010). A third species, *Aphanius dispar* (Rüppell, 1829), the Arabian Pupfish (Fig. 2), was collected in 1929 by the Franchetti exploration party in Lake Afdera and in the Gaharre water wells (Eritrea), about 20 km inland, west of the coastal oasis of Bēylul (Vinciguerra, 1931), and was also recorded from Lake Abaeded in 1998-2001 (Getahun and Lazara, 2001; Stiassny *et al.*, 2010) and in Lake Afrera and tributary creeks (Getahun and Lazara, 2001).



Fig. 2. Aquarium bred *Aphanius* cf. *dispar* from the Danakil Depression (photo: Jörg Freyhof).

To end with, Getahun and Lazara (2001) described a new species of cyprinodontid, the Lake Afrera Killifish *A. stiassnyae* (Getahun & Lazara, 2001), the fourth fish species of the Danakil Depression, from the hyperhaline lake Afrera. Indeed, this finding corroborates the assertion by Roberts (1975) who wrote “the Danakil desert has some species of the alkaliphile cyprinodont genus *Aphaniops*” hypothesizing the presence (at that time not supported with evidence) of more than one species of the genus *Aphanius* (= *Aphaniops* Hoedeman, 1951) in the area.

THE STUDY ORGANISMS

The biodiversity of fishes

The word “fish” is applied to a heterogeneous grouping of aquatic chordates (Phylum Chordata) comprised of hagfishes (Class Myxini) and lampreys (Class Petromyzontida), sharks, rays and chimaeras (Class Chondrichthyes), the lobe-finned bony fishes (Class Sarcopterygii), and the ray-finned bony fishes (Class Actinopterygii), the last two grouped in the Grade Teleostomi or Osteichthyes (Helfman *et al.*, 2009). That of bony fishes is by far the most diverse group among fishes and is well represented in freshwaters, while the others are predominantly marine groups.

The accurate number of extant fish species remains to be determined, but Nelson *et al.*, (2016) consider that more than a half of the known living vertebrates (60,000 species) are fishes (32,000 species). The Catalog of Fishes (Eschmeyer and Fong, 2017) lists 34,515 valid species with a total 7,732 fish species that were scientifically described in the 20-year period 1998-2017. This means an average 386.6 species/y (range: 295-523) and a rate of discovery unmatched in the Phylum Chordata, and in the vertebrates (Subphylum Vertebrata) in particular (Fig. 3). However, taxonomy experts think that the final number could be considerably higher. It is therefore evident that fishes represent the most successfully radiated group of vertebrates.

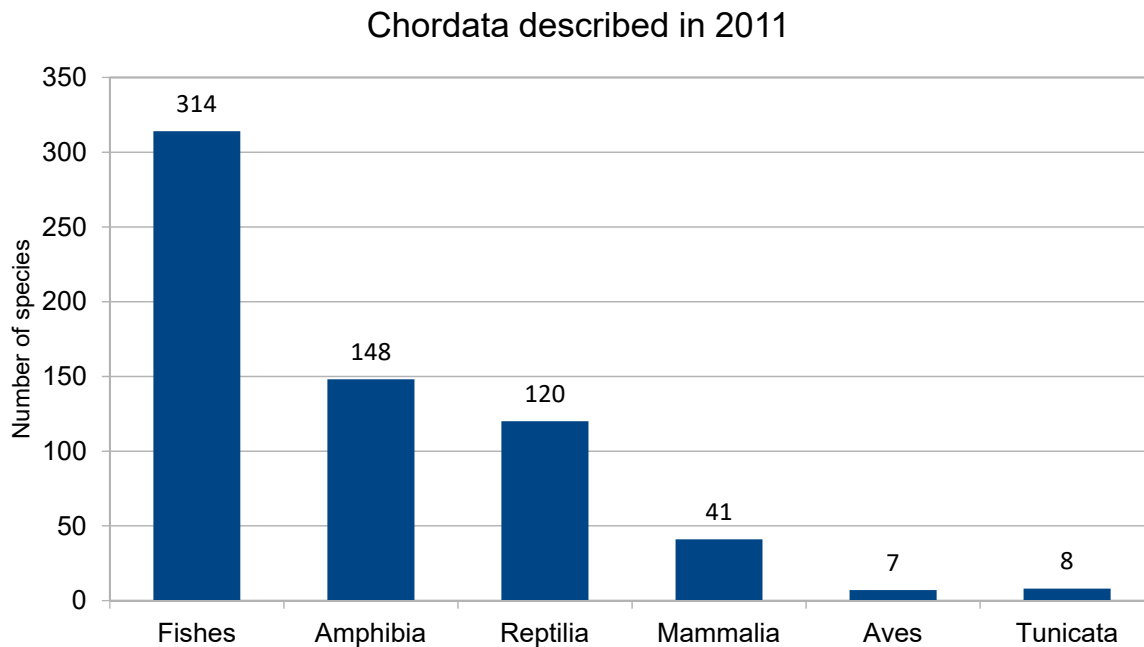


Fig. 3. Number of species described in 2011 in six classes of the phylum Chordata, based on last available annual report of the International Institute for Species Exploration (IISE, 2012).

Léveque *et al.* (2008), basing on the above mentioned numbers, reckon up to 15,000 species of

freshwater and brackish water fishes. The strictly freshwater species (13,000), they add, live in rivers and lakes that together account for only 1% of the Earth's surface, while the remnant species (16,000) live in salt and brackish waters covering a full 70% of the Planet's surface. Freshwater and, to a lesser extent, brackish water environments, with 15,000 species in 207 families and 2,513 genera, thus represent the most productive forge of fish biodiversity. This is particularly true for the major rivers and lakes of the inter-tropical regions of Africa, Asia and America.

Abell *et al.* (2008) analysis, that was aimed at the identification and definition of the freshwater ecoregions of the World for their conservation, refer as outstanding for their fish biodiversity and the number of endemism, a large part of the basin of the river Congo, the southern Gulf of Guinea drainage basins and Lakes Tanganyika, Malawi and Victoria (Africa), the Zhu Jiang (Pearl River) basin and neighboring systems (Asia), and large portions of the Amazon and Orinoco basins (South America). Besides, other areas stand out for globally high richness: Brahmaputra, Ganges, Yangtze, Mekong, Chao Phraya, Sitang and Irrawaddy basins (Asia), lower Guinea (Africa) and Paraná and Orinoco (South America). When richness is adjusted for ecoregion area, more freshwater systems emerge as especially noteworthy (e.g. Tennessee River; Niger River; New Caledonia, Vanuatu, and Fiji; Sumatra and Borneo).

The fish biodiversity of Africa and Madagascar is discussed in detail in Thieme *et al.* (2005).

The cichlids (family Cichlidae)

Cichlids are a group of fishes familiar even to lay people for their conspicuous presence in the aquarium hobby and in the food market. Two of the most known aquarium species are the Angelfish *Pterophyllum scalare* (Schultze, 1823) and the Discus *Symphysodon aequifasciatus* Pellegrin, 1904, both now successfully bred in tanks all over the World, but whose origin is the Amazon basin of South America. The success of cichlids among people is certainly due to the generally moderate size, the beautifully colored liveries of the breeding males of many species, to their fascinating breeding behaviors and courtship displays and, in many species, the dig of elaborated nests (bowers) and the parental care of eggs and fry.

On the other hand, it is in the food market that cichlids show their greatest importance in human life. In fact, the Nile Tilapia *Oreochromis niloticus* (Linnaeus, 1758) is one of the most important fisheries product in the world. Tilapias (including all farmed species) are the second most important group of aquaculture fish after carps (*Cyprinus carpio* Linnaeus, 1758), with an enormous and continuously increasing world annual production that reached about 3.7×10^6 t in 2014 (FAO: retrieved 28 Sep 2017

<http://www.fao.org/fishery/species/3217/en>).

Cichlids (Cichlidae) are a family of non-ostariophysan bony-fishes (Actinopterygii) sharing a large amount of homoplasy with the Labroidei, a group that include surfperches (Embiotocidae), the damselfishes (Pomacentridae), and the wrasses (Labridae), as discussed by Stiassny and Jensen (1987). Sparks and Smith (2004) discussed the molecular phylogeny of cichlids by considering a wider array of percomorph fishes and found that the Labroidei is not monophyletic and that the sister group to Cichlidae should include a large and diverse assemblage of percomorph lineages.

Following the systematic arrangement and data provided by Eschmeyer *et al.* (2017), the family Cichlidae is the third fish family for number of species (after Cyprinidae and Gobiidae) and the second biggest family of the Perciformes (after the Gobiidae). Perciformes is the fish largest order and, indeed, the largest order of all vertebrates. With 187 new species described in the decade 2008-2017 (Table 1), cichlids stand out as the second family of the order after the gobies (Gobiidae), for which 329 species were described in the same decade.

<i>Order</i>	<i>Family</i>	<i>Subfamily</i>	<i>Available</i>	<i>Valid</i>	<i>Described 2008-2017</i>	<i>Distribution</i>
Perciformes			21421	11570	1193	World
	Cichlidae		2246	1707	187	S Eurasia, Africa, America
		Etroplinae	20	16	3	Indian Peninsula, Sri Lanka, Madagascar
		Ptychochrominae	20	16	2	Madagascar
		Pseudocrenilabrinae	1407	1104	75	S Iran, Africa
		Cichlinae	796	571	107	America

Table 1. The taxonomic arrangement of cichlids (Cichlidae) from order to subfamilies, and species numbers at each taxonomic level after Eschmeyer and Fong (2017), modified. Species included in the count are those for which names are available for use. Subspecies are treated as available names at the species level and as synonyms at the species level. All new species described in the last 10 years are considered valid, unless status references show they are synonyms. Subfamily names after Van der Laan *et al.*, (2014). Distribution of subfamilies from FishBase (retrieved 28 Sep 2017, www.fishbase.org).

Cichlids are distributed in fresh- and brackish waters in Central and South America, S North America, West Indies, Africa, Madagascar, the Middle East (Syria, Israel, Iran), Sri Lanka, and coastal southern India (Fig. 4). Only one cichlid species occurs in true marine waters *Coptodon guineensis* (Günther, 1862). Some species were widely introduced outside their native range.

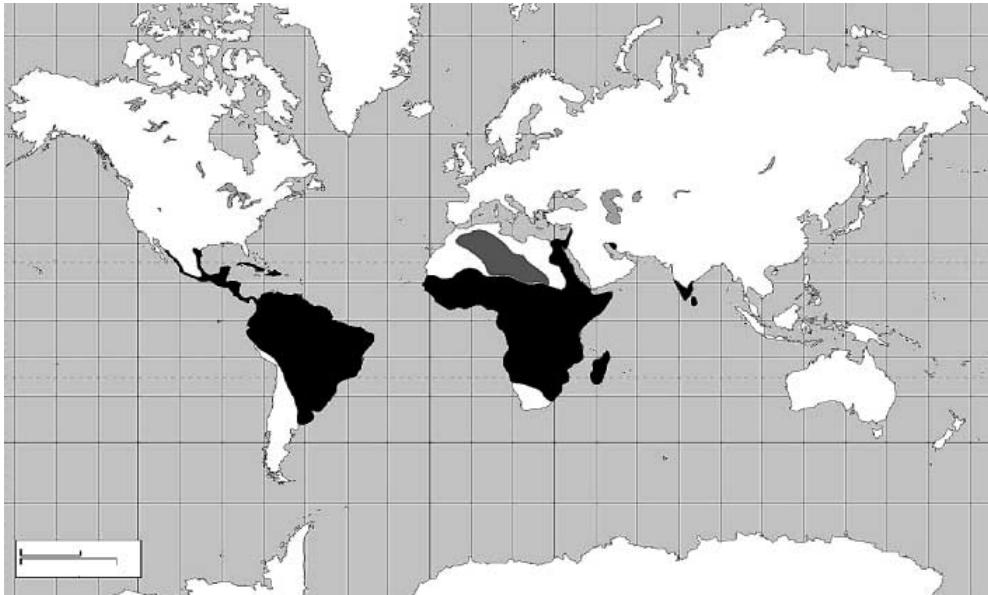


Fig. 4. Cichlid worldwide distribution from Chakrabarty (2004).

Origin and evolution

Cichlid fishes, along with Darwin's finches and a few other vertebrate systems, are considered since the description of the extraordinary adaptive radiation of the Great East African Lakes where they form species flocks, a key model for understanding the evolutionary patterns of biodiversity (e.g., to cite only some recent large-scale work: Kocher, 2004; Salzburger *et al.*, 2005; Santos and Salzburger, 2012; Wagner *et al.*, 2012; Meier *et al.*, 2017).

In general, bony fish are not considered the best candidates for good fossil preservation: 181 out of the 425 families lack a fossil record and, of the remaining 244 with fossil records, 58 had only otoliths (Azuma *et al.*, 2008). The oldest known genus of fossil cichlid is *Mahengechromis* from paleo-lake Mahenge, Eocene of Tanzania (45.83 ± 0.17 Ma), that provides a possible evidence of the ability of Cichlidae to form species flocks early in their evolution (Murray, 2000).

However, no agreement exists regarding the age of cichlids. Some estimates (e.g. Stiassny, 1991; Azuma *et al.*, 2008) support origins in the Early Cretaceous (based on the family's Gondwanan pattern of distribution between Africa and America and timing of Gondwanan break-up), while others (e.g., Near *et al.* 2012) consider much younger ages (Late Cretaceous). Disagreement is likely the consequence of poor fossil record and difference in the employment of calibrations and age estimation with molecular phylogenies (López-Fernández *et al.*, 2013).

In a recent paper, Friedman *et al.*, (2013) hypothesize that the separation between the African and American lineages of cichlids happened during the Eocene with the origin of the African lineage in the same period, thus well after the previous estimates. The traditional hypothesis of a Gondwanan

origin of Cichlidae explaining their biogeographic pattern by vicariance, is mostly linked to the present-day absence of obligate marine species and to the idea that cichlids cannot cross large tracts of open seawater due to their limited tolerance for high levels of salinity on the long run. This physiological constraint would have ruled out the possibility to reach America from Africa, about 1000 km distant in the Eocene. However, geological evidence during the Paleogene of a now submerged chain of islands between Africa and America, and the possible co-occurrence of continental freshwater plumes that provided surface brackish-water corridors, might be able to explain how cichlids succeeded in colonizing America.

Morphology

Cichlids can be distinguished for their external morphology from other percomorphs for the following characters:

- one nostril opening on each side of the head;
- the lateral line organ is interrupted and divided in two sections (upper and lower), the first running along the upper half of the body, and reaching about the end of the flank's second third, and the second running along the mid-line, from about the last third to the tail (with the exception for the genera *Teleogramma* and *Gobiocichla*);
- the sum of the scales along the two sections of the lateral lines usually 20-50;
- anal fin with 3-15 hard spinous rays (generally 3) and 4-30 flexible and branched soft rays;
- dorsal fin usually with 7-25 spiny rays and 5-30 soft rays;
- suborbital shelf absent.

However, the real morphological novelty that characterizes the family Cichlidae is in their skeletal anatomy and consists in the development of a synarthrosis between the lower pharyngeal jaws, a type of joint which permits very little or no movement under normal conditions between the two bones. This key innovation enabled cichlids not only to efficiently transport food items (deglutination) but freed the “anterior set of jaws” (the premaxillary and mandibular jaws) from the dual task of collection and preparation. Once this adaptive threshold was crossed, the premaxillary and mandibular jaws, leaving preparation to the lower pharyngeal jaw (Fig. 5), could evolve numerous specializations connected to the collection of dramatically diverse foods, thus opening the way for successful and rapid adaptation in new evolutionary scenarios (Liem 1973).

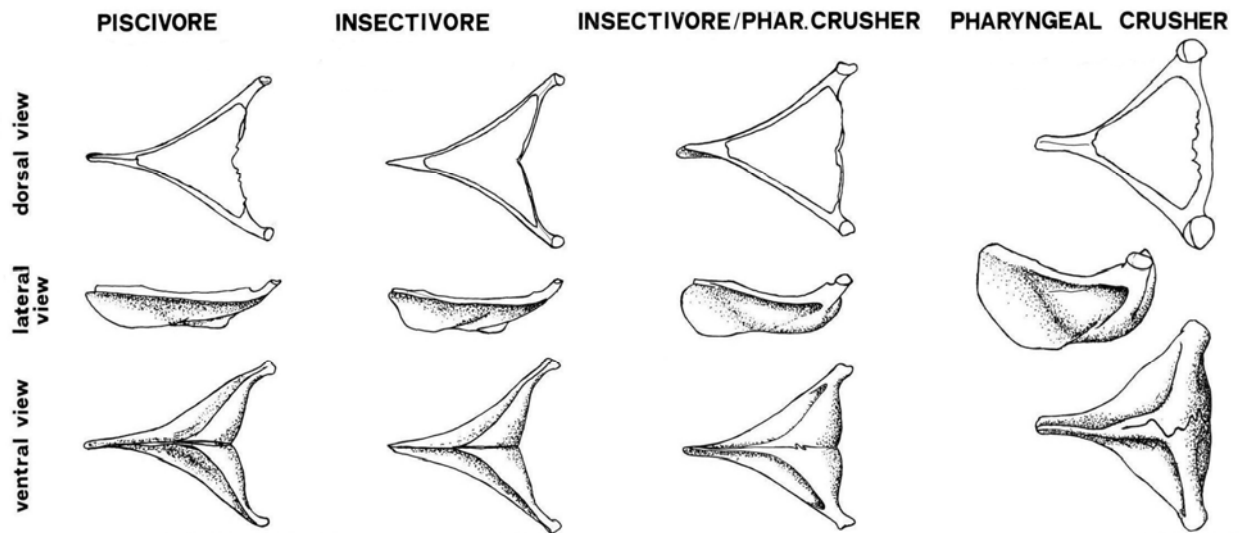


Fig. 5. The lower pharyngeal jaw apparatus in various cichlid species of Lake Victoria. Jaws are presented following a food specialization criterion (columns), and each one is showed in dorsal, lateral and ventral views (rows). From: Barel 1976, modified.

Breeding

The various mating systems found in the family Cichlidae and their variations are illustrated in Table 2. In general, monogamy is considered the ancestral type of mating system, while the most evolutionary recent forms include the polygamous mating systems (polygyny, polygynandry and polyandry). Parental care is present in three forms: mouthbrooding, substrate brooding, and substrate brooding of eggs then mouthbrooding of young.

Mating systems (general)	Subsets
<i>Monogamy</i> Male and female form enduring pairs	<i>Courtship</i> Male and female pair and court reciprocally for prolonged periods. Pairs separate after eggs are laid.
	<i>Parent</i> Male and female form pair that persists until offspring reach independence.
<i>Polygyny</i> One male fertilizes the eggs of more than one female	<i>Male territory</i> Male holds territory where he is visited by females to spawn. Female spawns with only one male at each reproductive cycle.
	<i>Bigamy</i> Two females hold breeding territories within that of the male, who may help care for offspring
	<i>Harem</i> As bigamy, but may have multiple females. Little or no male parental care
<i>Polygynandry</i> Each male fertilizes the eggs of more than one female. Each female has eggs fertilized by more than one male	<i>Male territory</i> Males hold well-spaced territories continuously. Males receive more than one female, and females may spawn with more than one male. Some species polygynous
	<i>Lekking</i> Females visit multiple males clustered on transient territories. Some species polygynous
<i>Polyandry</i> One female reproduces with multiple males. Each male spawns with only that female. Family cycles overlap	
<i>Extended family</i> Two or more group members of both sexes reproduce. Some offspring remain in the family	

Table 2. Mating systems observed in the family Cichlidae and their variations (from: Barlow 2000, modified).

The genus *Danakilia*

Taxonomy

The systematics of the isolated genus *Danakilia* in the context of the larger group of the tilapiines intrigued the ichthyologists since the description of *D. franchettii* (Vinciguerra, 1931). The Italian fish taxonomer Decio Vinciguerra (1856-1934), in his description of *D. franchettii*, recognized the peculiarity of this fish species, that he baptized *Tilapia franchettii*, in the general framework of the genus *Tilapia* Smith, 1840. In the opinion of contemporary taxonomists, *Tilapia* included a greater number of species than today, but while recognizing some similitude of *T. franchettii* with *T. sparrmanii* Smith, 1840 and *T. percivali* Boulenger, 1912 [= *Oreochromis spilurus* (Günther, 1894)], still Vinciguerra doubted of the validity of many species in the genus *Tilapia* that, in his opinion, were often merely based on very subtle differences and very low numbers of specimens (Vinciguerra, 1931).

In his annotated bibliography of the genus *Tilapia*, a milestone on the subject of tilapiine taxonomy, the Belgian ichthyologist Thys van den Audenaerde (1969), reevaluated the taxonomic importance of *T. franchettii* and first proposed *Danakilia* as a subgenus of *Tilapia*, thus recognizing the uniqueness of this species.

A few years after Thys publication, Roberts (1975), citing a personal communication by the influential British fish taxonomist Ethelwynn Trewavas (1900-1993) suggested a possible relationship of *Danakilia* with *Sarotherodon galilaeus* (Linnaeus, 1758), a piece of information that is also reported by Trewavas (1983).

The same Trewavas (1983), supporting and expanding Thys van den Audenaerde's conclusions, upgraded *Danakilia* to the genus level in consideration of the morphological peculiarities distinguishing it from other similar genera of Tilapiini, a tribe she baptized in the same publication, overlooking the subfamily name Tilapiinae already formalized by Hoedeman and de Jong (1947-1962). Furthermore, following the fresh description of *Iranocichla hormuzensis* Coad, 1982 (Coad, 1982), a species that shows many analogies with *Danakilia*, including to be a desert fish in an isolated genus, she postulated a close relationship of *Danakilia* with the Middle-Eastern genera *Iranocichla* Coad, 1982, of Southern Iran, and *Tristramella* Trewavas, 1942, of Lake Tiberias (Israel), based on morphological and ecological characters (Trewavas, 1983). Trewavas also stated she could not dismiss a possible phylogenetic connection with the East African genus *Alcolapia* Thys van den Audenaerde, 1969 of the highly alkaline Lake Magadi (Kenya) and Lake Natron (Tanzania). Albeit she could find morphological and ecological characters linking *Danakilia* and *Alcolapia*, she anyway regretted she could not compare their breeding biology, lacking information about the mating system of *Danakilia* (Trewavas, 1983), which we now know to be a maternal mouthbrooder.

A few years later, Stiassny (1991) provided a first cladistic analysis of cichlids based mainly on two additional morphological characters on the lower pharyngeal jaw (two prominent foramina at the back of the lower pharyngeal jaw and a median ridge on the dorsal aspect of the keel of the lower pharyngeal jaw) and included *Danakilia* in the monophyletic suprageneric clade tilapiine from which she excluded some of the genera included by Trewavas (*Pelmatochromis*, *Steatocranus* and *Pterochromis*).

Nagl *et al.*, (2001) provided a molecular phylogeny for 42 species of the tilapiine lineage based on the region encompassing the terminal part of the tRNA mitochondrial gene and the most variable part of the control region. In their conclusions, the authors well defined the separation among the genera *Oreochromis* Günther, 1889 and *Sarotherodon* Rüppell, 1852, on the one side, and *Tilapia*, on the

other. Besides, the clustering of *Oreochromis* with *Sarotherodon* corroborated the hypothesis that the mouthbrooding behavior of tilapiines evolved only once from substrate spawning behavior; conversely, adaptation to life in water with high salt concentration and elevated pH evolved independently at least twice in the lineage (Nagl *et al.*, 2001). *Danakilia* was excluded from the analysis for the unavailability, at that time, of DNA samples.

In a breakthrough study, Klett and Meyer (2002) provided a phylogeny of the “tilapias” based on the mitochondrial gene ND2 and on the parental care behavior (form of care and sex of caregiver); again, they did not include *Danakilia* in the analysis, lacking DNA material and indisputable information on the genus’ breeding system.

Takahashi (2003), following Trewavas (1983) and the morphological clues suggested by Stiassny (1991), grouped *Danakilia* within the Tilapiini, recovering Trewavas’ former tribe name.

Later, Sparks and Smith (2004) recovered the old subfamilial name Pseudocrenilabrinae to include all the African cichlids on the base of a strong family level phylogeny.

At the end of the first decade of this century, came the description of the second species of the genus, *Danakilia dinicolai* Stiassny, De Marchi & Lamboj, 2010, which provided new insights into the genus and its radiation in the Danakil Depression, plus some important observations on its ecology and breeding biology (Stiassny *et al.*, 2010). Unfortunately, as the same authors state in their paper, despite all their efforts, it was impossible to obtain DNA from the specimens, whose preservation was not optimal, subtracting *de facto* the research team (and the rest of the world) of the possibility to carry out the strongly needed molecular analyses (Stiassny *et al.*, 2010).

In an important paper, Dunz and Schlieven (2013) provided a comprehensive molecular phylogeny of almost all taxa formerly referred to as ‘*Tilapia*’ and related lineages. Again, DNA from the two species of *Danakilia* so far described was unavailable, as already stressed by Stiassny *et al.*, (2010) and the phylogenetic position of *Danakilia* remained unsolved. According to their molecular analyses, the tribe Tilapiini, was drastically downsized to three genera only (*Tilapia*, *Congolapia* and *Chilochromis*) forming a monophyletic clade and *Danakilia*, for the reason stated above, was then conditionally assigned to the new tribe Oreochromini (Dunz and Schlieven 2013).

Schwarzer *et al.*, (2016) recently assessed the genetic and phenotypic diversity among populations of *Iranocichla* across most of its geographical range in Southern Iran by sequencing two mitochondrial genes (ND2 and D-Loop) and comparing male nuptial coloration in different habitats. This study lately led to the description of a new species, *Iranocichla persa* Esmaili, Sayyadzadeh and Seehausen, 2016 (Esmaili *et al.*, 2016). What is more important in the light of the present study, Schwarzer *et al.*, (2016), could reconstruct a phylogenetic tree of *Iranocichla* encompassing 28

outgroups among which, thanks to my recently made collections, two Eritrean *Danakilia* populations were included for the first time. The analyses of the mtDNA sequences proposed a well-resolved separation between *Iranocichla* and *Danakilia* suggesting that the similarities between the two genera are probably attributable to parallel ecological adaptations to comparable desert environments, rather than to a close phylogenetic affiliation, as firstly suggested by Trewavas (1983).

Breeding

The details about the breeding biology of the cichlids of the genus *Danakilia*, object of the present study, were unknown until the description of *D. dinicolai* and then limited to episodic observations (Stiassny *et al.*, 2010). Recent observations in the field and the collection of living specimens for aquarium breeding carried out in the framework of the present research, provided evidence that *Danakilia* are mouthbrooders, as already guessed by Trewavas (1983) and Stiassny *et al.*, (2010), and a set of information hereafter described. The breeding male defends a territory around a pit (bower) chasing intruders of both sexes and even specimens of other species (*Aphanius cf. dispar*). Spawning females, that are possibly recognized as potential mates for their behavior and body pattern, are briefly courted and rapidly fertilized in the bower. Adult females lay up to 50 pear-shaped orange yellow eggs measuring about 3 mm that are almost immediately taken in the mouth while the circling male, with shivering movements of the body, repeatedly ejaculates. During the incubation, lasting about 20-22 days the female does not take food. Fry becomes independent soon after the release by the female. In aquarium, and presumably in the wild, fry is voraciously eaten by the adults. This last behavior is probably common to all observed populations, even if we have details only for the riverine populations, at present the only captive bred. In the wild, fry lives in schools formed by specimens about the same age and length in the shallow waters a few cm deep where the adults likely cannot reach. Field observations, also recorded on video, evidence that *D. franchettii* males do not dig a pit, but defend a portion of the bottom. Exhaustive information is unfortunately unavailable about *Danakilia's* mating system, which is anyway almost certainly sited in the range of polygyny or polygynandry.

Ecology

Danakilia morphospecies live in the brackish waterbodies of the Danakil Depression of Eritrea and Ethiopia. These include groundwater fed creeks flowing to Lake Afrera, the volcanic Lake Abaeded and temporary ponds along the bed of three endorheic temporary rivers subject to flash floods that flow from the Danakil Block towards the inner part of the Danakil Depression where they form alluvial fans (Shukoray, Zariga and Gali Colluli). *Danakilia* favors waters with lower salinity (4.41-

20.52 g/l: present study) than those found in the hypersaline Lake Afrera (130 g/l: present study) where only the more salt tolerant fishes of the genus *Aphanius* can thrive. However, *Danakilia* were observed and collected also in Lake Afrera (Trewavas, 1983), but exclusively in areas adjacent to the creek mouths and where they probably do not form distinct viable populations. Moreover, when sampling in the lake's waters during the present research, we could collect only *Aphanius*, at least in areas far from the creeks inhabited by *Danakilia*.

Danakilia are vegetarian fishes whose main food are algae growing abundant in shallow waters in all waterbodies (Trewavas, 1983; Stiassny *et al.*, 2010; this study). This is probably the reason why the Italian explorer Baron Raimondo Franchetti, could not find any fish in the middle of Lake Afrera, even after the repeated launch of dynamite cartridges (Franchetti, 1930). Moreover, SCUBA divers could not observe *D. dinicolai* specimens below the depth of 3 m during the exploration of the crystal-clear waters of Lake Abaeded (Ministry of Fisheries and Eritrean Diving Center 1998).

The pupfishes (family Cyprinodontidae)

The Family Cyprinodontidae that counts 138 valid species (Table 3) is one of the largest of the Order Cyprinodontiformes and the fourth family after the Cynolebiidae (439 species), the Poeciliidae (353 species), and the Notobranchiidae (275 species).

<i>Order</i>	<i>Family</i>	<i>Subfamily</i>	<i>Available</i>	<i>Valid</i>	<i>Described 2008-2017</i>	<i>Distribution</i>
Cyprinodontiformes			1864	1341	229	America, Africa, S Eurasia
	Cyprinodontidae		221	138	13	N, C and S America, N and NE Africa, S Europe, SW Asia
		Cubanichthyinae	3	3	0	Cuba, Jamaica, Colombia
		Cyprinodontinae	218	135	13	America, N and NE Africa, S Europe, SW Asia

Table 3. The taxonomic arrangement of pupfishes (Cyprinodontidae) from order to subfamilies after Van der Laan *et al.*, (2014), and species numbers at each taxonomic level after Eschmeyer and Fong (2017), modified. Species included in the count are those for which names are available for use. Subspecies are treated as available names at the species level and as synonyms at the species level. All new species described in the last 10 years are considered valid, unless status references show they are synonyms. Distribution after Nelson *et al.*, 2016.

These generally smallish fishes (most species do not exceed 5-10 cm, while the biggest known, *Orestias cuvieri* Valenciennes, 1846 reaches a length of 30 cm), are found in fresh, brackish and marine waters. They are more usually known with the common names of tooth-carps, killifishes, killies or pupfishes. A number of species entered the fish hobby but had not the same diffusion of cichlids, remaining confined to really focused enthusiasts.

The common and scientific names (tooth-carp and Cyprinodontidae) refer to the superficial resemblance of these fishes to the carps or minnows (Cyprinidae), with which the cyprinodonts share a fairly elongate and compressed body, and to the presence of jaw teeth (Cyprinidae have pharyngeal teeth) typically triscupidate in the subfamily Cyprinodontinae, and conical and placed in multiple rows in the Cubanichthyinae. The body and head are covered with scales and no spines are present in the fins. The caudal fin is truncate or rounded, the pectoral fins are set low on the body, the dorsal

and anal fins are short. The lateral line is absent or reduced to points.

The Cyprinodontidae are oviparous fish that usually lay their eggs on plants. No forms of egg resistance to drying are present, contrary to other families in the order Cyprinodontiformes (e.g. the annual Nothobranchiidae).

The genus *Aphanius*

The genus *Aphanius*, the only native Old World representative of the family Cyprinodontidae, is present in the Danakil Depression with two described species, *A. cf. dispar* (Rüppell, 1829) and *A. stiassnyae* (Getahun & Lazara, 2001). At present counting 28 valid species (Gholami *et al.*, 2015), this genus ranges in coastal brackish waters (rarely saltwater) and inland freshwater environments from the Mediterranean Sea, Red Sea, Persian Gulf, Arabian Sea and Indian Ocean, south to the Somali coast and east to southern Pakistan (Wildekamp, 1993, Teimori *et al.*, 2012a). The hotspot of *Aphanius* diversification is located in the area encompassing central Turkey (12 species: Froese and Pauly, 2016) and Iran (14 species: Gholami *et al.*, 2015).

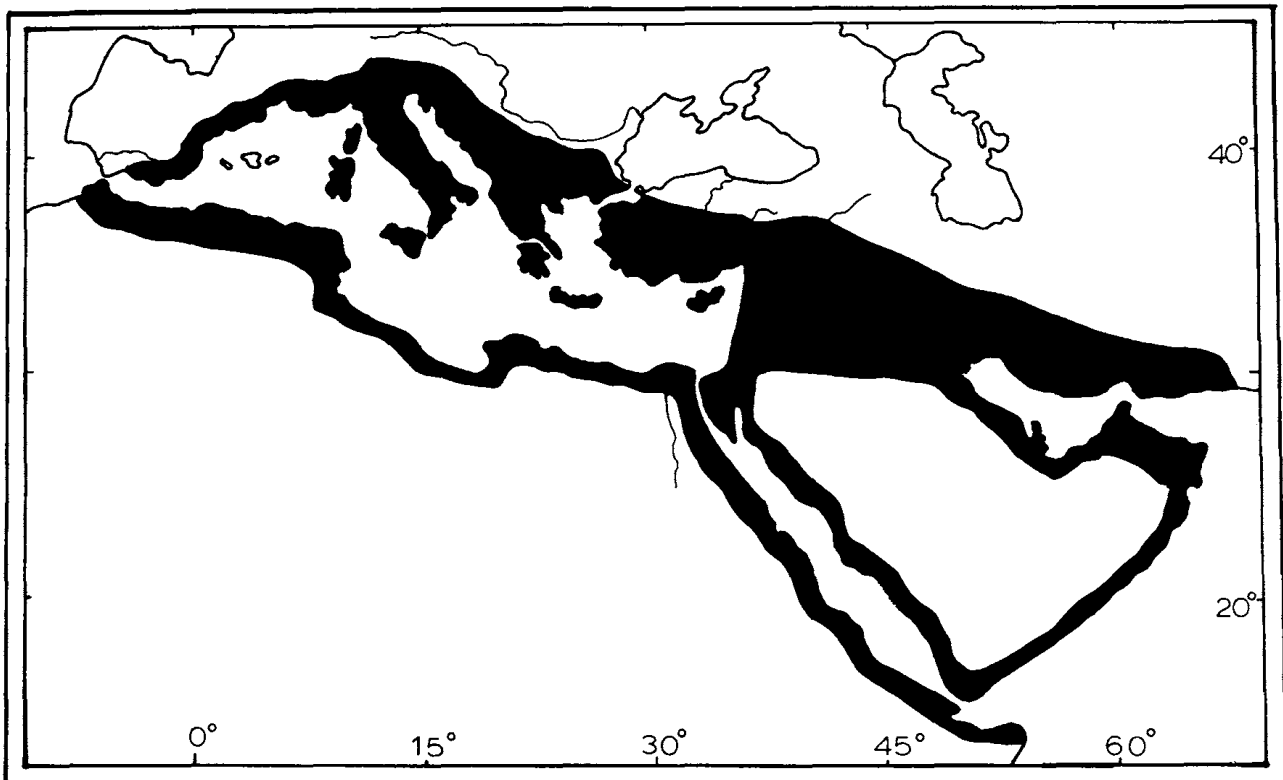


Fig. 6. Distribution of the “Anatolian” pupfishes of the genus *Aphanius* (from: Parenti 1981).

The present distribution of the genus *Aphanius* (Fig. 6) along the coastline of the late period Tethys Sea is likely the result of vicariant speciation of what was once a widespread group of fishes. The

radiation of *Aphanius* commenced with the formation of many new isolated geological units that followed the closing of the Tethys seaway near Oligocene/Miocene boundary (Hrbek and Meyer 2003) at about 14 MYA, in coincidence with the Middle Miocene Climatic Transition, a period of major climatic and oceanographic changes (Hamon *et al.*, 2013).

On a morphological basis, the genus is quite uniform, but as it is usual in cyprinodontids, all species show a clear sexual dimorphism, with the males showing patterns of bars and spot-like pigmentation and the females displaying vertical flank bars.

On an ecological basis, the genus *Aphanius* can be separated into two groups, one composed of species living in brackish and euhaline waters in coastal environments, and one inhabiting freshwaters in landlocked basins (Hrbek and Meyer 2003, Teimori *et al.*, 2012a).

Within the genus, *A. dispar* (Rüppell, 1829) is the most widespread taxon, with a native distribution ranging from the Red Sea and Persian Gulf to the Indian Ocean and southern coastline of Pakistan (Wildekamp 1993). *A. dispar* is a euryhaline species that can be found in coastal lagoons, hyperhaline lakes, fresh-water oases, endorheic drainage systems, hot sulphuric springs and estuarine rivers, its habitats including saline, hyperhaline, brackish and occasionally fresh water environments (Hrbek and Meyer 2003, Getahun and Lazara 2001, Teimori *et al.*, 2012a and references therein). In northeastern Africa, *A. dispar* ranges from Egypt to northern Somalia in coastal and inland zones (Wildekamp *et al.*, 1986).

Despite known to show great intraspecific variation for morphological characters and otolith traits, the species has received little scientific attention (Wildekamp, 1993; Reichenbacher *et al.*, 2009, Teimori *et al.*, 2012b). Hrbek and Meyer (2003) study based on molecular information (12s and 16s ribosomal RNAs, tRNAs and mtDNA NADH subunits I and II) concluded that this taxon should not be regarded as monophyletic; for this reason in the opinion of these authors, *A. dispar* cannot be considered as a species according to the phylogenetic species concept.

Teimori *et al.*, (2012b) found considerable evidence of geographical differentiation in three Iranian river populations of *A. dispar* not attributable to environmental differences among drainage systems. This suggested an effect of geographical isolation and allopatric divergence connected to separation events (migration barriers and changing hydrological networks) dating back to the Pleistocene period (1.8×10^6 - 8×10^3 years BP) and concluded that the three groups of *A. dispar* may represent subspecies or even different species.

Natural sympatry of *Aphanius* species is rare, and hybridization - a phenomenon that occurs in fishes more commonly than in other vertebrates (Scribner *et al.*, 2001) – is not always resulting from it.

Hybrids occur between *A. dispar* and *A. mento* in the Ein Fashkha wetland of the Dead Sea in Israel (Goren and Rychwalski 1978), between *A. dispar* and *A. fasciatus* (Lotan and Ben-Tuvia 1996) in the Bardawil Lagoon on the Mediterranean coast of Sinai (Egypt) and fishes of the *A. dispar* group and *A. mesopotamicus* living in sympatry in the Shadegan Wetland of SW Iran (Masoudi *et al.*, 2016). On the other hand, literature reports two cases of sympatry in the absence of hybridization for this species: *A. dispar* and *A. stiassnyae* in Lake Afdera (Getahun and Lazara 2001), and *A. dispar* and *A. furcatus* in the Hormuzgan Basin of southern Iran (Teimori *et al.*, 2014).

The current knowledge about *A. stiassnyae* is limited to the study of the holotype and paratype (two female specimens: males are undescribed) collected in 1996 along the southwestern shores of hyperhaline Lake Afdera in close proximity of a hot spring flowing to the lake (Getahun and Lazara 2001). The species description centers on morphological and meristic characters in comparison with the syntopic population of *A. dispar*: molecular analyses neither were performed on the type specimens, nor was fresh material collected after the species description. In general, the *Aphanius* populations from the Danakil Depression were never investigated before, either morphologically or molecularly.

Ecology of Aphanius in the Danakil Depression

The *Aphanius* populations living in the Danakil Depression were always found sharing the same habitats of *Danakilia* forming two-species fish assemblages in every situation. For this reason, I presume that their general ecology is possibly quite similar to that of the cichlids. In general, according to various authors, *Aphanius dispar* are eclectic fishes feeding on algae and small aquatic animals (copepods, rotifers and, rarely, small insects). The salt tolerance of *A. dispar* is elevated and these fishes are capable of swift changes from saltwater to freshwater. Also temperature tolerance is high, having been reported to survive in waters 38.4 °C and beginning to die at 45.1-46.0 °C (Coad, 2017 and references therein).

THE STUDY SETTING: THE DANAKIL DEPRESSION

Geology

The Danakil Depression (Chapter 2, Fig. 1) is situated at the northernmost apex of the Afar region, a triangular-shaped area of ~200,000 km² located at the separation point of the Arabian, Nubian and Somali tectonic plates (R-R-R triple rift: Mohr, 1978) and wedged among the Red Sea to the north, the Ethiopian Plateau to the west, and the Somali highlands to the south (Beyene and Abdelsalam, 2005). Geologically, Afar has its own structural individuality and is characterized by extensive volcanic and telluric activity (Rognon, 1972; Tazieff, 1971; Tazieff *et al.*, 1972). As a geological feature, southern Afar represents the northern extreme of the African Rift system, while the central and northern portions belong to the Red Sea and Gulf of Aden rifts, where new crust is generated (Gasse and Street, 1978). The Danakil Depression extends NNW-SSE for about 300 km from the Gulf of Zula to the north (15°N, 39°E) to Lake Afdera to the south (13°N, 41°E). To the west, the area is delimited by the continental crust block of the Eritrean-Ethiopian plateau, while to the east it is bounded by the Danakil Block, similarly of continental origin. In spite of being fully continental, ~10,000 km² in the Danakil Depression lay below the sea level.

Several authors provided different interpretations of the genesis of the Danakil Depression. In synthesis, a tensional and rotational movement detached the Danakil Block from the Ethiopian Plateau causing the formation of the depression (Bonatti *et al.*, 1971; Tazieff *et al.*, 1972; Beyene and Abdelsalam, 2005; Bosworth *et al.*, 2005; Abbate *et al.*, 2015). Most authors agreed on its Middle/Late Miocene origin above a thinned continental crust characterized by a Precambrian basement, Paleozoic to Mesozoic sedimentary deposits (1000-1500 m thick) and Oligocene trap basalts (Abbate *et al.*, 2004; Abbate *et al.*, 2015).

The Danakil Depression displays three stratigraphic formations. The 1000 m thick Danakil Formation (24-5 Ma) marks the boundaries of the depression: bright red conglomerates and sands with mudstones, occasionally gypsiferous, and rare freshwater gastropod-bearing limestones characterize it. The sedimentary scenery was that of alluvial fans, lotic streams and swamps (Abbate *et al.*, 2004). The Enkafala or Zariga Formation, fossil-dated 200,000-24,000 y (Bonatti *et al.*, 1971), is a few tens of meters thick and is mainly composed of gypsum and marls; it stays on top of the Danakil Formation and marks a marine introgression in the north of the depression, providing evidence of a seaway connecting the Red Sea to the depression during interglacial high sea levels (Abbate *et al.*, 2004). The Enkafala Formation blends towards the center of the basin into the 2200 m thick evaporitic salt formation, composed of halite, gypsum, potash salts and clays that, at the surface, forms an impressive bright-white salt plain. The upper levels of the salt formation are dated 80,000 years, but its thickness

suggests a possible Pliocene dating, that brings the presence of the sea back to 5 MYA. The connection with the southern Red Sea was interrupted by the end of Pleistocene for the formation of an axial volcanic range in the area of the Gulf of Zula, between Arafale and Buia (Alid volcanic center: Lowenstern *et al.*, 1999). Pleistocene volcanism is existing also in the southern part of the Danakil Depression: the NW-SE oriented Erta Ale massive axial volcanic range bisects the region (Mohr, 1978). The southern Danakil Depression is characterized by the presence of lacustrine sediments associated with present day saline lakes, such as Lake Afdera (Gasse, 1974; Abbate *et al.*, 2004; Abbate *et al.*, 2015).

Paleoenvironment

Barberi *et al.*, (1972) age determinations show that the Danakil rift began to form in the lower Miocene (23-25 MYA). According to Bonatti *et al.*, (1971), which base their hypothesis on the dating of a fossil coral atoll found in the area, part of the Danakil Depression was an intermittent marine basin until late Pleistocene, when it lastly separated from the Red Sea: final desiccation of the basin started 32,000 years ago and was relatively rapid, lasting ~4000 years.

Evidence collected in Eritrea in the middle 1990's in the fossiliferous strata of the Danakil Formation of the Buia section of the Dandiero Group (Early-to-Middle Pleistocene) testify the presence 1 MYA of a rich vertebrate fauna including mammals, birds, reptiles and fishes. The Buia vertebrate assemblage included several taxa with marked water dependence (i.e., *Hippopotamus amphibius*, *Tragelaphus* cf. *T. spekei*, *Kobus* cf. *K. ellipsiprymnus*, *Crocodylus niloticus*, water birds of the families Anhingidae and Rallidae and catfishes of the family Clariidae) and a number of taxa associated with semiarid environments (Abbate *et al.*, 1998; Ferretti *et al.*, 2003; Delfino *et al.*, 2004; Martinez-Navarro *et al.*, 2004; Rook *et al.*, 2013). Altogether, their ecological requirements indicate a fluvio-deltaic and lacustrine environment with moist grassland habitats adjacent to persistent waters (Rook *et al.*, 2013). Sedimentology and facies analysis reinforce this scenery (Abbate *et al.*, 2004). Supported by hints coming from fossil remains and ichnologic evidence still under study was the possible presence of a representative of the tilapiine cichlids in the Buia paleoenvironment (G. Carnevale: pers. comm.), a particularly important issue in the light of the present research and, in general, for the interpretation of Cichlidae radiation in northeastern Africa.

Gasse (1974) reconstructed the hydrologic and climatic evolution of the southern portion of the Danakil Depression during the Holocene by studying the fossil diatom flora found in the region of Lake Afdera. The collections assembled by Gasse did not contain oceanic planktonic diatoms. For

this reason, she excluded a marine origin of the lake. Lake depth changed from a few meters above the present level around 9800 years BP, to ~12 m above the present level between 8750-7800 years BP, to reach more than 50 m above the present level around 7800 years BP, when the lake occupied an area of about 200 km². Finally, the lake rapidly retreated around 7300 years BP. At the same time, the study of the diatom assemblages encountered in the various strata, made it possible to describe the fluctuations of salinity and pH of paleo-lake Afdera. In general, diatoms are highly tolerant of pH fluctuations, but typically form distinctive associations in function of water pH. The fossil diatoms found in the area are mainly alkalibiont and alkaliphile species developing in basic or neutral to basic water conditions. This find demonstrated that the Holocene lake had a higher pH (pH 9 or higher) than the present day lake, with near-neutral water conditions. Fossil diatom assemblages show that salinity was generally lower than today. However, in coincidence with the maximum expansion of the lake, possibly due to a dramatic change in the climatic conditions and/or a tectonic event (7800 years BP), it paradoxically grew, as shown by the diatom species assemblages. The current hypersaline condition derive from the accumulation of sodium chloride due to water evaporation not counterbalanced by inflow from subterranean sources.

Climate

The Danakil Depression is one of the hottest areas of Earth (Vinassa De Regny, 1931; Fantoli, 1940), but unfortunately recent data are not available to confirm this evidence (Fazzini et al, 2015). At Dallol (130 m b.s.l.), which is considered the hottest, inhabited place of the world, Pedgley (1967) measured a mean annual temperature of 34.7 °C, mean maximum temperatures of 41.2 °C, and a peak of 45.7 °C for the 1960–1966 interval. Moreover, Fazzini *et al.* (2015) report a personal communication by P. Billi of recent records (November 2007) of temperatures of 42–44 °C in the Samoti plain, in the northern Eritrean Danakil. The mean maximum at Dallol is so high not because the hottest months are especially hot, but because the coolest months are particularly warm: for example, many places over a wide part of northern Africa have temperatures in their hottest months comparable to those at Dallol, but their coolest months are commonly cooler by 2.8-5.5 °C (Pedgley, 1967). However, Pedgley (1967) hypothesizes that since Dallol is not quite at the lowest part of the Danakil Depression, it is possible that the annual mean is higher some 20 miles to the south, particularly where black lava flows replace the white salt and gypsum.

In the Danakil depression, annual precipitation is constantly less than 250 mm but can be as low as 50 mm and cloudiness, which along the Eritrean coast determines night advection fogs turning into

morning drizzle, is almost an exceptional phenomenon (Fazzini *et al.*, 2015). Extreme evapotranspiration conditions are found in the Danakil depression where values of as much as 4,000 mm can be estimated for Dallol. This figure, combined with inappreciable precipitation and extremely hot temperatures, makes this area as one of the most arid on the planet (Fazzini *et al.*, 2015). In the last three decades, the aridity index of the Danakil depression decreased beyond the critical value of 0.65; for this reason, the Danakil Depression have to be considered as a desertification prone area (Fazzini *et al.*, 2015).

Hydrology

The rivers existing in the Danakil area flow from the heights situated in the northern (Alid volcano area), eastern (Danakil Alps) and western (Ethiopian Plateau) parts of the depression. These rivers are endorheic watercourses flowing into the depression where they drain into alluvial fans. All rivers are ephemeral and dry for most of the year; intermittent pools form along their courses in the interval between rains and represent irreplaceable water resources for wildlife (including fishes) and livestock. These drainage systems collect the monsoonal downpours falling on the coastal heights and on the Ethiopian Plateau and are subject to sudden flash floods carrying huge volumes of water and sediments to the inner depression. For example, River Ragali collects water from the catchments of the Ethiopian-Eritrean Plateau and its annual runoff is estimated at $117 \times 10^6 \text{ m}^3$ with a peak discharge of $650 \text{ m}^3/\text{s}$ (Ghebremariam and Steenbergen, 2007). A significant amount of water has subterranean origin (Gasse, 1974; Gasse and Street, 1978).

Endorheic basins are also present, i.e. hypersaline lakes Afrera (in the south), Assale and Bachili (in the center), Badda (in the north). A small (10 ha) crater lake (Lake Abaeded) is situated in the northwestern portion of the Danakil Depression.

Biodiversity

Due largely to political instability in the region over the last 50 years, many elements of the fauna and flora remain poorly known. According to a reassessment and redefinition of the former terrestrial ecoregions indicated by Olsen *et al.* (2001), which included the Danakil Depression in the “Ethiopian xeric grasslands and shrublands”, this desert area is now part of the “Djibouti xeric grassland” newly recognized ecoregion (Dinerstein *et al.* 2017). This ecoregion extends inland from the Red Sea including the Dahlak Archipelago and the Howakil and Anfile bays islands, stretching from the

Sudanese-Eritrean border, well south through Ethiopia's Awash Valley and eastwards into Djibouti and Somaliland. Phytogeographically, White (1983) regarded this ecoregion as part of the Somali-Masai regional centre of endemism and mapped the vegetation as “Somalia-Masai semi-desert grassland and shrubland”. Along the coast, mangroves occur in muddy areas, primarily around wadi "outwashes" and inlets, while further inland, vegetation changes to grass shrub steppe.

The Danakil Depression, notwithstanding its paucity of water, sustains fish populations and the area is part of the “Western Red Sea Drainages” freshwater ecoregion (Abell *et al.*, 2008).

Levels of endemism are generally low. For vertebrates, while the reptile fauna is well represented, amphibians and fishes are scarce. Birds are abundant as passage migrants especially along the coast, being the area along the East Asia/East Africa fly way. Among mammals, desert ungulates are well represented. The Eritrean and Ethiopian population of the critically endangered African Wild Ass (*Equus africanus somalicus*) survives here possibly with the last viable groups. The vulnerable Dorcas Gazelle (*Gazella dorcas*) and Soemmerring's Gazelles (*Nanger soemmerringii*) still persist in Eritrea (Fig. 7) apparently in good numbers (F. Hagos, pers. comm.).



Fig. 7. Soemmerring's Gazelle (*Nanger soemmerringii*) in the Danakil desert (photo credit: G. De Marchi).

Populations of most large mammal species have been severely reduced through hunting by local people and by the government and resistance armies during 30 years of war.

The ichthyological exploration of the Danakil

Early information about fish presence in the Danakil Depression dates back to the beginning of the 20th century. The first narrative account of fish living in the creeks feeding Lake Afrera appeared in the writings of Ludovico Mariano Nesbitt (1891-1935), an Anglo-Italian explorer who, together the Italian Tullio Pastore and Giuseppe Rosina, 15 Eritreans and Ethiopians, 25 camels and four mules, crossed in the entire Afar Triangle south-north (1300 km), in a memorable expeditionary trip lasting three and a half months in 1927-1928. Though the approach to the fish was far from scientific, we anyway get some interesting hints that allow us to conclude that what they collected were cichlids “*In several of the water-holes, we now observed schools of little fishes. Using a bed-sheet as a net, we caught about four pounds weight of these creatures. Their fins were hard and needle-sharp, and it was difficult to take them with one's hand. I also shot some doves and partridges, as they came to the pools at dusk.*”. Unfortunately, the fate of these *Danakilia* is made clear in the next sentences “*That night, we had prepared for us a sumptuous meal of fried fish, roast birds, and boiled rice, and these things were provided for our sick men also.*” (Nesbitt, 1935).

The first multidisciplinary scientific expedition to the Danakil Depression was carried out in 1928-1929 by the Italian baron Raimondo Franchetti (1889-1935) that reached the Ethiopian highlands



Fig. 8. *Danakilia franchettii* collected during the Franchetti's expedition of 1928-1929 (courtesy Luca Lupi).

crossing east-west from Assab to Addis Abeba. The expedition, listing a dozen Italians, a hundred natives, 30 camels and 16 mules and armed with hand-guns, rifles and a machine-gun, reached and

explored Lake Afrera where it collected (and carefully preserved) the first specimens of *Danakilia* (Fig. 8), later studied and named *Tilapia franchettii* by the Italian ichthyologist Decio Vinciguerra (1856-1934). Vinciguerra, vice-director of the Museo Civico di Storia Naturale “G. Doria” of Genoa, studied all the ichthyological collections, which included also specimens of *Cyprinodon* (= *Aphanius*) *dispar* collected in Lake Afrera and in the Gaharre water wells, about 20 km inland from the Red Sea coast (Vinciguerra, 1931). Seventy years later, in April 1998, an Italo-Eritrean expedition organized by the Ministry of Fisheries of Eritrea and the Eritrean Diving Center of Massawa with the participation of Eritrean marine biology and SCUBA diving experts (Samson Tsegay and Yassin Adem) and Italian advisors (Dania Avallone and Giuseppe De Marchi) reached Lake Abaeded. The expedition was organized in order to verify the rumours about the presence of “colourful sea fishes” in the small lake. De Marchi immediately recognized they were cichlids belonging to the genus *Danakilia*. In a successive expedition, in 2001, De Marchi collected several specimens that he later sent out for study to the American Museum of Natural History. The study resulted in the publication of a new species: *Danakilia dinicolai*, named after Ernesto Di Nicola (1969-2001) a member of the 2001 collecting expedition died in a car accident on the way back from the lake (Stiassny *et al.*, 2010).

Outline of the study

Aim of the research is to study the phylogeography and diversification of the two poorly known actinopterygian desert fish genera inhabiting the Danakil Depression of north-eastern Africa: *Danakilia* (Cichlidae), endemic of the Danakil Depression, and *Aphanius* (Cyprinodontidae). The focus of the study is on the variation of the two desert fish genera, here investigated for the first time in a molecular and geometric morphometric approach, and on the interpretation of the observed variation in a geological and paleoclimatic framework that will take into account the historical evolution of this desert area. Three expeditions have been carried out during the years 2014-2016 (December 2014-January 2015, April 2015 and December 2015-January 2016) in the Danakil Depression of Eritrea and Ethiopia in the framework of international cooperative collaborations with American, Eritrean and Ethiopian scientific institutions. The research was carried out both in the field, with the collection of samples in six locations across the entire area, and in the molecular facilities of the Earth and Environmental Science Department of the University of Pavia and the Department of Ichthyology of the American Museum of Natural History.

Chapter 2 deals with the assessment of the within-genus observed morphological variability of the fish of the Danakil Depression by analyzing the mitochondrial genetic variation across samples of *Danakilia* and *Aphanius* using concatenated *COI* and *cytb* mt-DNA markers and infer the phylogeographic relationships within each genus giving an interpretation of the present distribution in the light of the geological and paleoclimatic knowledge of the Danakil Depression.

Chapter 3 examines variation in body and lower pharyngeal jaw of the *Danakilia* morphospecies using an advanced geometric morphometric approach in order to understand how body and lower pharyngeal jaw vary within and among *Danakilia* morphospecies and whether the observed variation in geographical space is founded on a genetic basis or on local plastic adaptation. Moreover, the analyses try to identify consistent patterns of co-variation which might point to pleiotropy or linkage as possible mechanisms accelerating diversification.

Chapter 4 is the presentation of research projects already commenced, but still under way and aimed at understanding aspects of the taxonomy, biology and conservation of the studied fishes. In particular, a dd-RAD sequencing genetic approach will allow a better understanding of the systematic placement of *Danakilia*. The fishes are under study also in a more classical taxonomic setting employing morphological and meristic characters. The adaptive significance of the nuchal hump present in *Danakilia* and in other cichlids, will be investigated across the entire family in a cause-effect statistical model. Finally, the accent is put on conservation of the Danakil Depression water-bodies, threatened by natural and anthropogenic causes.

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CHAPTER 2

RESEARCH PAPER

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Male *Aphantius* cf. *dispar* from the River Shukoray, Danakil Depression

Fishes in the desert: mitochondrial variation and phylogeography of *Danakilia* (Actinopterygii: Cichlidae) and *Aphanius* (Actinopterygii: Cyprinodontidae) in the Danakil Depression of northeastern Africa

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ABSTRACT

The Danakil Depression in northeastern Africa represents one of the harshest arid environments on Earth, yet two genera of fishes, *Danakilia* (Cichlidae) and *Aphanius* (Cyprinodontidae), share its sparse aquatic habitats. The evolutionary history of these fishes is investigated here in the context of genetic, geological and paleoenvironmental information. We collected samples from seven sites and assessed phylogeographic relationships using concatenated *COI* and *cytb* mtDNA genes. *Danakilia* morphospecies show low differentiation at mitochondrial markers, but variation is partitioned between a northern cluster containing *D. dinicolai* plus three undescribed riverine populations, and a southern cluster including two creek populations of *D. franchettii* separated by the hypersaline waters of Lake Afrera. *Aphanius* displayed four genetically distinct clades (*A. stiassnyae* in Lake Afrera; one distributed across the entire area; one in Lake Abaeded; and one in the Shukoray River), but without clear large-scale geographic structure. However, Danakil *Aphanius* are clearly differentiated from *A. dispar sensu stricto* from the Sinai Peninsula. Geological evidence suggests that after the Late Pleistocene closure of the Danakil-Red Sea connection, increased post-glacial groundwater availability caused the formation of a brackish paleolake flooding the entire region below the –50m contour. Fish populations previously isolated in coastal oases during glaciation were able to mix in the paleo-lake. Subsequently, in a more arid phase starting ~7300 BP, paleo-lake regression isolated

fishes in separate drainages, triggering their still ongoing diversification.

Keywords

Africa; *Aphanius dispar*; *Aphanius stiassnyae*; clade diversification; *Danakilia*; extremophile fishes

INTRODUCTION

Desert ecosystems represent some of the harshest environments for fishes on Earth, and the ichthyofaunas of such regions consequently mirror a history of extreme isolation and rapid adaptation. Of these regions, the northern Danakil Depression now partitioned between Eritrea and Ethiopia stands out as among the most isolated and arid, receiving a mean annual rainfall of less than 100 mm/a, with evaporation reaching 5 m/a (Faure *et al.* 2002).

In general, desert fish assemblages tend to reflect the faunal composition of the surrounding regions, suggesting that fish life in deserts evolved through isolation and adaptation of local populations whose presence predated the development of present-day conditions (Hillyard *et al.* 2015). The desert fish assemblage of the Danakil Depression is no exception, and is composed of species belonging to taxonomic groups represented in the nearby drainage basins of the Nile and Awash rivers and the Red Sea coast. Interpretation of the biogeographic history of extant fish species in the Danakil Depression requires an understanding of their evolutionary history in light of regional geomorphological and climatic changes over the Pleistocene and Holocene.

The present day fish fauna of the Danakil Depression is restricted to four described species: two in the endemic cichlid genus *Danakilia* Thys van den Audenaerde, 1969 (Oreochromini), and two in the cyprinodontid genus *Aphanius* Nardo, 1827 (Cyprinodontinae), which is widely distributed throughout the Mediterranean and Middle East. Of the two cichlid species, *Danakilia franchettii* (Vinciguerra, 1931) was first collected in 1929 in hot mineral springs feeding Lake Giulietti (now Afrera or Afdera), (Franchetti 1930, Vinciguerra 1931), while *D. dinicolai* Stiassny, De Marchi and Lamboj, 2010 was first collected in 1998 from Lake Abaeded, a small volcanic lake located some 170 km to the north of Lake Afrera (Stiassny *et al.* 2010).

Of the two cyprinodontids, the widely distributed *Aphanius dispar* (Rüppell, 1829) was collected in the Danakil Depression from Lake Afrera and tributary creeks (Getahun and Lazara 2001, Vinciguerra 1931) and from Lake Abaeded (Stiassny *et al.* 2010). A third *A. dispar* population was collected in 1928 outside the Danakil Depression, in the Gaharre water wells (13.248421°, 42.129956°) of adjoining coastal Eritrea (Vinciguerra 1931). The second species, *A. stiassnyae* (Getahun and Lazara, 2001), was collected in Lake Afrera, where it is considered to be endemic.

Biogeography and taxonomy of Danakilia

To date, within-genus diversity of *Danakilia* is poorly known and its sister-group relationships remain unresolved. Morphological studies have been inconclusive, although a possible relationship was proposed with the desert genera *Iranocichla* Coad, 1982, and *Alcolapia* Thys van den Audenaerde, 1969, as well as with the Levantine genus *Tristramella* Trewavas, 1942 (Trewavas 1983, Stiasny *et al.* 2010). A number of molecular studies have investigated ‘tilapiine’ relationships, however the placement of *Danakilia* is still undefined due to the lack of tissue samples for the genus (Nagl *et al.* 2001, Klett and Meyer 2002, Dunz and Schlieven 2013). Most recently, Schwarzer *et al.* (2016) assessed genetic and phenotypic diversity among populations of *Iranocichla* across its range in Iran, and although *Danakilia* was included among outgroup taxa, the study was unable to unambiguously resolve interrelationships among the oreochromines (*sensu* Dunz and Schlieven 2013) in their sample.

Biogeography and taxonomy of Aphanius

Across its range, *A. dispar* shows marked morphological variation (Wildekamp 1993, Reichenbacher *et al.* 2009, Teimori *et al.* 2012). Results from a previous mtDNA-based study indicate that *A. dispar* is not monophyletic and cannot be considered a single species under the phylogenetic species concept (Hrbek and Meyer 2003).

Current knowledge of *A. stiassnyae* is limited to the type series composed of two females only (Getahun and Lazara 2001). The species description compared morphological and meristic characters of *A. stiassnyae* to a syntopic population of *A. dispar* with which *A. stiassnyae* apparently did not hybridize, but no molecular analyses were performed.

Climate, hydrology and geological history of the Danakil Depression

An endorheic basin, the Danakil Depression (~10,000 km²) mostly lies below sea level, with the lowest point at ~120 m (Beyene and Abdelsalam 2005). Climatically, the area holds the world record for the highest average annual temperature ever recorded in an inhabited place (34.7 °C in Dallol, from 1960 to 1966: Pedgley 1967), and the region is characterized by low and irregular precipitation. Temporary brackish rivers ending in alluvial fans converge towards the center of the depression. These rivers are fed by the rains falling seasonally on the Eritrean-Ethiopian Plateau and along the coastal Red Sea highlands. Moreover, some brackish or hypersaline lakes, mostly fed by groundwater, are present in the area at various elevations below sea level: Lake Abaeded (~90 m), Lake Āsāle (~118 m), Lake Bakili (~120 m) and Lake Afrera (~111 m).

The Danakil Depression is located in a geologically young region, the Afar Triangle, where extensive volcanic and telluric activity continues to occur (Rognon 1972, Tazieff *et al.* 1972). The Afar Triangle is located on a triple junction (R-R-R triple rift: Mohr 1978) at the separation of the Arabian, Nubian and Somali tectonic plates. While southern Afar lies at the northern extreme of the African Rift system, the central and northern portions (including the Danakil Depression) belong to the Red Sea and Gulf of Aden rifts, where new crust is generated (Gasse and Street 1978).

The Danakil rift began to form in the lower Miocene, 23-25 Mya (Barberi *et al.* 1972) and the Danakil Depression was an intermittent marine basin until Late Pleistocene.

Fossils from Buia (northern Danakil Depression) record the presence, around 1 Mya, of several markedly water dependent vertebrate taxa as well as taxa associated with semiarid environments, indicating a fluvio-deltaic and lacustrine environment with moist grassland habitats adjacent to persistent waters (Abbate *et al.* 1998, Ferretti *et al.* 2003, Delfino *et al.* 2004, Martinez-Navarro *et al.* 2004, Rook *et al.* 2013). Further ichthyological fossil remains, currently under investigation, point to the presence of an oreochromine cichlid in this paleoenvironment (G. Carnevale: pers. comm.), a particularly important finding in the light of the present research and more generally for the interpretation of cichlid historical biogeography in northeastern Africa.

Thick deposits of evaporites reaching a depth of at least 3 km (Bonatti *et al.* 1971) attest to formerly marine conditions and profoundly affect present-day geochemistry and ecology of the region. The Danakil basin separated from the Red Sea ~32,000 BP with the uplift of the Alid volcanic center (Lowenstern *et al.* 1999), an event that triggered the final desiccation of the marine basin, which lasted only ~4000 years (Bonatti *et al.* 1971).

The drying of the marine basin of internal Danakil coincided with a relatively short stage of dry climate in Northern Africa during the Last Glacial Maximum (LGM) event of ~20,000 BP (Gasse *et al.* 1990), when waters were trapped in the ice sheets in both hemispheres, and sea-level dropped to 120 m below present level (Rohling *et al.* 1998). Nevertheless, evidence from several desert areas in northern Africa suggest that an immense amount of fossil groundwater persisted in aquifers even during the most arid climatic phases, and during the current arid phase the Nubian Sandstone aquifer of eastern Sahara is estimated to contain upwards of 150,000 km³ of water. During the Early Holocene, steepening of the water table gradient and the removal of 120 m of hydrostatic pressure produced by the sea-level drop, is thought to have created groundwater fed springs on the exposed continental shelves. Local water abundance in an otherwise hyperarid environment facilitated the onset of "coastal oases" that became refugia for a wide-range of plants and animals, including hominids (Faure *et al.* 2002).

The next stage in the history of the region was studied by Gasse (1974) who reconstructed the

hydrologic and climatic history of the southern portion of the Danakil Depression during mid-Holocene by studying the fossil diatom flora of lacustrine deposits near Lake Afrera. During a wet phase starting at the beginning of Early Holocene, increased water availability from the aquifer and an increased contribution of runoff, account for higher levels of Lake Afrera. Lake depth changed from a few meters above the present level around 9800 BP, to ~12 m above between 8750-7800 BP, and reached more than 50 m above the present level around 7800 BP when, according to Gasse (1974), Lake Afrera doubled its present-day area occupying a surface of about 200 km². The lake rapidly shrank around 7300 BP.

Research aims

Given the dynamic geology and dramatic past climatic and hydrologic changes that have occurred in this region, we hypothesize that the phylogeography of Danakil fish assemblages were shaped by these recent extreme events, resulting in recurrent isolation and mixing. To investigate this hypothesis, we assessed genetic variation across populations of the four currently known species of *Danakilia* and *Aphanius* and their radiation and current distribution in the Danakil Depression.

Results are based on extensive sampling from throughout the Danakil Depression during three expeditions from December 2014 to January 2016. We sampled most known localities, as well as other waterbodies where fish presence was reported by local collaborators (Futsum Hagos, pers. comm.). We analyzed mitochondrial genetic variation across these samples in order to:

1. assess genetic diversity and infer the phylogeographic relationships within each genus;
2. test the effectiveness of a mitochondrial-based DNA approach to recover the observed morphological variability;
3. interpret the present distribution of genetic and morphological diversity in the light of the geological, paleontological and paleoclimatic knowledge of the Danakil Depression.

MATERIAL AND METHODS

Study area

Our study area encompassed most of the Danakil Depression of Eritrea and Ethiopia (northern Danakil Depression: Fig. 1) of northeastern Africa, a desert region extending 300 km from the Gulf of Zula (15.266667°, 39.750000°) to Lake Afrera (13.26664°, 40.903355°).

The northern Danakil Depression is wedged between the Red Sea to the north, the Danakil Block of continental origin (maximum elevation <1400 m: Mohr 1978) to the east, the Eritrean-Ethiopian Plateau, also of continental origin, to the west, and merges to the south with the south-western and

east-central Afar region (Beyene and Abdelsalam 2005).

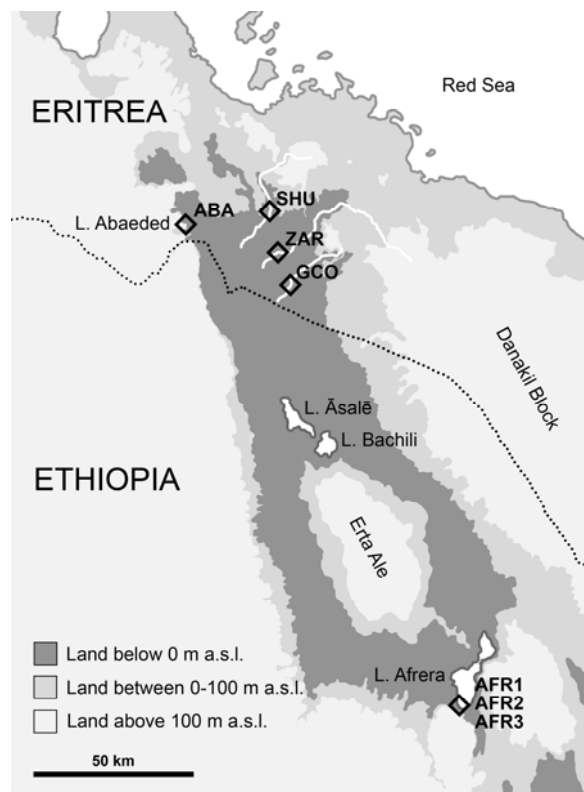


Figure 1. Study area indicating collection sites: Lake Abaeded (ABA), river Shukoray (SHU), river Zariga (ZAR), river Gali Colluli (GCO), Afreera “spring 3” (AFR1), Afreera “spring 8” (AFR2), Lake Afreera (AFR3). Major geographical landmarks are also shown. International borders in the map are not authoritative.

Sampling

Specimens were collected during three expeditions (2014-2016) organized within the framework of collaborations among the University of Pavia (Italy), the American Museum of Natural History (USA), the Ministry of Marine Resources of Eritrea, and the Ethiopian Biodiversity Institute.

Three new geographically separated populations of *Danakilia* from three ephemeral internal drainage rivers were discovered during these expeditions (Table 1). These new populations can be easily discriminated from *D. dinicolai* and *D. franchettii* by male breeding coloration that is rather uniform in the three rivers and is substantially different from the lacustrine populations, according to both field and aquarium observations (Fig. 2).

Six populations of *Danakilia* (5 specimens *per* population) were sampled from the ephemeral rivers Shukoray (SHU), Zariga (ZAR), and Gali Colluli (GCO) in the northeastern portion of the study area, in crater lake Abaeded (ABA) in the northwestern part of the study area, and in two springs flowing into Lake Afreera in the southernmost portion of the genus range: Afreera “spring 3” (AFR1)

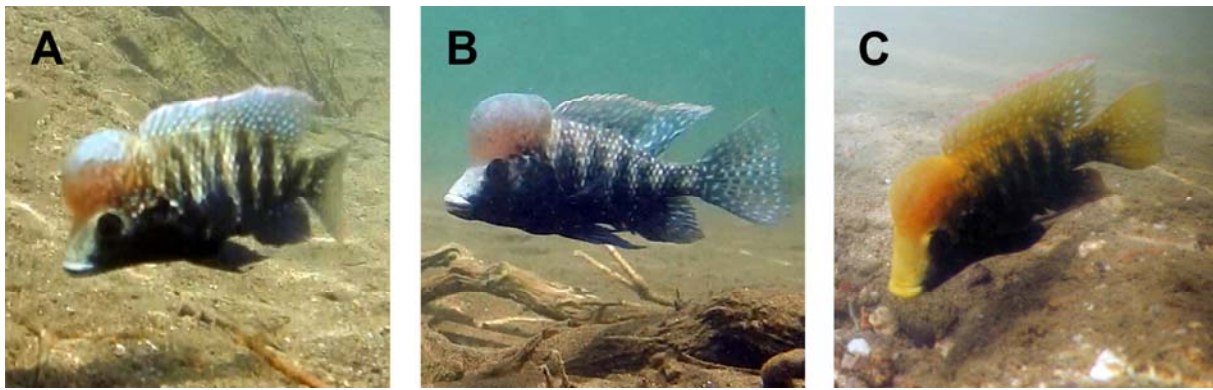


Figure 2. *Danakilia* morphospecies. From left to right, males in nuptial coloration: *Danakilia franchettii*, Lake Afrera springs (A); *Danakilia dinicolai*, Lake Abaeded (B); *Danakilia* sp., Shukoray, Zariga and Gali Colluli rivers (C). All riverine populations exhibit similar male nuptial coloration, as verified in aquarium-bred individuals. Images cropped from underwater videos recorded in the field in similar light conditions and water depths. *D. franchettii* is here shown in life and color for the first time since its discovery in 1929.

and Afrera “spring 8” (AFR2) (Fig. 1, Table 1). Specimens were collected by seine and hand net and kept in plastic buckets containing water drawn from capture sites. Frequent water changes were performed, each specimen was photographed and fin samples were collected using sterilized tools. Fin clips were immediately preserved in 95% ethyl alcohol, and kept at -20 °C until genetic analysis. Voucher specimens of the Eritrean populations were euthanized with clove oil (Erdmann 1999) and, after death, were fixed in 10% formalin for 5 days, then rinsed in water for 2-3 days before transfer to 70% ethanol for final preservation. Because of legal restrictions in Ethiopia, no voucher specimens of *D. franchettii* were preserved, but photographs were taken of each specimen next to a scale. To maintain good condition after sampling, specimens were retained in clean water for 15-25 minutes prior to release in the collection site.

Similarly, 27 *Aphanius* were sampled at the same 6 localities (from a minimum of 2 to a maximum of 5 samples *per* population, Table 1), and ascribed to *Aphanius* cf. *dispar* according to the current taxonomic knowledge (Hrbek and Meyer 2003), and a population of *A. stiassnyae* (a species also protected by the Ethiopian law) was fin-sampled in Lake Afrera (AFR3) (Fig. 1).

Water temperature (°C), conductivity (mS/cm) and pH were measured at each location with a portable meter (WTW Multi 340i™). Dissolved oxygen (mg/l) was measured either with the portable meter or, when not available, with a JBL™ O₂ aquarium test.

Water samples were collected in PVC bottles wrapped in aluminum foil for light protection, then

N°progr	Sample	Genus	Morphosp	Site	Map	Latitude	Longitude	COI	Accn ¹ _{COI}	cytb	Accn ¹ _{cytb}	COI+cytb
1	DANA1-1	Danakilia	sp.	Shukoray river	SHU	14.62488	40.33170	D1x	MG013816	D1b	MG013876	D1xb
2	DANA2-1	Danakilia	sp.	Shukoray river	SHU	14.62488	40.33170	D1x	MG013817	D2b	MG013877	D2xb
3	DANA3-1	Danakilia	sp.	Shukoray river	SHU	14.62488	40.32170	D1x	MG013818	D2b	MG013878	D2xb
4	DANA4-1	Danakilia	sp.	Shukoray river	SHU	14.62488	40.32170	D1x	MG013819	D2b	MG013879	D2xb
5	DANA5-1	Danakilia	sp.	Shukoray river	SHU	14.62488	40.32170	D1x	MG013820	D2b	MG013880	D2xb
6	DANA1-2	Danakilia	sp.	Zariga river	ZAR	14.49276	40.33669	D1x	MG013821	D2b	MG013881	D2xb
7	DANA2-2	Danakilia	sp.	Zariga river	ZAR	14.49276	40.33669	D1x	MG013822	D2b	MG013882	D2xb
8	DANA3-2	Danakilia	sp.	Zariga river	ZAR	14.49276	40.33669	D1x	MG013823	D2b	MG013883	D2xb
9	DANA4-2	Danakilia	sp.	Zariga river	ZAR	14.49276	40.33669	D1x	MG013824	D2b	MG013884	D2xb
10	DANA5-2	Danakilia	sp.	Zariga river	ZAR	14.49276	40.33669	D1x	MG013825	D2b	MG013885	D2xb
11	DANA1-3	Danakilia	sp.	Gall Coliuli river	GCO	14.40216	40.38466	D1x	MG013826	D2b	MG013886	D2xb
12	DANA2-3	Danakilia	sp.	Gall Coliuli river	GCO	14.40216	40.38466	D1x	MG013827	D2b	MG013887	D2xb
13	DANA3-3	Danakilia	sp.	Gall Coliuli river	GCO	14.40216	40.38466	D1x	MG013828	D2b	MG013888	D2xb
14	DANA4-3	Danakilia	sp.	Gall Coliuli river	GCO	14.40216	40.38466	D1x	MG013829	D2b	MG013889	D2xb
15	DANA5-3	Danakilia	sp.	Gall Coliuli river	GCO	14.40216	40.38466	D1x	MG013830	D2b	MG013890	D2xb
16	DANA1-4	Danakilia	<i>ditricolal</i>	Lake Abaeded	ABA	14.58901	40.08428	D1x	MG013831	D2b	MG013891	D2xb
17	DANA2-4	Danakilia	<i>ditricolal</i>	Lake Abaeded	ABA	14.58901	40.08428	D1x	MG013832	D2b	MG013892	D2xb
18	DANA3-4	Danakilia	<i>ditricolal</i>	Lake Abaeded	ABA	14.58901	40.08428	D2x	MG013833	D2b	MG013893	D3xb
19	DANA4-4	Danakilia	<i>ditricolal</i>	Lake Abaeded	ABA	14.58901	40.08428	D2x	MG013834	D2b	MG013894	D3xb
20	DANA5-4	Danakilia	<i>ditricolal</i>	Lake Abaeded	ABA	14.58901	40.08428	D2x	MG013835	D2b	MG013895	D3xb
21	DANA1-5	Danakilia	<i>franchetii</i>	Athera spring "3"	AFR1	13.22551	40.87514	D3x	MG013836	D2b	MG013896	D4xb
22	DANA2-5	Danakilia	<i>franchetii</i>	Athera spring "3"	AFR1	13.22551	40.87514	D3x	MG013837	D3b	MG013897	D5xb
23	DANA3-5	Danakilia	<i>franchetii</i>	Athera spring "3"	AFR1	13.22551	40.87514	D2x	MG013838	D3b	MG013898	D6xb
24	DANA4-5	Danakilia	<i>franchetii</i>	Athera spring "3"	AFR1	13.22551	40.87514	D2x	MG013839	D4b	MG013899	D7xb
25	DANA5-5	Danakilia	<i>franchetii</i>	Athera spring "3"	AFR1	13.22551	40.87514	D2x	MG013840	D4b	MG013900	D7xb
26	DANA1-6	Danakilia	<i>franchetii</i>	Athera spring "8"	AFR2	13.18258	40.89041	D2x	MG013841	D4b	MG013901	D7xb
27	DANA2-6	Danakilia	<i>franchetii</i>	Athera spring "8"	AFR2	13.18258	40.89041	D2x	MG013842	D4b	MG013902	D7xb
28	DANA3-6	Danakilia	<i>franchetii</i>	Athera spring "8"	AFR2	13.18258	40.89041	D4x	MG013843	D4b	MG013903	D8xb
29	DANA4-6	Danakilia	<i>franchetii</i>	Athera spring "8"	AFR2	13.18258	40.89041	D4x	MG013844	D4b	MG013904	D8xb
30	DANA5-6	Danakilia	<i>franchetii</i>	Athera spring "8"	AFR2	13.18258	40.89041	D2x	MG013845	D5b	MG013905	D9xb
31	APHA1_1	Aphanis	ct. dispar	Shukoray river	SHU	14.62488	40.33170	A1x	MG013846	A1b	MG013906	A1xb
32	APHA2_1	Aphanis	ct. dispar	Shukoray river	SHU	14.62488	40.33170	A1x	MG013847	A1b	MG013907	A1xb
33	APHA3-1	Aphanis	ct. dispar	Shukoray river	SHU	14.62488	40.33170	A1x	MG013848	A1b	MG013908	A1xb
34	APHA1-2	Aphanis	ct. dispar	Zariga river	ZAR	14.49276	40.33669	A7x	MG013849	A1b	MG013909	A7xb
35	APHA2-2	Aphanis	ct. dispar	Zariga river	ZAR	14.49276	40.33669	A7x	MG013850	A1b	MG013910	A7xb
36	APHA3-2	Aphanis	ct. dispar	Zariga river	ZAR	14.49276	40.33669	A8x	MG013851	A1b	MG013911	A8xb
37	APHA4-2	Aphanis	ct. dispar	Zariga river	ZAR	14.49276	40.33669	A9x	MG013852	A1b	MG013912	A9xb
38	APHA5-2	Aphanis	ct. dispar	Zariga river	ZAR	14.49276	40.33669	A7x	MG013853	A1b	MG013913	A7xb
39	APHA1-3	Aphanis	ct. dispar	Gall Coliuli river	GCO	14.40216	40.38466	A2x	MG013854	A1b	MG013914	A2xb
40	APHA2-3	Aphanis	ct. dispar	Gall Coliuli river	GCO	14.40216	40.38466	A3x	MG013855	A1b	MG013915	A3xb
41	APHA3-3	Aphanis	ct. dispar	Gall Coliuli river	GCO	14.40216	40.38466	A4x	MG013856	A1b	MG013916	A4xb
42	APHA4-3	Aphanis	ct. dispar	Gall Coliuli river	GCO	14.40216	40.38466	A5x	MG013857	A1b	MG013917	A5xb
43	APHA5-3	Aphanis	ct. dispar	Gall Coliuli river	GCO	14.40216	40.38466	A6x	MG013858	A1b	MG013918	A6xb
44	APHA1-4	Aphanis	ct. dispar	Lake Abaeded	ABA	14.58901	40.08428	A10x	MG013859	A2b	MG013919	A10xb
45	APHA2-4	Aphanis	ct. dispar	Lake Abaeded	ABA	14.58901	40.08428	A10x	MG013860	A2b	MG013920	A10xb
46	APHA3-4	Aphanis	ct. dispar	Lake Abaeded	ABA	14.58901	40.08428	A10x	MG013861	A2b	MG013921	A10xb
47	APHA4-4	Aphanis	ct. dispar	Lake Abaeded	ABA	14.58901	40.08428	A10x	MG013862	A3b	MG013922	A11xb
48	APHA5-4	Aphanis	ct. dispar	Lake Abaeded	ABA	14.58901	40.08428	A10x	MG013863	A2b	MG013923	A10xb
49	APHA1-5	Aphanis	ct. dispar	Athera spring "3"	AFR1	13.22551	40.87514	A7x	MG013864	A1b	MG013924	A7xb
50	APHA2-5	Aphanis	ct. dispar	Athera spring "3"	AFR1	13.22551	40.87514	A11x	MG013865	A1b	MG013925	A12xb
51	APHA1-6	Aphanis	ct. dispar	Athera spring "8"	AFR2	13.18258	40.89041	A12x	MG013866	A1b	MG013926	A13xb
52	APHA2-6	Aphanis	ct. dispar	Athera spring "8"	AFR2	13.18258	40.89041	A13x	MG013867	A4b	MG013927	A14xb
53	APHA1-7	Aphanis	<i>stassnyae</i>	Lake Athera	AFR3	13.22092	40.87407	A14x	MG013868	A5b	MG013928	A15xb
54	APHA2-7	Aphanis	<i>stassnyae</i>	Lake Athera	AFR3	13.22092	40.87407	A15x	MG013869	A6b	MG013929	A16xb
55	APHA3-7	Aphanis	<i>stassnyae</i>	Lake Athera	AFR3	13.22092	40.87407	A16x	MG013870	A7b	MG013930	A17xb
56	APHA4-7	Aphanis	<i>stassnyae</i>	Lake Athera	AFR3	13.22092	40.87407	A11x	MG013871	A1b	MG013931	A12xb
57	APHA5-7	Aphanis	<i>stassnyae</i>	Lake Athera	AFR3	13.22092	40.87407	A14x	MG013872	A5b	MG013932	A15xb
Outgroups												
58	-	<i>Oreochromis niloticus</i>	<i>niloticus</i>	Myanmar	-	-	-	O1x	LC189947	O1b	AB018989	O1xb
59	-	<i>Oreochromis niloticus</i>	<i>niloticus</i>	Egypt	-	-	-	O1x	LC189947	O2b	GU477625	O2xb
60	Aph_dis1	Aphanis	<i>dispar</i> s.s.	Egypt (Sinai)	-	28.18284	34.44224	A17x	MG013873	A8b	MG013933	A18xb
62	Aph_dis2	Aphanis	<i>dispar</i> s.s.	Egypt (Sinai)	-	28.18284	34.44224	A18x	MG013874	A9b	MG013934	A19xb
63	Aph_dis3	Aphanis	<i>dispar</i> s.s.	Egypt (Sinai)	-	28.18284	34.44224	A19x	MG013875	A10b	MG013935	A20xb

Table 1. Samples of *Danakilia* and *Aphanis* and outgroups. The table includes sequential number, sample code, ascribed morphological species (when available), sampling sites (including map codes and geographic coordinates), GenBank accession numbers and haplotype codes for both COI, cytb fragment and concatenated genes.

temporarily stored in the shade at ambient temperature until final preservation in fridge (4 °C) for subsequent laboratory analyses that provided values for salinity (g/l), ionic composition (ppm) and carbonate hardness (°F). Water quality results are summarized in Table S1.

Choice of outgroups

Danakilia: To investigate phylogenetic relationships within *Danakilia*, we included 2 sequences of

Oreochromis niloticus as outgroup. Based on the recent study of Schwarzer *et al.* (2016) this taxon is an African and Middle-Eastern representative of the tribe Oreochromini to which *Danakilia* belongs (see Table 1 for accession numbers and references).

Aphanius: To assess genetic differentiation between *A. cf. dispar* and *A. stiassnyae* populations from our study area and recognized samples of *A. dispar* (Hrbek and Meyer 2003) we obtained 3 ethanol-preserved specimens of *A. dispar sensu stricto* from the southeastern coast of the Sinai Peninsula (Gulf of Aqaba, Egypt, Table 1). Von Rüppell described *A. dispar* from unspecified localities in the Red Sea region, but did not designate a holotype (Rüppell 1828-1830). Subsequently, Villwock *et al.* (1983) selected one of Rüppell's specimens (SMF 821), as the lectotype reporting "Red Sea" as its collection locality. More detailed geographical information is given in Rüppell's text stating that the species is found "in all areas of the Red Sea" and "also in the Red Sea thermal fresh water source of Hadjer Elme near Tor", an Egyptian locality now corresponding to the harbor town of El Tûr (Sinai Peninsula, Gulf of Suez) and situated ~80 km from our specimen locality.

Laboratory protocol

Total genomic DNA was extracted using the GenUP™ Tissue DNA Kit (biotechrabbit GmbH, Hennigsdorf, Germany) following manufacturer's instructions. As our primary aim was to describe molecular variation of desert fish populations inhabiting the Danakil Depression, and genetically characterize their morphologically diverse phenotypes, we selected two mitochondrial markers, both of which have been widely used to elucidate relationships among populations of vertebrates and particularly fish. In order to check the current morphology-based taxonomy and describe the pattern of genetic variation of the three newly discovered riverine populations, the "barcoding" fragment of the cytochrome *c* oxidase subunit I (*COI*) gene was chosen for investigating species-level identification. Universal primers LCO1490 and HCO2918 (Folmer *et al.* 1994) were used to amplify approximately 600 bp of *COI* in a 20 µl reaction volume containing 10X PCR buffer with 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 0.2 µM of each primer and 0.5 U of Hot Start *Taq* DNA Polymerase (biotechrabbit GmbH). The thermal profile consisted of 60 s initial denaturing cycle at 94°C, followed by 5 cycles of denaturation at 94°C (60 s), annealing at 45°C (90 s) and extension at 72°C (90 s), 35 cycles of 94°C (60 s), 50°C (90 s) and 72°C (60 s), and final extension of 72°C for 7 min. In addition, a ~400bp fragment of the mitochondrial gene encoding for cytochrome *b* (*cytb*) protein was selected. The latter has frequently been used in identification of genetic discontinuity among distinct intra-specific lineages resulting from alternative colonization patterns, and the recognition of spatially distinct haplogroups in vertebrates (e.g. Johns and Avise 1998, Zardoya and Doadrio 1999, Galván-Quesada *et al.* 2016). Amplifications were performed as for *COI* but using the universal

primer pair L14725-H15149 (Kocher *et al.* 1989), and a thermal profile involving a 3 min initial denaturing cycle at 94°C and 30 cycles of denaturation at 94°C (30 s), annealing at 47°C (30 s) and extension at 72°C (30 s) with a final extension at 72°C (7 min). Sequencing was carried out for both markers on an ABI 155 3730 XL (Macrogen Europe Inc., Amsterdam, The Netherlands), using the same amplification primers. According to standard “barcoding” procedures, *COI* fragments were sequenced in both directions.

Sequences were corrected by visual inspection of automated sequencer chromatograms in BIOEDIT 7.1 (Hall 1999). Codons were translated into amino acid sequences to check for the presence of nuclear pseudogenes using MEGA 6 (Tamura *et al.* 2013). To avoid the inclusion of *COI* nuclear pseudogenes of mitochondrial origin (i.e. NUMTs, Bensasson *et al.* 2003) we also followed the guidelines proposed in Song *et al.* (2008) and Buhay (2009).

Mitochondrial variation and genetic distance estimation

Identical sequences were collapsed into unique haplotypes using the online webtool Fabox 1.41 (available at <http://users-birc.au.dk/biopv/php/fabox/>). Genetic distances were estimated for each marker (*COI*, *cytb*) and genus (*Danakilia*, *Aphanius*) to infer molecular variation between and within populations, morphospecies, and clusters identified by phylogenetic analyses. For *COI*, genetic distances were estimated with Kimura-2-parameter (K2P) correction, whereas standard *p*-distance was applied for *cytb*. We assumed the 2% sequence divergence as a consensus threshold for *COI* to identify putative species within our datasets following a standard “barcoding” approach (Hebert *et al.* 2003). Noteworthy, this threshold was shown to be a reliable way to delineate distinct species within freshwater fish assemblages of the Mediterranean Biodiversity Hotspot (Geiger *et al.* 2014). All analyses were performed in MEGA 6 (Tamura *et al.* 2013).

Phylogenetic inference

Phylogenetic analyses using Bayesian Inference (BI) were performed on concatenated *COI+cytb* alignment and selected outgroups as described above. Partitioned phylogenetic analyses were run using the best-partitioned scheme suggested for the concatenated dataset by PartitionFinder 1.1.0 (Lanfear *et al.* 2012). An input configuration file was created containing 6 partitions, corresponding to individual codon positions for the two mitochondrial fragments, which was the most finely partitioned scheme possible for our dataset. We used the “greedy” algorithm (heuristic search) with branch lengths estimated as unlinked to search for the best-fit scheme. To run Bayesian analysis, a total of 24 a priori schemes with varying degrees of complexity and the best-fit and the worst-fit schemes were statistically compared in PartitionFinder using the BIC. Bayesian analyses were

performed in MrBayes 3.2 (Ronquist *et al.* 2012) sampling two runs and four chains for each run for 5×10^6 generations (started on random trees) and four incrementally heated Markov chains (using default heating values), and sampling every 100th tree. The first 20% of generations were discarded as burn-in after checking tracer plots in Tracer 1.5 (Rambaut and Drummond 2007). Convergence of chains, upon a stationary distribution, was checked also by monitoring the standard deviation of split frequencies (= 0.0034) and the Potential Scale Reduction factor (PSRF=1.000). Resulting phylograms and posterior probabilities were visualized in FIGTREE 1.3 (Rambaut 2009). Nodes were considered strongly supported if posterior probability values were ≥ 0.95 (Wilcox *et al.* 2002, Huelsenbeck and Rannala 2004).

Population genetic structure of Danakil Depression fish

Evolutionary relationships among *Danakilia* and *Aphanius* lineages were visualized by haplotype network analysis using TCS 1.21 (Clement *et al.* 2000). A parsimony criterion following the algorithm by Templeton *et al.* (1992) was used to estimate the number of mutational steps by which pairwise haplotypes differ, assessing also the minimum number of connections required to join all haplotypes in a single gene network.

Genetic diversity indexes (haplotypes diversity, Hd , nucleotide diversity, π , and average number of nucleotide differences, k) were computed at different hierarchical levels (within populations, morphospecies, and spatially distinct haplogroups, the latter identified by phylogenetic and haplotype network reconstructions) using DNAsp5 (Librado and Rozas 2009). Geographic structuring of populations was assessed by means of Spatial Analysis of Molecular Variance (SAMOVA) using the software SAMOVA 2.0 (Dupanloup *et al.* 2002). This analysis is based on a simulated annealing procedure that defines k continuously homogeneous but genetically differentiated populations, where the proportion of total genetic variance (F_{CT}) is maximized. In addition, we performed Mantel tests to investigate the possible pattern of isolation by distance among populations, using the total dataset and groups accounting for the geographic clustering of samples in both *Danakilia* and *Aphanius* (see Results). This method was used to estimate possible correlations between genetic distances (pairwise F_{ST}) estimated using Arlequin 3.11 (Excoffier *et al.* 2005) with 1000 permutations and geographic distances computed using GenAlEx 6.5 (Peakall and Smouse 2012) directly from sampling localities coordinates. Using this method, we evaluated the extent of isolation across different scales, from local to regional.

GIS modeling

Paleontological findings document a substantial water level rise of up to 55 m which inundated the

area of Lake Afrera during the mid-Holocene (Gasse 1974), and resulted in a lake water surface elevation between -50 and -40 m (Gasse and Street 1978). To investigate how these rising of lake Afrera levels would have affected the rest of the Danakil Depression, we extended the model to the entire region by defining the extension of the paleo-lake through a Digital Elevation Model (DEM) of the study area. In particular, we applied a spatial resolution of 30 m building all the contour lines with a 5m interval and, considering the most conservative estimate by Gasse and Street (1978), we selected the isolines equal to or lower than -50 m. All the spatial analyses were performed by means of a GIS platform (QGIS v2.14.12 Essen).

RESULTS

We obtained >1000 bp of mitochondrial sequence data across sampled *Danakilia* and *Aphanius*. No gaps, stop codons or ambiguous positions were detected when sequences were translated into amino acids (see Table 1 for accession numbers). Overall, genetic results indicate disparate patterns of variation in the two desert fish genera, with higher variability in *Aphanius* compared to *Danakilia*.

Genetic variation in sampled mtDNA markers

Within *Danakilia*, four closely related *COI* haplotypes were found (D1x-D4x, Table 1). Average genetic distance estimated between populations currently ascribed to different species (*D. dinicolai* and *D. franchetti*) was estimated at 0.1%, far below the standard “barcoding” threshold of 2% for vertebrates (Table 2). *Aphanius* were considerably more variable in *COI* sequences returning 16 distinct *COI* haplotypes (A1x-A16x, Table 1). No haplotype sharing was detected between rivers (haplotypes A1x-A9x), and all the samples from Lake Abaeded share a single and private *COI* haplotype (A10x). The Lake Afrera (AFR3) population share only haplotype A11x with AFR1, while no haplotype sharing was detected with AFR2 (Table 1). The most striking difference was the differentiation of SHU from all the others, ranging from 2.2% (with GCO) to 3.4% (with ABA and AFR1, Table 2a). It is notable that the observed variation in this sample was comparable to the differentiation of Danakil *Aphanius* from the *A. dispar* outgroup sampled from Sinai (average distance=3.7%, see Table 2b).

Five distinct *cytb* haplotypes were found in *Danakilia* populations, D3b, D4b and D5b being private to the Lake Afrera populations (Table 1). Within *Aphanius*, six *cytb* haplotypes were recovered, A1b being common to all populations except ABA (Lake Abaeded), whereas haplotypes A2b-A6b were private to single populations/species (Table 1). According to this marker, *Danakilia* populations appear more diversified according to spatial distance between them (Table 3a), whereas differentiation among *Aphanius* populations was less striking than for *COI*. High intra-population

variation was recovered within AFR2 (2.5%), and overall Danakil *Aphanius* appeared deeply divergent from the outgroup, *A. dispar s.s.* ($\geq 4\%$, see Table 3b).

a) *Danakilia*

pop	SHU	GCO	ZAR	ABA	AFR1	AFR2
SHU	<i>n/c</i>					
GCO	0	<i>n/c</i>				
ZAR	0	0	<i>n/c</i>			
ABA	0.001	0.001	0.001	<i>0.001</i>		
AFR1	0.002	0.002	0.002	0.002	<i>0.001</i>	
AFR2	0.002	0.002	0.002	0.002	0.002	<i>0.001</i>

sp	[r]	[d]	[f]
Rivers [r]		<i>0.002</i>	
<i>D. dimicolai</i> [d]		0.002	<i>0.001</i>
<i>D. franchettii</i> [f]		0.003	0.001 <i>n/c</i>

b) *Aphanius*

pop	SHU	GCO	ZAR	ABA	AFR1	AFR2	AFR3	[DIS]
SHU	<i>n/c</i>							
GCO	0.022	<i>0.014</i>						
ZAR	0.027	0.012	<i>0.013</i>					
ABA	0.034	0.015	0.013	<i>n/c</i>				
AFR1	0.034	0.011	0.007	0.009	<i>0.002</i>			
AFR2	0.033	0.012	0.009	0.008	0.004	<i>0.006</i>		
AFR3	0.033	0.013	0.011	0.008	0.006	0.006	<i>0.006</i>	
<i>A. dispar s.s.</i> Sinai [DIS]	0.025	0.033	0.036	0.042	0.041	0.040	0.040	<i>0.003</i>

sp	[r]	[d]	[s]	[st]
Rivers [r]		<i>0.015</i>		
<i>Aphanius</i> "Abaeded" [d]		0.016	<i>n/c</i>	
<i>Aphanius</i> "springs" [s]		0.013	0.008	<i>0.004</i>
<i>A. stiassnyae</i> [st]		0.014	0.006	0.006 <i>0.006</i>

Table 2. Genetic distances estimated for *COI* with K2P correction. Distances between/within populations (pop) and morphospecies (sp) of *Danakilia* (a) and *Aphanius* (b) are reported below/along the diagonal, respectively. For *Aphanius* the genetic distances were estimated with reference to the outgroup (*A. dispar s.s.* from Sinai Peninsula, in bold).

Resolution of phylogenetic relationships within fish populations

We used concatenated *COI*+*cytb* alignments in phylogenetic analyses, corresponding to 1014 bp and 1029 bp (for *Danakilia* and *Aphanius*, respectively). The best partition scheme for concatenated alignments as estimated using Partitofinder, was (*COI_pos1*, *cytb_pos1*) (*COI_pos2*, *cytb_pos2*) (*COI_pos3*) (*cytb_pos3*) for *Danakilia*, while only three partitions were implemented for *Aphanius* (*COI_pos1*, *cytb_pos1*) (*COI_pos2*, *cytb_pos2*) (*COI_pos3*, *cytb_pos3*) (refer to Table S2 for all evolutionary models). Within *Danakilia*, overall phylogenetic resolution was relatively weak, as

expected from the low mtDNA variation detected. The two Afrera spring populations (hereafter referred as the “southern cluster”) group together in a moderately supported clade (bpp=0.80), while all other populations group together as a “northern cluster” although only a strongly supported relationship among riverine lineages was recovered (bpp=0.94) (Fig. 3).

a) *Danakilia*

pop	SHU	GCO	ZAR	ABA	AFR1	AFR2
SHU	0.003					
GCO	0.001	<i>n/c</i>				
ZAR	0.001	0	<i>n/c</i>			
ABA	0.001	0	0	<i>n/c</i>		
AFR1	0.006	0.005	0.005	0.005	0.005	
AFR2	0.010	0.008	0.008	0.008	0.005	0.005

sp	[r]	[d]	[f]
Rivers [r]		0.007	
<i>D. dimicolai</i> [d]		0.001	<i>n/c</i>
<i>D. franchettii</i> [f]		0.008	0.006 0.003

b) *Aphanius*

pop	SHU	GCO	ZAR	ABA	AFR1	AFR2	AFR3	[DIS]
SHU	<i>n/c</i>							
GCO	0	<i>n/c</i>						
ZAR	0	0	<i>n/c</i>					
ABA	0.006	0.006	0.006	0.002				
AFR1	0	0	0	0.006	<i>n/c</i>			
AFR2	0.012	0.012	0.012	0.015	0.012	0.025		
AFR3	0.015	0.015	0.015	0.017	0.015	0.014	0.013	
<i>A. dispar s.s.</i> Sinai [DIS]	0.040	0.040	0.040	0.041	0.040	0.045	0.045	0.003

sp	[r]	[d]	[s]	[st]
Rivers [r]		<i>n/c</i>		
<i>Aphanius</i> “Abaeded” [d]		0.006	0.002	
<i>Aphanius</i> “springs” [s]		0.012	0.025	0.025
<i>A. stiassnyae</i> [st]		0.015	0.017	0.014 0.013

Table 3. Genetic distances estimated for *cytb* with p-distance. Distances between/within populations (pop) and species (sp) of *Danakilia* (a) and *Aphanius* (b) are reported below/along the diagonal, respectively. For *Aphanius* the genetic distances were estimated with reference to the outgroup (*A. dispar s.s.* from the Sinai, in bold).

Stronger phylogenetic resolution was recovered for *Aphanius*. All Danakil individuals fell within a single clade, deeply divergent from specimens of *A. dispar s.s.* collected from the Sinai (Fig. 4). The Danakil *Aphanius* specimens clustered in several distinct clades representing recently derived lineages within the two lakes (Afrera/*A. stiassnyae* and Abaeded) and are supported by high posterior probabilities (bpp=1.00 for both). In contrast, riverine haplotypes (particularly SHU) represented deeply differentiated lineages with moderate support (Fig. 4b).

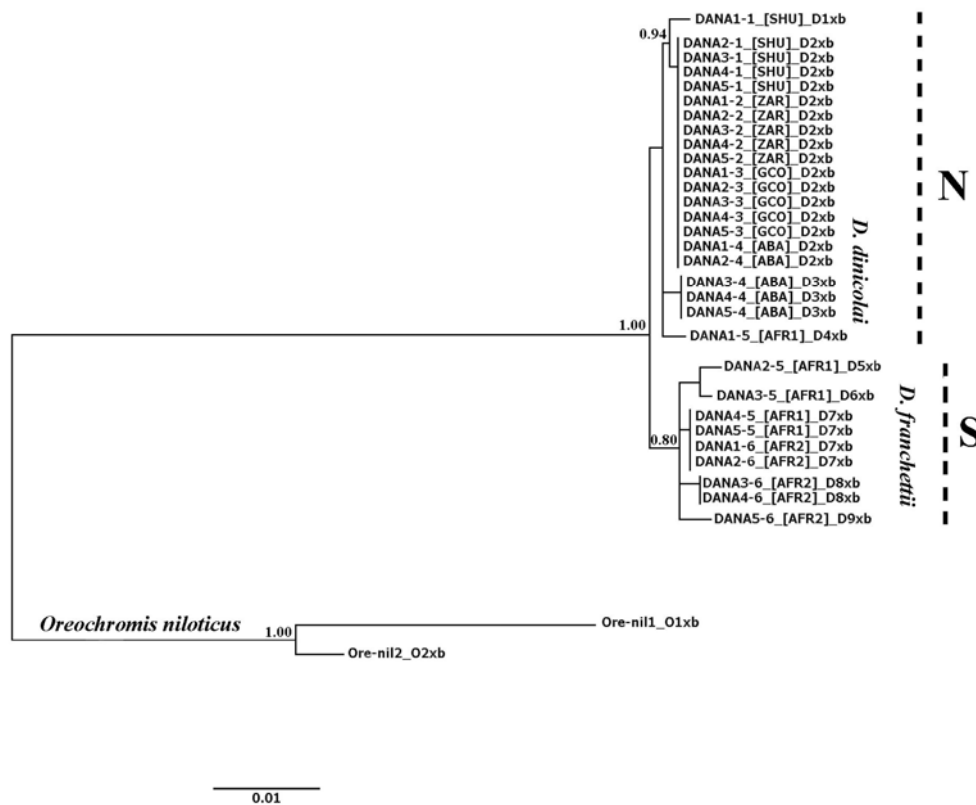


Figure 3. Bayesian phylogenetic trees for mitochondrial COI+cytb sequences of *Danakilia* populations. Numbers above nodes refer to Bayesian posterior probabilities. Mitochondrial clusters (dashed line) and clades (solid line) identifying main phylogenetic relationships among individuals are also shown.

mtDNA gene genealogies

***Danakilia*:** Haplotype network reconstructions based on the concatenated alignment suggest recent and rapid diversification (Fig. 5b). Indeed, only one or two mutational steps were required to join most haplotypes. Nonetheless, two closely related haplogroups could be detected in the concatenated network (95% connection limit fixed to 13 steps), one of mainly northern riverine samples (SHU, GCO, ZAR) and ABA (haplotypes D1xb-D3xb), and the other of southern haplotypes from Lake Afrera (haplotypes D5xb-D9xb). The only exception concerns haplotype D4xb (corresponding to sample DANA1-5 from AFR1, see Table 1) which differ from D3xb by a single mutation step) (see Fig. 5b).

***Aphanius*:** A single network joining all haplotypes (95% connection limit fixed to 14 steps) indicates more structured variation (Fig. 6b). Indeed, four haplogroups could be identified according to the parsimony criterion, when the 99% connection limit (5 steps) was applied: 1) one including deeply differentiated riverine haplotypes (A1xb, A4xb, A6xb, A8xb), 2) one including well differentiated haplotypes from Lake Afrera and its affluent creeks (A14xb-A17xb), 3) two private haplotypes from

Lake Abaeded (A10xb-A11xb) and 4) remaining haplotypes widespread across the entire Danakil region.

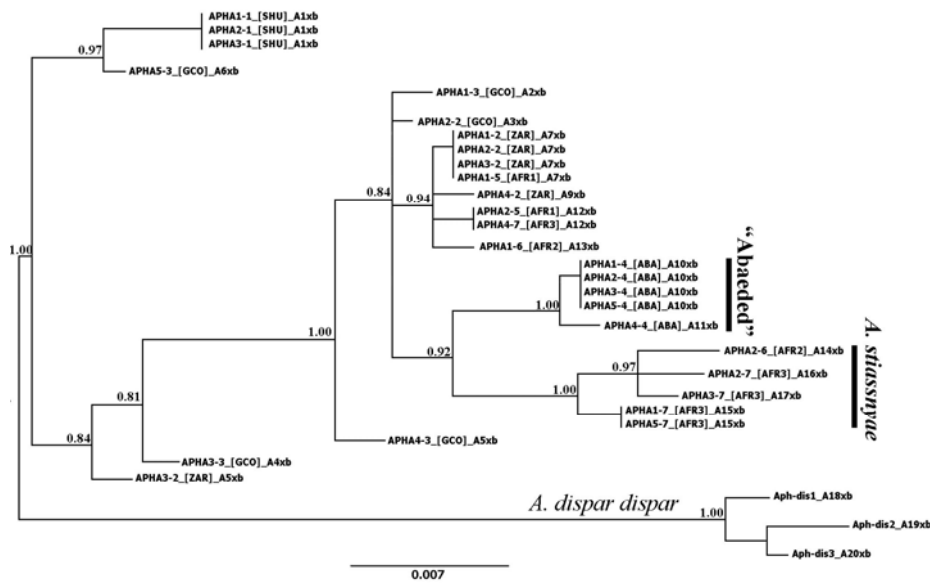


Figure 4. Bayesian phylogenetic trees for mitochondrial COI+cytb sequences of *Aphanius* of the Danakil Depression. Numbers above nodes refer to Bayesian posterior probabilities. Mitochondrial clusters (dashed line) and clades (solid line) identifying main phylogenetic relationships among individuals are also shown.

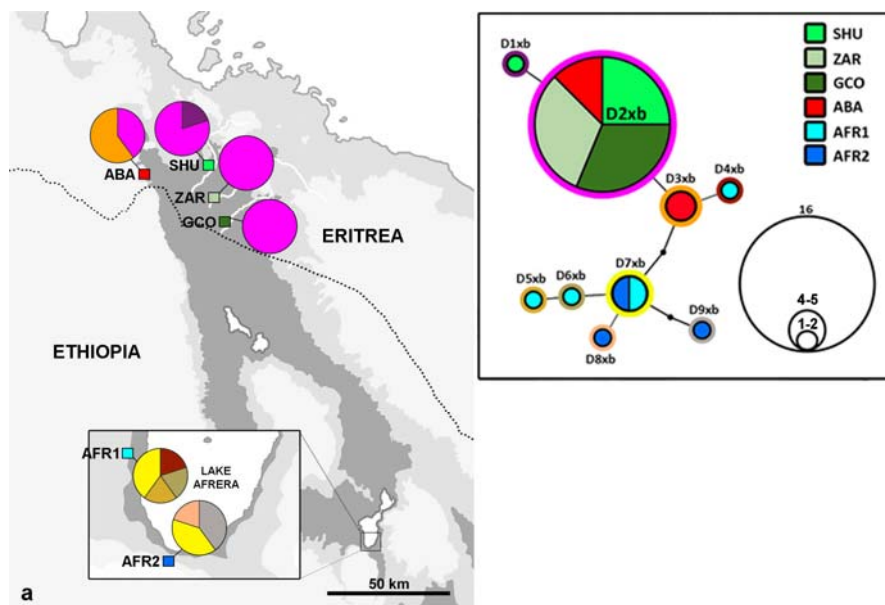


Figure 5. Spatial distribution of mitochondrial haplotypes found in *Danakilia* populations. Each color identifies a different haplotype. Diagrams (a) indicate the proportion of each haplotype in the populations. Haplotype networks are also illustrated in the insets (b): here colors refer to populations, while strokes refer to the haplotype (as indicated in the accompanying map). Size of circles are proportional to the number of individuals sharing the haplotype. International borders in the map are not authoritative.

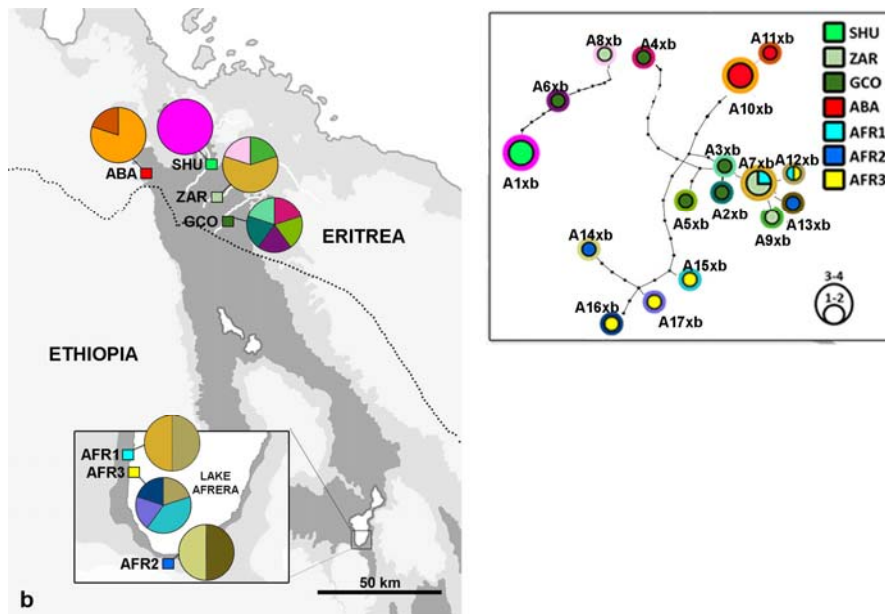


Figure 6. Spatial distribution of mitochondrial haplotypes found in *Aphanius* populations. Each color identifies a different haplotype. Diagrams (a) indicate the proportion of each haplotype in the populations. Haplotype networks are also illustrated in the insets (b): here colors refer to populations, while strokes refer to the haplotype (as indicated in the accompanying map). Size of circles are proportional to the number of individuals sharing the haplotype. International borders in the map are not authoritative.

Population genetic structure of Danakil Depression fish

Diversity indices show a difference between northern and southern lineages within *Danakilia*. Specifically, northern riverine populations appear homogeneous in term of mitochondrial variation, and only slight variation (i.e. 2 haplotypes) was recovered in populations SHU and ABA. Conversely, Afrera populations show higher haplotype diversity (> 0.800), but little population structure (Table 3a). A completely different pattern of genetic structuring was observed for *Aphanius*, and higher haplotype diversity values were recovered in both riverine and Afrera populations (reaching maximum value of 1.000 in GCO, AFR1, and AFR2). Additionally, nucleotide diversity was high in several populations reaching particularly high values in GCO (0.01375 ± 0.00336) and AFR2 (0.01361 ± 0.00996), as multiple divergent haplotypes were detected at these sites (Table 4b).

SAMOVA analysis for *Danakilia* was replicated for $k=2$ to 5 and variance between spatial groups was maximized at $k=4$ ($F_{CT}=0.76$), separating riverine populations (SHU, GCO, ZAR) vs. ABA vs. AFR1 vs. AFR2. These results indicate an unexpectedly high differentiation between the two geographically proximate Lake Afrera spring populations, which appears higher than the one observed between populations inhabiting different rivers in the north. Correlation between genetic and spatial distance (i.e. Mantel test) was high and significant ($r = 0.850$, $P < 0.05$). In contrast, no

clear geographic structure was recovered by SAMOVA for *Aphanius*, and the correlation between genetic and spatial distances was not significant (data not shown).

a) <i>Danakilia</i>							
Population/groups	N	N _k	S	H _d ±SD	π±SD	k	
SHU	5	2	1	0.400±0.237	0.00041±0.00024	0.400	
ZAR	5	1	0	0	0	0	
GCO	5	1	0	0	0	0	
<i>All rivers</i>	15	2	1	0.133±0.112	0.00014±0.00031	0.133	
<i>ABA/dinicolai</i>	5	2	1	0.600±0.175	0.00061±0.00049	0.600	
<i>"N lineage"</i>	20	3	2	0.353±0.123	0.00038±0.00057	0.368	
AFR1	5	4	4	0.900±0.161	0.00204±0.00059	2.000	
AFR2	5	3	3	0.800±0.164	0.00143±0.00049	1.400	
<i>"S lineage"/franchettii</i>	10	6	7	0.844±0.103	0.00190±0.00049	1.867	
<i>All Danakilia</i>	30	9	9	0.701±0.085	0.00200±0.00031	1.963	

b) <i>Aphanius</i>							
Population/groups	N	N _k	S	H _d ±SD	π±SD	K	
SHU	3	1	0	0	0	0	
ZAR	5	3	12	0.700±0.218	0.00766±0.00411	4.800	
GCO	5	5	16	1.000±0.126	0.01375±0.00336	8.600	
<i>All rivers</i>	13	9	21	0.923±0.057	0.00974±0.00117	10.026	
ABA	5	2	1	0.400±0.237	0.00039±0.00023	0.400	
AFR1	2	2	1	1.000±0.500	0.00097±0.00049	1.000	
AFR2	2	2	14	1.000±0.500	0.01361±0.00996	14.000	
AFR3	5	4	17	0.900±0.161	0.00719±0.00225	7.400	
<i>All Afrera</i>	9	7	21	0.944±0.070	0.00831±0.00101	8.556	
<i>All Aphanius</i>	27	17	43	0.952±0.023	0.01121±0.00135	11.538	

Table 4. Genetic diversity indexes calculated for *Danakilia* (a) and *Aphanius* (b) populations of the Danakil Depression. The table includes population codes and groupings delineated according to phylogenetic and phylogeographic analyses, number of samples belonging to each population or group (N), number of haplotypes belonging to each population or group (N_h), number of segregating sites (S), haplotype (H_d) and nucleotide (π) diversity values with standard deviations (±SD), and the mean numbers of substitutions (k).

Mid-Holocene flooding

Our DEM indicates that an increase of the water surface elevation of −50 to −40 m of Lake Afrera during mid-Holocene extrapolated to the entire Danakil Depression below the present-day −50 m contour, would result in a massive paleo-lake with a surface of 5720 km² (Fig. 7). This paleo-lake would have extended for about 200 km from present-day Badda swamp (14.67000°, 40.08000°) in the north, to the Mount Afdera area (13.08000°, 40.95000°) in the south.

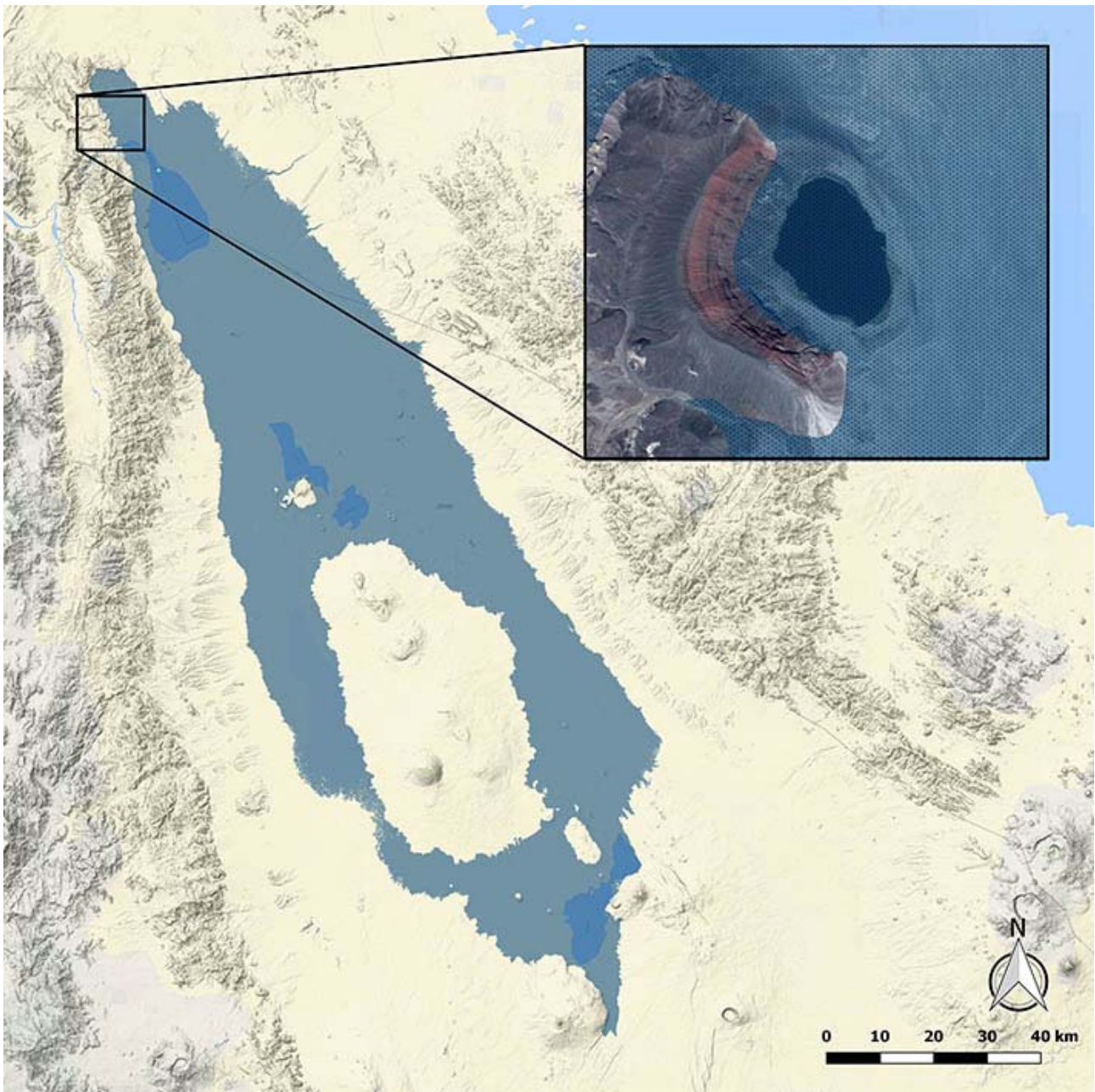


Figure 7. The paleo-lake (blue tinge) hypothesized to have flooded the Danakil Depression below the -50 m contour during mid-Holocene. In the inset, a reconstruction of how the paleo-lake would have flooded volcanic Lake Abaeded due to the geomorphology of the eastern sector of the cone (more details in the discussion). Image ©2017 Digital Globe, Map data ©2017 Google.

DISCUSSION

Genetic analyses of the fish populations of the Danakil Depression provide a number of insights into the radiation of the genera *Danakilia* and *Aphanius* in the region. However, while our study provides the first population genetic insights into the fishes of the Danakil Depression, it also underscores the need for additional genome-wide data to more precisely delineate the phylogeographic history of *Danakilia* and *Aphanius* in the region. Recent small-scale radiations, like those found in these two

genera, are often best explored using genomic approaches employing large sets of SNPs, which are considered more reliable for the taxonomic delimitation of such “young” lineages early in the process of speciation (Shaffer and Thomson 2007).

Nonetheless, a number of findings, based on the analysis of mitochondrial DNA, form a foundation for further investigation employing genome-wide data:

- The two described species, *Danakilia franchettii* and *D. dinicolai*, and three undescribed riverine populations, all morphologically distinguishable based on coloration of breeding males (a characteristic often associated with reproductive isolation in cichlids) (Seehausen *et al.* 2008), showed low mitochondrial differentiation. Surprisingly, the riverine lineages share a single haplotype that is also found in *D. dinicolai*. This pattern likely resulted from a very recent separation of the four populations. Whereas *D. dinicolai* is now entirely isolated in the endorheic crater-lake Abaeded, we cannot rule out a possible connection between the three riverine populations at their outflows during exceptional flash floods. Phylogenetic and haplotype network analyses recovered the presence of two weakly differentiated population clusters: one in the north, including *D. dinicolai* and the riverine populations, and one in the south, containing the two sampled *D. franchettii* populations. These patterns are consistent with the geographical distance between the two areas and the present-day absence of suitable habitat for cichlids in the desert areas separating them.
- In contrast to *Danakilia*, the Danakil Depression *Aphanius* show high mitochondrial variability, with some haplotypes private to single sampling locations and some that are shared even among geographically distant areas. The geographic distribution of these haplotypes reveal some interesting patterns. In particular, the populations inhabiting the Eritrean rivers and the Lake Afrera creeks share a number of widespread haplotypes indicating possible past interconnections among the northern and southern sectors of the Danakil. Overall, the “generalized” *Aphanius* populations of the Danakil Depression are clearly differentiated from *A. dispar s.s.* from the Sinai Peninsula and should probably be considered as separate species. However, a deeper study involving more sampling effort over a wider area than the one investigated here is needed before any taxonomic and nomenclatural revision can be undertaken.
- The *Aphanius* population from the Shukoray River (SHU) is markedly different both from the rest of the Danakil Depression population and from the Sinai population. Despite the short distance between this and the other sites in the northern study area (13-26 km), this riverine population may be a candidate for species status, a finding clearly warranting further investigation.

- The isolated *Aphanius* population of Lake Abaeded also shows deep differentiation from the other *Aphanius* of the region, and should be considered a putative new species based on mitochondrial variation.
- The population from Lake Afrera, likely *A. stiassnyae*, represents a distinct and well-supported clade within Danakil *Aphanius*, supporting the specific status of this taxon (Getahun and Lazara 2001). Nevertheless, some haplotypes seem to be more related to northern riverine populations (i.e. A12xb), probably as a result of past introgression or the retention of ancestral polymorphisms.

Interpreting Danakilia and Aphanius radiations within the Danakil Depression

A mid-Holocene paleo-lake flooding the Danakil Depression

Gasse's (1974) reconstruction of the hydrologic and climatic evolution of the southern portion of the Danakil Depression during mid-Holocene indicates a change in depth of Lake Afrera from a few meters to more than 50 m above the present level, and provides an important basis for interpreting the differentiation of lineages now present in the desert Danakil Depression. The increase of water surface elevation of -50 to -40 m of Lake Afrera (Gasse and Street 1978), with an inflow from subterranean springs and runoff greater than the current one, suggests the existence of a paleo-lake occupying, at least for a short period, the entire Danakil Depression below the present-day -50 m contour. Curiously, an Afar legend tells that the region was once an immense lake that a man would have emptied simply by displacing a pebble (Rognon 1972). At the same time, the study of the Lake Afrera diatom assemblages indicates fluctuations of salinity and pH. During mid-Holocene, Lake Afrera had a higher pH than today, with near-neutral water conditions, and salinity was generally lower (Gasse 1974). Coincident with the maximum expansion of the lake (7800 BP), salinity rose, probably as a consequence of increased availability of groundwater with elevated salt content that started flowing through new faults generated by tectonic events (Gasse 1974).

Detailed sedimentary evidence is unfortunately lacking for the region. However, in the context of the general rise of mid-Holocene lake levels in northern Africa (Gasse and Street 1978, Hoelzmann *et al.* 1998), Gasse's 1974 reconstruction accounts for the wide diffusion of both *Danakilia* and *Aphanius* in the Danakil Depression. The brackish paleo-lake flooding the depression provided a favorable environment for both *Danakilia* and *Aphanius* to disperse freely over the entire area. The relatively recent desiccation of the paleo-lake is consistent with the low diversification of *Danakilia*, presumably a young colonizer of the Danakil Depression catchment with an original distribution possibly limited to a restricted area of the Awash or Nile drainage basins. Conversely, high diversification of *Aphanius* may be the consequence of its potential for rapid speciation

(Reichenbacher *et al.* 2009) and to its former segregation in geographically and ecologically separated “coastal oases” (Faure *et al.* 2002) situated along the ancient coastline of the desiccated Danakil branch of the Red Sea (Bonatti *et al.* 1971). Similar to that hypothesized for *A. dispar* in the SE Arabian Peninsula (Reichenbacher *et al.* 2009), we suggest random genetic drift rather than selective adaptation may be responsible for the observed high diversification of *Aphanius* in the Danakil Depression.

Fish isolation in Lake Abaeded

The colonization of Lake Abaeded by both *Danakilia* and *Aphanius* was facilitated by the collapse of a sector of the volcanic cone (Fig. 7: inset). Such a catastrophic event was likely caused by a seismic episode at the fault intersecting the crater (Ernesto Abbate, pers. comm.) and/or to a hydrothermal explosion primed by the penetration of water from the subterranean hot springs that still feed the lake. A phreatomagmatic origin is also suggested for craters found in the nearby Piano del Sale-Dallol area (Tazieff *et al.* 1969), and is well-documented elsewhere (López and Williams 1993). The crater would thus had been covered by ~20 m of water of the paleo-lake that flowed over the lower rim (–85 m) of the collapsed cone (Fig. 7: inset). The subsequent drying of the paleo-lake starting from ~7300 BP (Gasse 1974), resulted in the isolation of the fishes in volcanic Lake Abaeded, uninterruptedly fed by the aquifer. The higher mitochondrial differentiation of the *Aphanius* population of Lake Abaeded compared to *A. dispar s.s.* of the Sinai Peninsula and the rest of the Danakil populations is again the consequence of *Aphanius*’ potential for rapid speciation (Reichenbacher *et al.* 2009) combined with the relatively long segregation of this population in the small endorheic basin where founder effect and bottlenecks may have played crucial roles.

Hypersaline Lake Afrera: an ecological barrier?

A slightly different hypothesis, although still based on Gasse’s observations, is required to interpret the diverging lineages of *Danakilia* and *Aphanius* found in Lake Afrera and its inflowing thermal springs. As documented by Gasse (1974), the paleo-lake Afrera was more alkaline, but had a lower salinity than today’s lake. The current hypersaline conditions of Lake Afrera (158 g/l: Martini 1969; 130.25 g/l: present study) result from the long accumulation of various salts (NaCl, KCl, MgCl₂, MgSO₄) washed from the evaporitic substrate by the inflowing springs, and of the strong evaporation not counterbalanced by water inflow and precipitation (Martini 1969). It is probable that the ecological conditions of the paleo-lake were suitable for both *Danakilia* and *Aphanius*, but current conditions are more favorable for *Aphanius*. While *Aphanius* generally shows a wide tolerance for salinity extremes (6.50-130.25 g/l: present study), *Danakilia* favors waters with lower salinity (4.41-

20.52 g/l: present study). The exclusive presence of *D. franchetti* in waters adjacent to the point of entry of the streams to the lake, but not in the lake itself (Franchetti 1930, Trewavas 1983, Getahun and Lazara 2001, present study) supports this notion. Contemporary lake hypersalinity appears then to present an ecological barrier to the diffusion of *D. franchetti* into the lake, and between springs and affluent streams. The finding that there are diversified mitochondrial haplotypes in two, geographically proximate springs feeding Lake Afrera is also consistent with this idea. It is probable that, at the time when the paleo-lake rapidly fell to its present level (~7300 BP: Gasse 1974) and its salinity increased, *Danakilia* gradually retreated to the springs where it met more suitable ecological conditions than those in the saltier lake (salinity of springs ranging 7.45-10.33 g/l vs. 130.25 g/l of Lake Afrera: present study), thus initiating the process of genetic divergence between populations within the lake.

The Lake Afrera endemic *Aphanius stiassnyae*, with its high tolerance of extreme salinity (a preadaptation common to other *Aphanius*), its large size (possibly an evolutionary solution to counteract the strong wave motion encountered in the lake), and characteristic oral dentition and mouth shape may represent ecomorphological adaptations enabling it to take advantage of a novel and unexploited habitat.

Isolation and differentiation of Danakil Aphanius populations

The Red Sea coastal distribution of *A. dispar s.s.* and its absence from inland Horn of Africa, suggest that the first colonizers likely followed a coastal corridor of diffusion. The overall high diversification of *Aphanius* in the Danakil Depression may be explained by an initial colonization of the region by one or more lineages referable to *A. dispar s.s.* of Tethyan origin (Hrbek and Meyer 2003). *Aphanius* could then have spread into the Danakil Depression when its northern connection with the Red Sea was still open. The closing of the seaway connection with the Red Sea, the rapid desiccation of the internal marine basin and the dropping of 120 m of the sea level during the LGM, would all have served to isolate *Aphanius* within the depression, resulting in differentiation from *A. dispar s.s.* living along the northern Red Sea coast. The presence of “coastal oases” fed by groundwater (Faure *et al.* 2002) along the (120 m lower than today) shores of the ancient Red Sea in general, and its Danakil branch in particular, could have shaped a mosaic of separated habitats providing ecological conditions favorable to the diversification of local *Aphanius* lineages in the otherwise arid and hypersaline landscape encountered during the LGM. According to Reichenbacher *et al.* (2009), rapid genetic diversification may account for otolith variation found in populations of *A. dispar* from SE Arabian Peninsula that were geographically isolated since the Late Holocene (4000 years ago) and concluded that *A. dispar* populations may be capable of evolving into new species within short periods of time.

The Shukoray River Aphanius

The SHU *Aphanius* can be reliably discriminated with the barcoding approach both from the rest of the populations of the Danakil Depression and from *A. dispar s.s.* from the Sinai. This suggests a long period of isolation from the neighboring riverine populations. We hypothesize that the ancestral SHU *Aphanius* invaded recently from the Red Sea coast to the inner Danakil Depression. This requires postulating the existence of water connections with the coast, features now absent in northeastern Danakil where the rivers drain to the center of the depression or to the Red Sea. However, river bifurcation is a common geomorphological phenomenon in ephemeral braided rivers such as those of the Danakil Depression, and occurs when a distributary stream branches off permanently, or temporarily, from the main channel. Bifurcations can be the result of tectonics or of sediment transport mechanisms that determine the partitioning of sediment discharge into the two downstream branches (Kleinhans *et al.* 2012). The Shukoray River originates on a 400m hill (14.78000°, 40.45000°) and presently flows along the steeper slope of a valley towards the depression. At about 14.80000°, 40.37000°, the valley enlarges and branches, with the main channel continuing to the west and a distributary stream heading to the north, towards the coast. We hypothesize that river bifurcation of the Shukoray River could have been responsible for the diffusion of the ancestral SHU population from the coast, to the inner part of the Danakil Depression. The hypothesis of a recent coastal origin of the SHU population should be tested in future genetic and morphological investigations of the *A. dispar s.s.* populations living along the African coast of the southern Red Sea, such as that of the Gaharre water wells (Vinciguerra 1931).

High diversification of Aphanius compared to Danakilia in rivers

A complementary explanation for the high diversification of *Aphanius* compared to *Danakilia* riverine populations involves their differing ecological requirements, lifespans and breeding biology, and the differential impacts of dramatic changes of regime in temporary rivers. Ecological conditions can vary rapidly and drastically in these environments, and are important stressors influencing the life of desert fishes. Species living in pools along dry riverbeds experience wide seasonal and daily fluctuations affecting water temperature and dissolved oxygen content in relation to vegetative photosynthetic activity. The stochasticity of inundations, despite the predictable seasonality of rainfall, and the isolation of pools precludes fishes from moving when their habitat desiccates or becomes inhospitable (Polačik and Podrabsky 2015). Survival in temporary pools permits the persistence of both *Danakilia* and *Aphanius* in the sampled rivers, but during extraordinarily long dry climate periods would have created a mosaic of disconnected environments, determining bottlenecks

that favored genetic drift and founder effects. Yet, the distinct ecological requirements of the two genera (with *Aphanius* more tolerant than *Danakilia* of extreme water conditions), the shorter lifespan of *Aphanius* and their breeding biology (the first is a maternal mouthbrooder, the second is a substrate spawner) would underlie different responses to such conditions. Populations of *Danakilia* are usually extirpated from shallower temporary pools (pers. obs.) while *Aphanius* thrives even in hypersaline pools the size and depth of a puddle (pers. obs.) and this, over time, will lead to fewer extirpation events and possibly higher diversification rates due to the intermittent isolation of such ephemeral habitats.

CONCLUSIONS

The exploration of remote lakes and rivers in the Danakil Depression carried out in the present study have shed light on the extremophile fish fauna of this area. In an attempt to provide an interpretation of the hydrological history of the region, we suggest a model that fits well with our biogeographical findings. We believe that this first attempt to integrate the biogeographic, geological and hydrological studies carried out by numerous authors over the last 50 years will provide a framework for further multidisciplinary research into the evolution of the Danakil Depression since its separation from the Red Sea. Nonetheless, further efforts are needed in order to sample additional *Aphanius* populations known to exist in the region (for example, in the Gaharre water wells) and *Danakilia* populations possibly present in some still ichthyologically unexplored corners of the Danakil Depression (i.e. Badda swamp, River Ragali, the northern coast of Lake Afrera). In the meantime, our ongoing collaborative projects are utilizing genomic approaches employing large sets of SNPs to more comprehensively describe the evolutionary patterns and processes that have shaped the fish fauna of this extraordinary ecosystem.

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DISCLOSURE STATEMENT

The authors report no conflicts of interest and are alone responsible for the content and writing of the paper.

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SUPPLEMENTARY TABLES

Sampling site	Salinity (g/l)	pH	T (°C)	Carbonate hardness (°F)	O ₂ (mg/l)	<i>Danakilia</i>	<i>Aphanius</i>	Population code	Latitude	Longitude	Date (dd/mm/yyyy)
Shukoray River	9.10	7.60	30.5	362.29	5	+	+	SHU	14.62488	40.33170	05/04/2015
Lake Abaeded	20.52	7.80	32.8	876.95	9	+	+	ABA	14.58901	40.08428	03/04/2015
Zariga River	70.60	8.09	30.3	2234.35	10	-	+	-	14.55745	40.45169	04/04/2015
Zariga River	13.33	8.06	25.9	551.51	9	+	+	ZAR	14.49276	40.33669	05/04/2015
Gali Colluli River	3.72	7.97	25.5	-	5	+	+	GCO	14.40216	40.38466	29/12/2014
Afrera spring	6.51	7.01	38.9	232.31	5	+	+	-	13.22578	40.87503	05/01/2016
Afrera spring	7.45	7.05	38.5	276.38	8	+	+	AFR1	13.22551	40.87514	05/01/2016
Lake Afrera	130.25	7.38	30.5	3518.71	5	-	+	AFR3	13.22092	40.87407	05/01/2016
Afrera spring (*)	4.41	7.16	40.9	140.05	6	+	-	-	13.22053	40.87356	05/01/2016
Afrera spring	6.82	7.06	43.5	231.00	10	-	-	-	13.21497	40.87367	06/01/2016
Afrera spring	6.71	7.31	42.5	252.73	10	-	-	-	13.21430	40.87351	06/01/2016
Afrera spring	8.11	7.27	46.8	343.78	9	-	-	-	13.21071	40.87355	06/01/2016
Afrera spring	10.34	7.80	29.6	387.12	10	+	+	AFR2	13.18258	40.89041	06/01/2016

Table S1. Principal physical and chemical parameters of water sampled at different locations in the Danakil Depression. Sampling sites order follows a north to south criterion. Presence/absence of both *Danakilia* and *Aphanius* is indicated with + and - respectively. When a water-sampling site matches with a fish population collecting-site, the corresponding three-letter code used throughout the paper is shown. The continuous presence of *Danakilia* in Afrera spring (*) is dubious since no adults could be sampled and only a small school of very young specimens (1.5-2 cm) was observed loitering close to the creek mouth, where water is slightly colder than at the spring source (T = 42.5 °C).

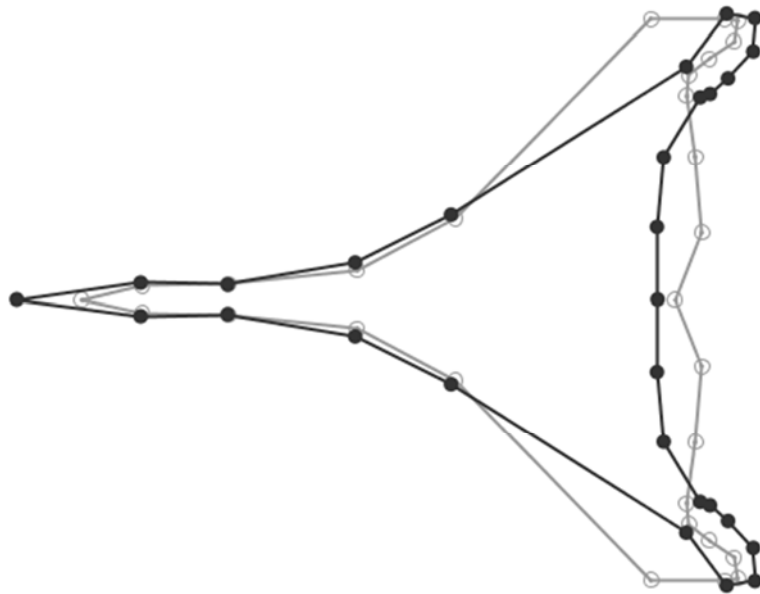
Taxon	Criterion	Partition	Model selected
<i>Danakilia</i>	BIC	(COI_pos1, cytb_pos1)	K80
		(COI_pos2, cytb_pos2)	F81
		(COI_pos3)	TrN
		(cytb_pos3)	TrN+I
<i>Aphanius</i>	BI	(COI_pos1, cytb_pos1)	K80
		(COI_pos2, cytb_pos2)	HKI
		(COI_pos3, cytb_pos3)	HKI+I

Table S2. Summary of evolutionary models applied to *Danakilia* and *Aphanius* for phylogenetic inference.

CHAPTER 3

RESEARCH PAPER

(advanced draft)



Lower pharyngeal jaw shape change in Danakilia

Deserts require unique fishes: phenotypic variation in the endemic extremophile cichlid genus *Danakilia* of the Danakil Depression

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ABSTRACT

The Danakil Depression in northeastern Africa represents one of the harshest arid environments on Earth and one of the least explored for its biodiversity. Despite the paucity of water, this desert region, where the environment is threatened by mining interests, is inhabited by the endemic extremophile cichlid genus *Danakilia*. The genus includes at least two populations of *D. franchettii* (Afr) from groundwater springs feeding Lake Afrera, *D. dinicolai* (Aba) from volcanic Lake Abaeded, and three recently discovered and still undescribed riverine populations (Shu, Zar, GCo). We here analyze for the first time the morphological variation in body and lower pharyngeal jaw (LPJ) shape of *Danakilia* across its entire known range with a geometric morphometrics approach. In particular, we ask how body and LPJ, two evolutionarily important traits in cichlids, vary within and among *Danakilia* populations; if these traits display genetic or plastic local adaptation or a neutral model of variation in geographic space; and if it show consistent patterns of co-variation that might point to pleiotropy or linkage. We found distinctiveness of Afr compared to Aba confirming current taxonomy; a sharp separation of Afr from northern populations for both traits; a less pronounced separation among the northern populations; a deeper body shape in Afr and Zar compared with more elongated populations Aba, Shu and Gco; slender LPJ in Afr than in the northern populations. This significant variation – which cannot be explained by phenotypic plasticity – suggests that each population might be an

evolutionary significant unit deserving conservation measures.

INTRODUCTION

The hot and arid Danakil Depression of north-eastern Africa, a geomorphological region (Billi, 2015) partitioned between southern Eritrea and northern Ethiopia, is a land of climatic, ecological and geological extremes and it is one of the least explored and poorly studied desert regions of the World. This desert region, extending 300 km from the Gulf of Zula (15.27°, 39.75°) to Lake Afrera (13.27°, 40.90°), is wedged between the Red Sea to the north, the Danakil Block of continental origin (maximum elevation <1400 m: Mohr, 1978) to the east, the Eritrean-Ethiopian Plateau, also of continental origin, to the west, and merges to the south with the south-western and east-central Afar region (Beyene and Abdelsalam, 2005).

Most of the Danakil Depression (~10,000 km²) lays below the sea level, with the lowest point at -120 m in Piano del Sale-Dallol (Beyene and Abdelsalam, 2005) or, alternatively, at -190 m, if we consider the lowest point to be the bottom of Lake Afrera (Bonatti *et al.*, 2017).

The climate is punitive for most of the year and the area still holds the world record for the highest average annual temperature ever recorded in an inhabited area (34.7 °C, from 1960 to 1966: Pedgley, 1967). Precipitation is scarce or even absent for years and the negative balance between precipitation (less than 100 mm/a) and evaporation (5000 mm/a) creates the conditions for its extreme present-day aridity (Faure *et al.*, 2002).

The Danakil Depression is a geologically young region characterized by an intense volcanic and seismic activity (Rognon, 1972; Tazieff *et al.*, 1972). The depression originated during Lower Miocene, 23-25 Mya (Barberi *et al.*, 1972) and the ~3 km thick deposits of evaporites (Bonatti *et al.*, 1971) attest its intermittent marine connection to the Red Sea until Late Pleistocene (~32,000 BP) when the rise of the Alid volcanic center blocked its northern waterway (Lowenstern *et al.*, 1999) and triggered its rapid (~4000 a) desiccation (Bonatti *et al.*, 1971). After the Last Glacial Maximum event of ~20,000 BP, with a rise in aridity, the Early Holocene saw an increased amount of groundwater due to deglaciation (~9800 BP: Gasse, 1974), which likely caused the formation of an extensive paleo-lake flooding the area below the -50 m contour until ~7300 BP (Chiozzi *et al.*, *submitted*).

The Danakil Depression is an endorheic basin. Temporary brackish rivers ending in alluvial fans drain the monsoonal rains that fall on the Eritrean-Ethiopian Plateau and on the Danakil Block towards its central part. Some brackish to hypersaline endorheic lakes, mostly fed by groundwater, are also present in the area at various elevations below the sea level.

Notwithstanding the extreme environmental conditions, the localized presence and stochastic

availability of water, the Danakil Depression's waterbodies sustain fish populations and the area is part of the Western Red Sea Drainages freshwater ecoregion (Abell *et al.*, 2008). Four described species of fish (Actinopterygii) belonging to the genera *Aphanius* (Cyprinodontiformes, Cyprinodontidae) and *Danakilia* (Perciformes, Cichlidae) are known to inhabit this region (Vinciguerra, 1931; Getahun and Lazara, 2001; Stiassny *et al.*, 2010).

In particular, the endemic genus *Danakilia* includes: at least two populations of *D. franchettii* (Vinciguerra, 1931) from groundwater springs feeding Lake Afrera (Vinciguerra, 1931; Trewavas, 1983), *D. dinicolai* Stiassny, De Marchi & Lamboj, 2010 from the tiny volcanic Lake Abaeded (Stiassny *et al.*, 2010), and three recently discovered riverine populations (Chiozzi *et al.*, *submitted*). Breeding male colouration allows easy discrimination in the field among *D. franchettii*, *D. dinicolai* and the three riverine *Danakilia* sp. considered as a whole (Chiozzi *et al.*, *submitted*). At closer examination, *D. dinicolai* can be discriminated from *D. franchettii* on the basis of body proportions, longer pectoral fins, more robust oral dentition, hypertrophied lower pharyngeal jaw with finer and more packed teeth on the posterior field of the jaw (Stiassny *et al.* 2010). Preliminary data show that the three *Danakilia* sp. are morphologically closer to *D. dinicolai* than to *D. franchettii* (G. Chiozzi and A. Lamboj, unpublished data). Despite the evident variation in colour and a suite of more subtle differences in certain morphological traits, *Danakilia* morphospecies show low differentiation at mitochondrial markers (concatenated *COI* and *cytb*). This subtle neutral genetic variation is partitioned between a northern cluster, including *D. dinicolai* and the three riverine populations, and a southern cluster, including two creek lineages of *D. franchettii*, ecologically separated by the hypersaline waters of Lake Afrera (Chiozzi *et al.*, *submitted*). The northern and southern cluster are separated by ~150 km of arid lands, while *D. dinicolai* and the three river populations live ~10-37 km apart from each other. The low neutral genetic differentiation between these populations is attributable to the young origin of the two lineages. Indeed, these likely originated from a common ancestor widespread in the brackish paleo-lake flooding the Danakil Depression until ~7300 BP, when the paleo-lake started drying. With drying, this ancestral cichlid separated in populations, thus originating the two lineages. (Chiozzi *et al.* *submitted*).

The extraordinary ability of cichlids to colonize freshwater habitats reaches an extent unmatched by any other vertebrate family (Liem, 1973). This is superbly exemplified by the booming adaptive radiations in the African Great Lakes (Tanganyika, Malawi and Victoria), each one containing hundreds of mostly endemic species. The surprising speed at which cichlids can explosively radiate is also renowned. In particular, some cichlid adaptive radiations developed in very short amounts of time, such as that of Lake Victoria (12,400-18,000 a depending on the study: Johnson *et al.*, 1996;

Seehausen, 2002; Salzburger *et al.*, 2014) or the small adaptive radiation of *Alcolapia* in Lakes Natron and Magadi (~10,000 a ago: Ford *et al.*, 2015 and references therein).

In cichlids, extensive morphological variation of potential adaptive value has been described, including in trophic morphology, external body shape, and coloration. In particular, the adaptive value of variation in pharyngeal jaws – an additional set of jaws used to crush hard food and present in a derived condition (pharyngognathy) in cichlids and other perciforms (Wainwright *et al.*, 2012) – has long been recognized (Barel *et al.*, 1977). Similarly, many studies have highlighted the importance of body shape for locomotion and differential adaptation to benthic or limnetic habitats (Barlow *et al.*, 1976). These and many other traits are today routinely analyzed using geometric morphometrics methods (Rohlf & Marcus, 1993), as much in cichlids as in other taxa (reviewed in Kerschbaumer & Sturmbauer, 2011). One of the key advantages of geometric morphometrics is that it allows to quantify even subtle variation in shape. This makes geometric morphometric tools particularly appropriate for intra-specific studies and studies of closely-related species (Loy, 1996).

Paradoxically, the most spectacularly diverse and species-rich cichlid radiations such as the ones which occurred in East African Great Lakes, are the most challenging to study. The ecological complexity of these environments makes it difficult to assess the relative contributions of geographical isolation, ecological specialization, sexual selection and hybridization as driving forces of speciation. Far from the extreme ecological complexity of the African Great Lakes, smaller isolated aquatic systems are a more simplified and useful model, similar to some extent to the one offered by island ecosystems, thus allowing clearer insight into the main forces driving biological diversification of which ecomorphological differentiation is the main manifestation (Wellborn and Langerhans, 2015). In fact, the ecological opportunity provided by isolated or depauperate ecosystems and their unexploited ecological niches, where colonizers are released from interspecific competition, is one of the foremost forces driving phenotypic diversification and adaptive radiation (reviewed in Yoder *et al.*, 2010). Well known examples come from the Nicaraguan (Lake Nicaragua, Lake Managua and satellite crater lakes: Barluenga *et al.*, 2006; Elmer *et al.*, 2010; Franchini *et al.*, 2016) and Cameroonian lakes (Lake Barombi Mbo, Lake Bermin and Lake Ejagham: Schliewen *et al.*, 1994; Schliewen *et al.*, 2001; Schliewen & Klee, 2004; Martin *et al.*, 2014), the desert marshes of northern Mexico (Cuatro Ciénegas: Magalhaes *et al.*, 2015), and the smaller lakes of the East African Rift, such as the crater lakes of Uganda (Machado-Schiaffino *et al.*, 2015) and the East African soda lakes Natron (Tanzania) and Magadi (Kenya) (Ford *et al.*, 2015; Kavembe *et al.*, 2016).

Similarly, isolated springs, lakes or waterholes along temporary rivers in desert areas can be assimilated to islands of water in a sea of dry land (Faulks *et al.*, 2010). Nonetheless, these

environments show various degrees of environmental stability, from desert springs being the most stable at seasonal and daily timescales, to ephemeral waterholes along dry riverbeds being subject to fluctuations of their physical (temperature, pH, turbidity) and chemical (conductivity, dissolved oxygen) parameters with dramatic daily and seasonal shifts of temperature, variation of dissolved oxygen following the circadian rhythms of photosynthetic activity and seasonal flash floods (Hillyard *et al.*, 2015; Polačik *et al.*, 2015). For these reasons, desert fishes, quite often endemic species deriving from ancestors once more widespread than today and belonging to taxonomic groups present in the nearby regions (Hillyard *et al.*;2015), can represent profitable “natural experiments” for the study of phenotypic variation in geographical space.

Here, variation in body and lower pharyngeal jaw of the extremophile cichlids of the genus *Danakilia* are studied for the first time using a geometric morphometric approach in order to answer the following questions. How do body and lower pharyngeal jaw vary within and among *Danakilia* populations and species? Do body and lower-pharyngeal jaw shape display variation in geographic space which indicates genetically based or plastic local adaptation? Or do these two traits rather conform to a neutral or quasi-neutral model of variation in geographic space? Do body and pharyngeal jaws display consistent patterns of co-variation, which might point to pleiotropy or linkage, as recently identified in other cichlids (Fruciano *et al.*, 2016) and which might accelerate diversification?

MATERIALS AND METHODS

Sampling

For the purpose of this research, we utilized 62 *Danakilia spp.* specimens from five sampling sites (Fig. 1). These include both the previously described population of *D. dinicolai* and three undescribed riverine populations (n=47) collected during two field expeditions (December 2014-January 2015, April 2015: Chiozzi *et al.*, *submitted*), complemented by museum specimens of *D. franchettii* (n=12) and *D. dinicolai* (n=3) preserved in the ichthyological collection of the American Museum of Natural History, New York (USA).

Specimens, collected by seine and hand net, were euthanized with clove oil (Erdmann, 1999) and fixed in 10% formalin for 5 days, then rinsed in water for 2-3 days before transfer to 70% ethanol for final preservation. The lower pharyngeal jaws (n=40) were later dissected from a subset of the ethanol-preserved specimens included in the sample.

Morphometric data acquisition and preparation

Images of the left side of the body of each specimen were taken using a Nikon™ digital camera

equipped with a 55 mm macro lens and mounted on a copy stand. A subset of 8 specimens, which was selected for a preliminary assessment of measurement error, was photographed two times in two different sessions.

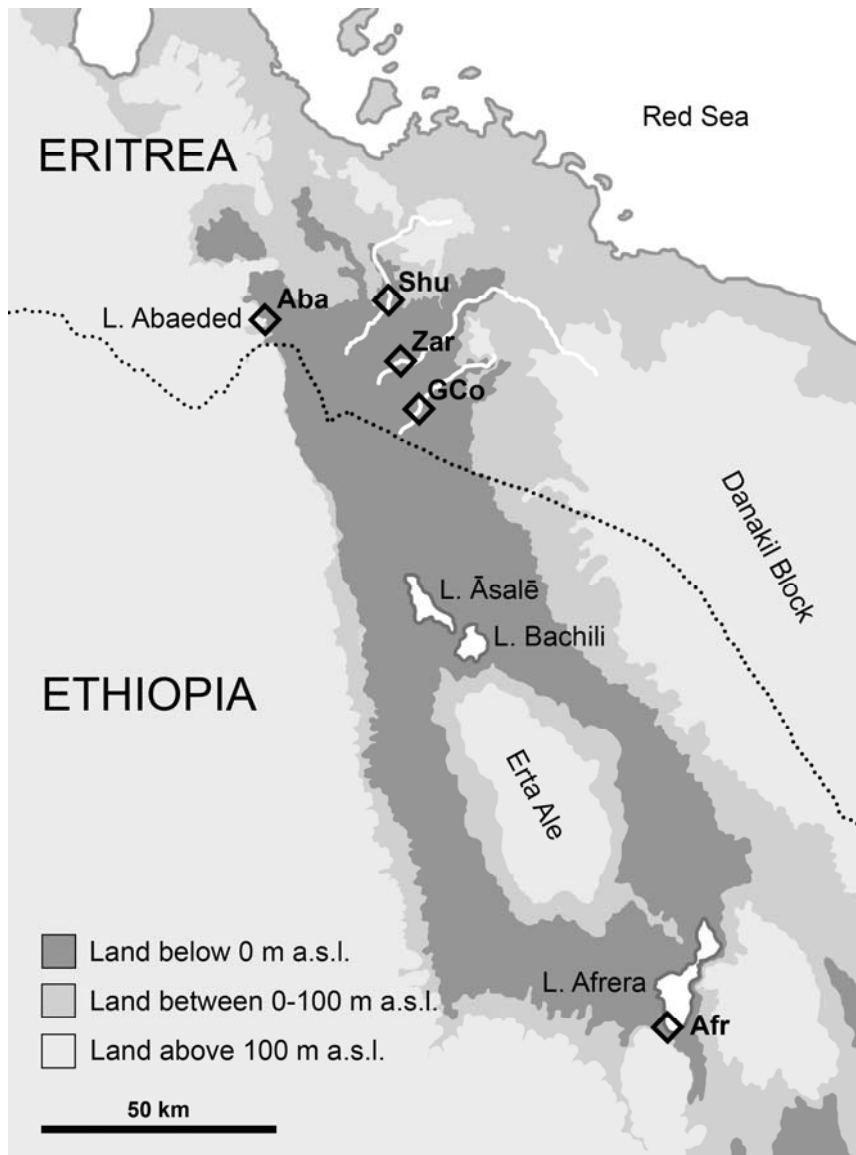


Figure 1. Study area indicating collection sites: Lake Abaeded (Aba), river Shukoray (Shu), river Zariga (Zar), river Gali Colluli (GCo), Lake Afrera (Afr). Major geographical landmarks are also shown. International borders in the map are not authoritative.

Because of their small size, the lower pharyngeal jaws were photographed in dorsal view using a Nikon™ stereoscope designed for digital imaging. To improve the detail and sharpness of the final images, they were obtained by photographing each jaw 10 times, slightly changing the focal plane on every shot, from the base of the jaw to its top. Finally, the resulting images were combined in a single “sandwich” picture by using a free stacking software (CombineZP© by Alan Hadley).

The landmark configurations used in morphometric analyses of body and pharyngeal jaw shape (Fig.

2) included landmarks, semilandmarks, and – for body shape – “helper points”. “Helper points” (Zedlitch *et al.*, 2004) were used to assist the alignment of semilandmarks around the eye, but were later removed from the analysis as they do not provide additional information other than the one provided by the two eye semilandmarks retained (i.e., relative eye size). Points were digitized on body and pharyngeal jaw photographs using tpsDig 2.26 (Rohlf, 2015).

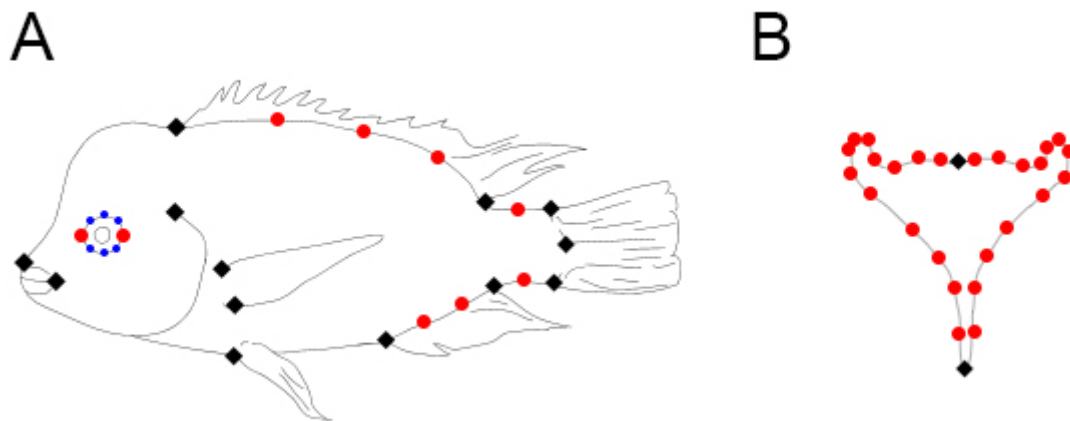


Figure 2. Configurations of points used in the morphometric analyses of body (A) and lower pharyngeal jaw (B) shape. Black diamonds = landmarks; red circles = semilandmarks; blue circles = “helper” semilandmarks.

For a subset of seven specimens presenting damage on one side of the pharyngeal jaw which prevented the digitization of two to four semilandmarks, we obtained estimates of the missing points by reflecting their counterparts on the other side across the symmetry axis. Previous studies on the lower pharyngeal jaw (Fruciano *et al.* 2016b) have used a reflection across the axis identified by the two fixed landmarks (one on the tip of the keel and the other medio-posteriorly). Here, because of poor performance of the previous method, we used a custom Matlab script (available from CF upon request) which exploits all the bilateral points present on both sides and the points on the midline to estimate the symmetry axis, using reduced major axis regression to minimize the effect of outliers. The obtained configurations of points (x, y coordinates) were subjected to a generalized Procrustes analysis with sliding of semilandmarks (Bookstein, 1997) in tpsRelW 1.65 (Rohlf, 2015). The same software was used to compute centroid size. Asymmetry was not of interest in the present study; therefore, all the subsequent analyses on pharyngeal jaws were performed on the symmetric component of shape variation (Klingenberg *et al.*, 2002), as in similar work on pharyngeal jaws (Fruciano *et al.*, 2011; Fruciano *et al.*, 2016a, 2016b; Gunter *et al.*, 2013).

A rough estimate of measurement error in body shape due to presentation was obtained performing a Procrustes ANOVA (Klingenberg and McIntyre 1998) on the subset of specimens photographed two

times and computing an analogous of the intraclass correlation coefficient (as described in Fruciano, 2016; often called “repeatability”). This revealed moderate levels of error with presentation error explaining only 5.99% of total variance and a “repeatability” of 0.89. For this reason, we deemed sufficient to perform all subsequent analyses on a single digitization of a single picture.

However, fish body arching can have a substantial effect on results while often having negligible effect on repeatability estimates (Fruciano, 2016). As in other studies on fish, (Fruciano *et al.*, 2011; Fruciano *et al.*, 2012; Fruciano *et al.*, 2014; Fruciano *et al.*, 2016a; Franchini *et al.*, 2014; Ingram, 2015) here we used the procedure to reduce variation due to body arching proposed by Valentin *et al.* (2008). Briefly (see Fruciano, 2016 for a more in-depth discussion), this procedure consists of modelling body arching and then removing the variation thus modelled – considered as artefactual – from the dataset using Burnaby’s projection (Burnaby, 1966). To create the body arching model, we used eight different specimens in our sample each recorded at five different levels of body arching. We also performed a preliminary set of analyses where we fit linear models using shape variables as dependent variables and location, sex and centroid size as predictors. As tests revealed potential for multi-way interactions between sex and the other two factors but inconsistently (because of the small number of females), we removed the females from our dataset. Conversely, a similar preliminary linear model on males only (body shape, first 36 principal components, pharyngeal jaws first 26 principal components; dimension reduction to remove near-zero dimensions) showed no significant interaction between location and size (body shape $p=0.32$, pharyngeal jaw shape $p=0.39$). The same linear model revealed significant or near-significant effects of location and size (body shape, location $p<0.001$, centroid size $p=0.07$; pharyngeal jaw shape, location $p<0.001$, centroid size 0.048). For these reasons, we performed in MorphoJ (Klingenberg, 2011) a linear regression of shape on centroid size (pooled within sampling site) and used residuals in subsequent analyses.

Body and pharyngeal jaw shape variation among sampling sites

To study variation among and within species and sampling sites, we subjected the dataset of male fish obtained after the steps detailed above a set of complementary approaches: exploratory ordinations, tests of differences in mean shape, supervised and unsupervised machine learning.

In detail, to explore variation between sampling sites using ordinations we performed a between-group principal component analysis (Boulesteix, 2005). This is an ordination technique which is increasingly used in geometric morphometrics (Firmat *et al.*, 2012; Franchini *et al.*, 2014; Franchini *et al.*, 2016; Fruciano *et al.*, 2014; Fruciano *et al.*, 2017; Schmieder *et al.*, 2015) because the ordinations do not exaggerate separation between groups, as instead happens with scatterplots of canonical variate scores (Mitteroecker & Bookstein, 2011). Between-group principal components

were computed in R using the package *Morpho* (Schlager *et al.*, 2016) and scores along the first two between-group principal components were visualised using the R package *ggplot2* (Wickman, 2016). *Morpho* was also used to compute Procrustes distances between sampling sites and to test for significance using the permutational procedure implemented in the package (10,000 permutations), as well as to perform a canonical variate analysis (using sampling site as group) with leave-one-out cross-validation. Further, we visualised differences in shape among populations by producing, in MorphoJ, wireframe graphs depicting the difference between each population mean shape and the grand mean. Finally, we used the same unsupervised machine learning approach described in Fruciano *et al.* (2016b), which is in turn largely derived from the one proposed by Ezard *et al.* (2010). In other words, we asked ourselves how many groups could be distinguished in our body and pharyngeal jaw shape datasets without knowing the groups a priori. The approach used here consists of a dimension reduction step based on the variance expected under the broken stick model (Jackson, 1993) and an unsupervised clustering step based on Gaussian mixture models, as implemented in the R package *mclust* (Fritsch, 2012). We also identified multivariate outliers in the data after dimension reduction using the method (Filzmoser *et al.*, 2008) implemented in the package *mvoutlier* (Filzmoser and Gschwandtner, 2012). Using a custom Matlab implementation (code available by CF upon request) on those specimens for which we had both body and pharyngeal jaw data, we quantified the congruence of the classification obtained using body and pharyngeal jaw by computing the adjusted Rand index (Hubert and Arabie, 1985) and tested its significance using a permutation test (Qannari *et al.*, 2014) (1,000 permutations).

As preliminary observations (G. Chiozzi, unpublished data) and its geographical position suggested that Lake Afrera could be distinct from the others, we performed the analyses of variation among sampling sites described above both with and without specimens from Afrera.

Integration and modularity

To study covariation between body and pharyngeal jaw shape, we used partial least squares analysis (Rohlf and Corti, 2000) and tests of differences in modularity (Fruciano *et al.*, 2013).

In particular, we used partial least squares (PLS) in *Morpho* to identify and test for patterns of covariation between body and pharyngeal jaws. We performed these analyses both including and excluding specimens from Afrera and on the data containing variation between sampling sites and after computing residuals from sampling site means. This latter choice is motivated with the fact that co-variation of body and pharyngeal jaw across sites does not have to have the same direction as covariation within sampling sites. For instance, if one or more populations (e.g., Afrera) have distinctive body and pharyngeal jaw shape, this might drive patterns of co-variation.

Based on the results from other analyses and previous knowledge on the population from lake Afrera (see above), we compared the level of body/pharyngeal jaw shape association between Afrera and the other sampling sites using procedures proposed by Fruciano *et al.* 2013. These include computing an estimate of Escoufier RV (Escoufier, 1973) rarified to the lowest sample size (Fruciano *et al.*, 2013) and a permutation test for difference in Escoufier RV.

RESULTS

Body and pharyngeal jaw shape variation among sampling sites

The scatterplots of scores along the first two between-group principal components (using sampling site as a group) reveal a good separation among sampling sites for both body and pharyngeal jaw shape. In particular, when all sampling sites are included, the population from Afrera is clearly distinct from the others which, in turn, show a higher degree of overlap (Fig. 3). Clearly, excluding Afrera from the analysis reveals a clearer separation of the other populations (Fig. S1).

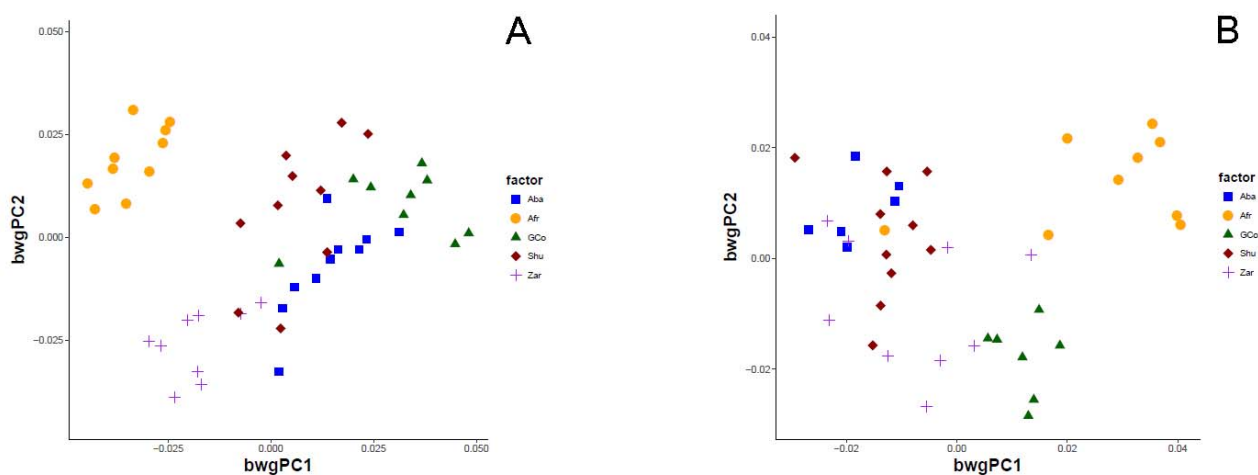


Figure 3. Scatterplots for the first two principal components of between-group principal component analyses for body shape (A) and lower pharyngeal jaw (B). Sampling sites define groups.

The distinctiveness of the Afrera population is also confirmed when computing pairwise Procrustes distances between population means and testing for their significance (Tab. 1). Indeed, while all pairwise comparisons are significant for body shape, when analysing pharyngeal jaw shape the population from Afrera is the only one for which all comparisons with other populations are significant. This is further confirmed when performing canonical variate analysis.

In fact, overall cross-validated correct classification (Tab. S1) is very poor for body shape (52.08%)

Body shape – Procrustes distances				
	Aba	Afr	Gco	Zar
Afr	0.05581498			
GCo	0.02925620	0.06641862		
Sar	0.04221376	0.04823782	0.05953397	
Shu	0.03125374	0.04579564	0.02898216	0.04209690

Body shape – p values				
	Aba	Afr	Gco	Zar
Afr	0.0001			
GCo	0.0439	0.0001		
Sar	0.0004	0.0001	0.0001	
Shu	0.0168	0.0002	0.0473	0.0004

LPJ shape – Procrustes distances				
	Aba	Afr	Gco	Zar
Afr	0.04499404			
GCo	0.04088990	0.03487250		
Sar	0.02095342	0.04123815	0.02320375	
Shu	0.01636221	0.04155358	0.03435479	0.01853183

LPJ shape – p values				
	Aba	Afr	Gco	Zar
Afr	0.0001			
GCo	0.0002	0.0012		
Sar	0.1265	0.0001	0.0596	
Shu	0.3300	0.0001	0.0012	0.1260

Table 1. Pairwise comparison of Procrustes distances between group means for both body and lower pharyngeal jaw shape. Significance was assessed with 10,000 permutation runs (T-square test).

but good for pharyngeal jaw shape (82.93%). However, Afrera is the only population whose members are consistently classified correctly across body and pharyngeal jaw shape (90% and 100% correct classification, respectively). The visualization of mean population shape relative to the grand mean for body shape (Fig. 4) shows a general trend of populations with a deeper body (Afrera and Zariga; negative scores on between-group PC1) and against the others with a more elongated body. It is noteworthy that the two deeper-bodied populations – and Afrera in particular – show a pronounced “hump” in the posterior portion of the body under the dorsal fin, just in front of its posterior limit. The two deeper bodied populations (Afrera and Zariga) also show a mouth placed more dorsally and a more anterior pectoral fin. Zariga also shows a pronounced shortening of the caudal peduncle, whereas Afrera has a more upturned mouth and attachment of the pectoral fin.

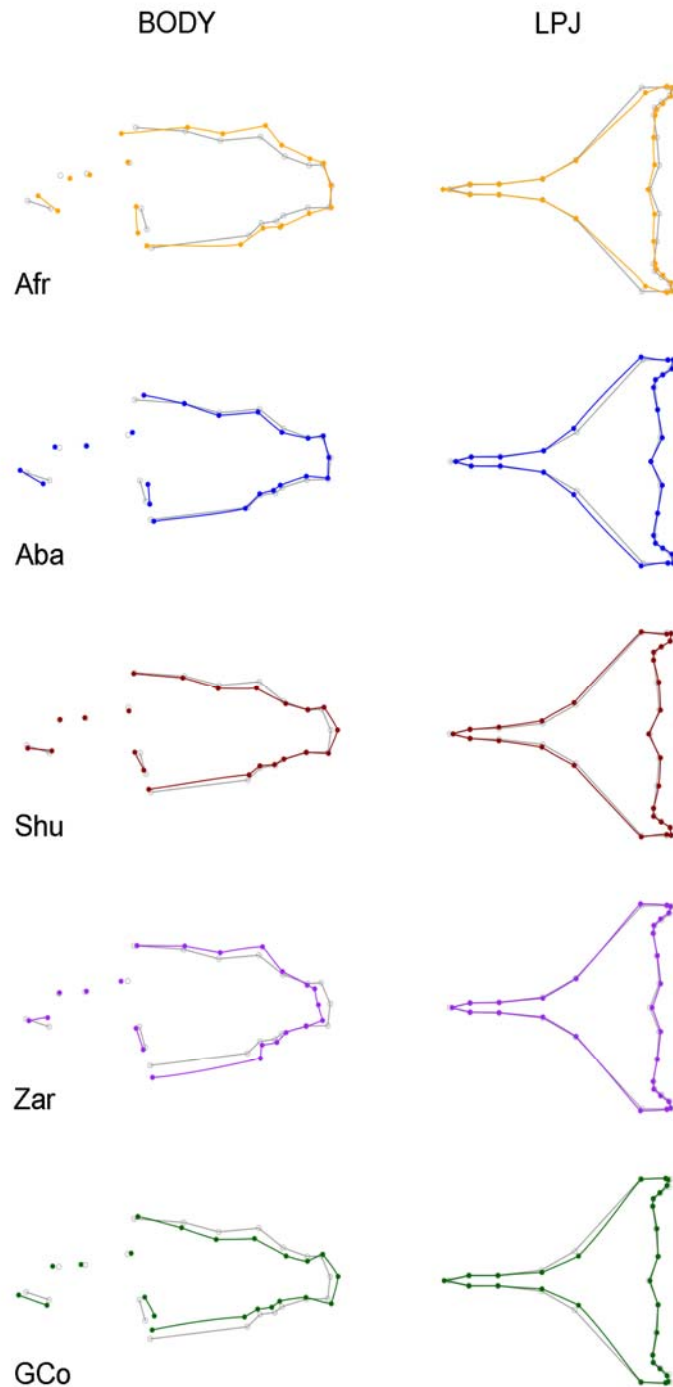


Figure 4. Wireframe graphs of mean population shapes for body (left column) and lower pharyngeal jaw (right column) relative to the grand mean. Matching colours indicate the same population and grey the grand mean (rows).

Among the slenderer populations, GCo is the most elongated and also shows a slightly more ventral mouth and more posterior pectoral fin.

Variation in pharyngeal jaw mean shape among populations occurs mainly along the anterior margin of its main portion, whereas the keel varies only slightly. Interestingly, here Afrera and GCo, which

had quite different body shape, have similar, more slender (positive between-group PC1 scores), pharyngeal jaw. However, these two populations are different from each other in the posterior margin of the pharyngeal jaw (which is less pronounced in Afrera). Most importantly, the main distinction between Afrera and all the other populations (including GCo) can be seen in the shape of the horns, which are much less robust in Afrera.

The samples from Afrera proved distinctive also when we used an unsupervised clustering approach. In fact, our unsupervised clustering approach recovered three clear groups both for body and pharyngeal jaw shape (Fig. 5).

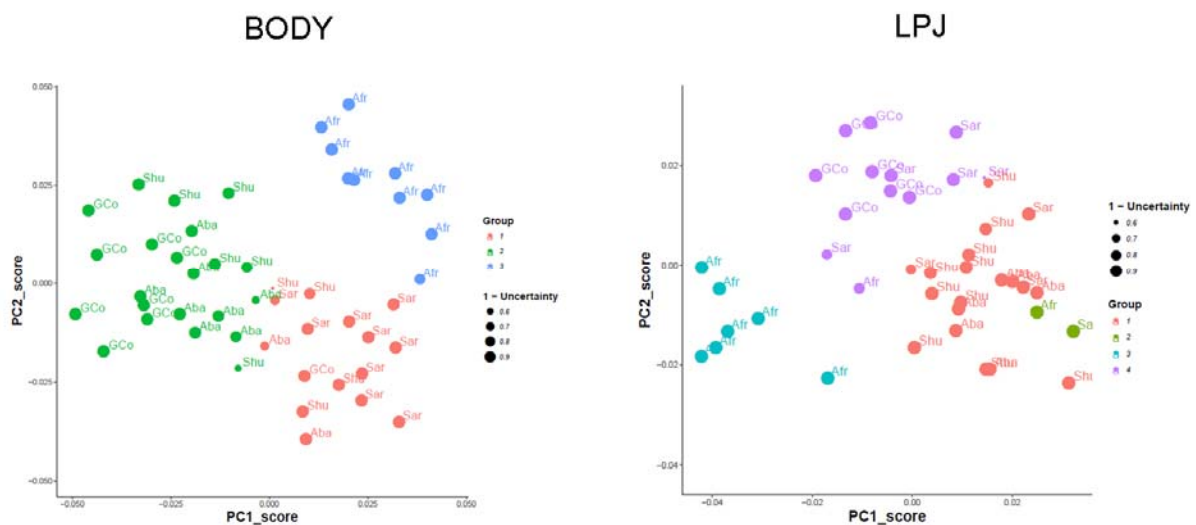


Figure 5. Scatterplots showing the variation in shape between sampling sites using an unsupervised clustering approach (see Fruciano et al., 2016b). This analysis recovers how many groups can be distinguished in our body (left) and pharyngeal jaw (right) shape datasets without knowing the groups *a priori*. Dot colours indicate the group (sampling locations), and dot size the level of uncertainty in the assignment (dimension: 1 – uncertainty, where a value of 1 indicates no uncertainty).

One of the two groups in both analyses almost identifies with the population from Afrera (i.e., only a few specimens from Afrera are not in the group, and the group contains almost exclusively specimens from Afrera). On the other hand, the other two groups contain specimens from multiple sampling sites. Unsupervised clustering on the pharyngeal jaw dataset identifies a fourth group of only two specimens, however these are also identified as potential multivariate outliers. When comparing the classification obtained using body and pharyngeal jaw, we obtain a moderate (adjusted Rand index 0.21) but highly significant ($p < 0.001$) agreement between the two datasets and similar results are obtained excluding those specimens identified as potential multivariate outliers. However, testing for agreement between the two classifications without including the specimens from Afrera shows no

agreement (adjusted Rand index -0.02, $p=0.6$). Similarly, when performing unsupervised clustering on our datasets not containing specimens from Lake Afrera, no clustering is identified.

Integration and modularity

When performing PLS on all the sampling sites and maintaining variation among them, the first singular axis is significant ($p<0.0001$) and accounts for 88.07% of covariance. The shape changes predicted by the first pair of singular axis corresponds to a deepening of the body and increase of the “hump” at the caudal edge of the dorsal fin attachment associated to a reduction in LPJ horn thickness, a less marked posterior margin of the pharyngeal jaw and an elongation of the keel (Fig. 6).

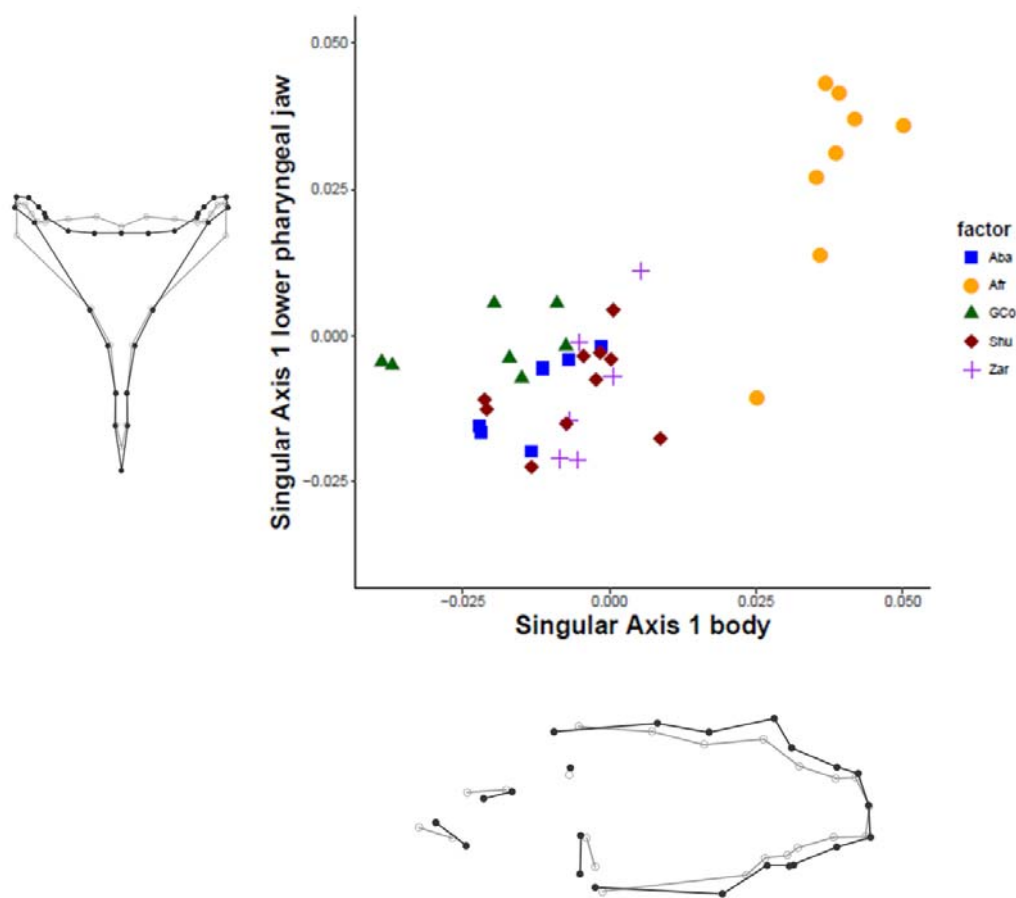


Figure 6. Partial least squares analysis (PLS: Rohlf and Corti, 2000) for body and lower pharyngeal jaw datasets. The scatterplot shows the level of association between the two datasets. The wireframes (LPJ, upper right; body lower left) show the amount of variation between the extremes of shape within each dataset.

An inspection of the scatterplot of the scores on the first pair of singular axes reveals that Afrera occupies a distinct region. When performing the same analysis on residuals from sampling site means, the first pair of singular axes accounts for 51.07% of covariance and is not significant ($p=0.27$). The

following pairs of singular axes from the second to the fifth are, instead, significant, but they are difficult to interpret because of the way they are constructed (orthogonality) and relatively little explained covariance (31.25% or less). When performing the same analyses excluding fishes from Afrera, the first pair of singular axis of the dataset containing variation explains 54.56% of covariance between body and pharyngeal jaw shape and is not significant ($p=0.54$). Removing variation between population means results in higher explained covariance (64.42%) for the first pair of singular axes, which is also significant ($p=0.037$). However, a scatterplot of the scores along the first pair of singular axes for this dataset reveals a very large spread of a single population (Shu), probably driving covariance.

Levels of association between body and pharyngeal jaws are similar between Afrera (mean rarefied RV 0.56, sd 0.19) and the other samples (mean rarefied RV 0.44 sd 0.16) and the permutation test for the difference in RV is not significant ($p=0.488$).

DISCUSSION

Here, we carried out the first comprehensive study of body and lower pharyngeal jaw shape variation across all known species and populations of the desert endemic fish genus *Danakilia* by employing a geometric morphometric approach. Overall, our findings show significant phenotypic divergence along the genus' entire geographical range in the Danakil Depression of Eritrea and Ethiopia.

In particular, our findings indicate:

- distinctiveness of *D. franchettii* (Afr) compared to *D. dinicolai* (Aba) both for body and LPJ shapes;
- a sharp separation of *D. franchettii* (Afr) from the northern populations as a whole (Aba, Shu, Zar and Gco) both for body and LPJ shapes;
- a relatively clear – but less pronounced – separation among the northern Danakil Depression populations (Aba, Shu, Zar and Gco);
- a body shape pattern contrasting deeper bodied populations (Afr and Zar) with more elongated populations (Aba, Shu and Gco);
- more robust lower pharyngeal jaws in the northern populations.

Geometric morphometric analyses thus quantitatively substantiate previous qualitative observations on variation among populations in colouration of breeding adult males both in the wild and in aquarium (Chiozzi *et al. submitted*) and confirm the current taxonomy (Stiassny *et al.* 2010). This structure of variation among populations and alternative morphs is consistent with a previous study of mitochondrial markers (Chiozzi *et al. submitted*). This kind of morphometric information can prove useful not only in future taxonomic studies, but it can also shed light on the evolutionary

mechanisms that led to the diversification of the *Danakilia* lineages.

In particular, *D. franchettii* (Afr) appears as clearly distinct from fish from other sampling sites both for body and lower pharyngeal jaw shape. To the trained eye, male preserved *D. franchettii* appear visually different from males of the other populations for their deeper body, less sloping back and deeper caudal peduncle. These features are confirmed by the visualization of mean population shape relative to the grand mean. *D. franchettii* also shows a pronounced “hump” in the posterior portion of the body. A similar hump is found in the River Zariga population (Zar), however the caudal peduncle in Zariga is shorter and thinner than in Afr. Fish body shape has been observed to evolve rapidly even across small spatial scales (Woods, 2007; Janhunen *et al.*, 2009; Tobler *et al.*, 2011). In the small and isolated populations of *Danakilia*, it is possible that processes such as bottlenecks or sexual selection, could have produced shifts in phenotype distributions across populations. In particular, the pronounced hump on the back and the thicker caudal peduncle that characterize the Afr and Zar populations can possibly be explained in this way.

In general, according to Webb (1984), a deep, flattened body together with lateral insertion of pectoral fins, anterior ventral-lateral insertion of pelvic fins and extended dorsal and anal fins (common traits in cichlids), indicate slow swimming and precise manoeuvrability, which are characteristic of Median and Paired Fin propulsion swimmers (MPF) eating non-evasive food in structurally complex habitats. All examined *Danakilia* populations share these morphological characters and a vegetarian diet, facts that allows us to classify this genus as a MPF species, *sensu* Webb (1984). However, while the thicker caudal peduncle observed in Afr and Zar can in principle have a functional significance, this can hardly be postulated about the “hump” on the back of the dorsal fin attachment. In this case, the effect of sexual selection or its use in competitive interactions seems a more parsimonious explanation. In general, the pectoral fin base in *Danakilia* is set obliquely on the flank, but it is more vertical in Afr and Aba than in the other populations. According to Thorsen *et al.* (2005), a more vertical insertion of the pectoral fin indicates a rowing specialist, compared to flapping specialists, where flapping specialists are designed to achieve and maintain higher swimming speeds than rowing specialists (Walker and Westneat 2002). The more vertical pectoral fin present in Afr and Aba, may be suggestive of ecological adaptation to the more stable conditions found in creeks (Afr) and in lake Abaeded (Aba), compared to those found in rivers subject to flash floods.

It is common textbook knowledge that mouth position in fishes correlates with where a fish lives and feeds in the water column. The vast majority of fishes have terminal mouths, which is also the situation we find in all populations of *Danakilia*, with minor average shape shifts from the grand mean in different populations: in particular, Afr presents a more dorsal terminal mouth with an upward gape; Zar shows a downward sloping terminal mouth; Aba and Shu have terminal mouths; GCo a

more ventral terminal mouth. Any hypothesis suggesting adaptive significance of these subtle differences would be, however, far-fetched, as current knowledge of the biology of these fish suggest that all the populations have a vegetarian diet based on filamentous algae, ubiquitous in the sampling locations (Trewavas 1983, Stiassny *et al.* 2010, G. Chiozzi, G. De Marchi, A. Lamboj and M. Fasola pers. obs.). In fact, episodic ecological observations in the field and anatomical dissections of some specimens do not support differences among populations in general habitat characteristics and food preferences. These sampling sites are also rather uniform in relation to physical and chemical water parameters (Chiozzi *et al. submitted*) while the inspection of the digestive tract of the dissected specimens shows a small stomach and an elevated Relative Gut Length (intestinal length/Standard Length: average=8.04, range=6.80-10.43, n=4, G. Chiozzi & M.L.J. Stiassny unpublished data) indicating a vegetarian diet (Zihler, 1982). This apparent uniformity of environment and feeding habits also renders an adaptationist interpretation of the differences we observed in pharyngeal jaw shape extremely difficult.

The lower pharyngeal jaw in cichlids is a feature that is strongly linked to dietary specialization (Liem 1973). Phenotypic plasticity can contribute to variation in fish body (Pakkasmaa & Piironen, 2001; Eklöv & Jonsson, 2007; Burns *et al.*, 2009) and lower pharyngeal jaw shape (Muschick *et al.* 2011; Huysseune *et al.*, 1994; Gunter *et al.* 2013). In particular, a number of studies have shown through common-garden experiments that fish fed a diet of hard items more robust and heavier (more dense) lower pharyngeal jaws, with broader horns (more robust) and a more pronounced posterior margin (Muschick *et al.*, 2011; Gunter *et al.*, 2013). However, more recent studies have also highlighted similar shape changes associated to variation at quantitative trait *loci* (Fruciano *et al.*, 2016). Interestingly, while Afr and GCo, show divergent body shapes and mouth positions, they have quite similar “slender” lower pharyngeal jaws. Yet, the lower pharyngeal jaw shape of Afr differs slightly from all the other populations in having less robust horns and a flatter posterior outline. The differences observed in the lower pharyngeal jaw shape of *Danakilia* could thus possibly reflect differences in diet, with populations showing more robust horns and more undulate posterior outline likely eating harder food items. As the available information points to a uniform diet across the genus *Danakilia*, it is hard to postulate that the variation of pharyngeal jaw shape we observe is the product of phenotypic plasticity or adaptation to different food sources. While, in the absence of a year-round knowledge of the environmental variability, we cannot completely rule out these explanations, a more parsimonious explanation is that the effect of neutral and stochastic processes – such as bottlenecks – have contributed to sorting of ancestral polymorphism.

The comparison among the Lake Afrera sample (Afr) and the rest of the Danakil Depression populations suggests a north-south geographic pattern in which the most remote and isolated

Danakilia population (Afr) is also the most differentiated both for body and lower pharyngeal jaw shapes. Indeed, while we did not observe any meaningful pattern of co-variation between body and pharyngeal jaws – or variation of the strength of their association between the two species of the genus – these analyses substantially confirmed the distinctiveness of the population from Afr. In fact, the only easily interpretable pattern of covariation we found was obtained subjecting all the populations to partial least squares. The apparent pattern of covariation, however, was driven by the distinctiveness of Afrera, which is different in both body and pharyngeal jaw shape.

On the other hand, the northern Danakil Depression populations (Aba, Shu, Zar and GCo), appear more uniform and partially overlapping. This is the same pattern observed at neutral genetic *loci* (Chiozzi et al. submitted).

Chiozzi et al. (*submitted*) have also presented an hypothesis for the evolutionary history of this genus: a not yet identified ancestral tilapiine from the Nile or Awash drainage basins dispersed in the Danakil Depression following the formation of a paleo-lake flooding the area (5720 km²) below the –50 m contour during mid-Holocene (about 7800-7300 BP). Climatic evolution towards drier conditions then caused the dessication of the paleo-lake and the segregation of *Danakilia* populations in their present-day locations, endorheic lakes and rivers separated by wide extensions of scorching arid-land. In particular, *D. franchettii* (Afr) lives in groundwater fed brackish thermal springs that flow in the hypersaline endorheic Lake Afrera, at about 150 km from its nearest neighbour population (GCo); *D. dinicolai* (Aba) is enclosed in the endorheic crater lake Abaeded, at about 170 km from the nearest known Afr population and 20 km from the nearest riverine population (Shu); the riverine populations (Shu, Zar, GCo) inhabit temporary pools along the bed of ephemeral rivers distant less than 10 km one another.

Higher shape similarity between *D. dinicolai* (Aba) and the riverine populations (Shu, Zar, GCo) can be explained by a longer-lasting contact between these populations in the paleo-lake. Also, we cannot rule out that the riverine populations (Shu, Zar, GCo) can occasionally exchange migrants at the rivers' alluvial fans in case of exceptional flash floods (Chiozzi *et al. submitted*), thus contributing to their morphological similarity.

CONCLUSIONS

Our study, the first on the cichlid genus *Danakilia*, provides strong evidence that *Danakilia* is a unique desert fish for both body and lower pharyngeal shapes.

While the observed discrimination between *D. franchettii* and the remaining populations does contribute to support its specific status, the taxonomic position of *D. dinicolai* with respect to the remaining populations of *Danakilia* is more uncertain and these might represent different species or,

alternatively, local variants of the same species. All in all, our findings point to a recent separation of the different populations that is coherent with the low level of discrimination found at mitochondrial markers in a previous study (Chiozzi *et al. submitted*). This encourages in-depth investigation with a genomic approach (dd-RADseq, Peterson *et al.*, 2012) that, by considering large sets of SNP, is advised as the most appropriate to study young radiations (Shaffer and Thomson, 2007).

Significant population differentiation in *Danakilia* could have important implications for the conservation effort. The endemic genus *Danakilia* has a fragmentary and limited range in the Danakil Depression that can in itself influence its vulnerability to extinction (Fagan *et al.*, 2005). Even if at present we lack a complete conservation assessment of the genus (limited to *D. franchettii*, classified as Endangered by IUCN: Getahun, 2010), once more the main threats to an ecosystem come from human activity. Growing interests for industrial salt extraction affect the Lake Afrera area since about two decades with important effects on fish habitats: water pumped from the lake for salt production is suspected to affect its level, while salt pans are believed to have covered many thermal springs (Getahun, 2001). In the Danakil Depression of Eritrea, a multinational potash mining project is in progress, with potential high impact on the environment for land use (plants, evaporation ponds, roads) and large scale surface and groundwater consumption. Moreover, in a scenario of global climate change and increasing desertification in the Sahel (Mitchell 2013), it is not treacherous to think that also the delicate water ecosystem of the Danakil Depression may suffer from heavy repercussions, as it already happened in other desert areas (Trape 2009, Hillyard *et al.* 2015) and is expected to happen in the next decades in many drainage basins of the World (Xenopoulos *et al.* 2005). Among the first to suffer these repercussions could be the cichlids of the genus *Danakilia*, in which we identify significant phenotypic variation in key traits among all known populations. This phenotypic variation – which cannot be explained by phenotypic plasticity and despite of limited differentiation at low-resolution genetic markers – suggests that each population might be an evolutionary significant unit (Ryder, 1986; Moritz, 1994), in need of attention and protection.

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SUPPLEMENTARY FIGURES AND TABLES

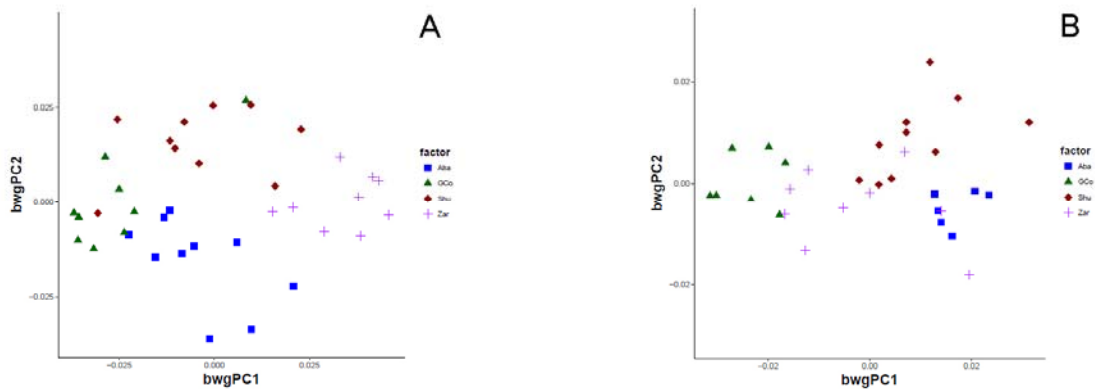


Figure S1. Scatterplots for the first two principal components of between-group principal component analyses for body shape (A) and lower pharyngeal jaw (B). Sampling sites define groups. The Lake Afrera population (Afr) is here excluded from the analyses.

Body shape CVA – cross-validation

cross-validated classification results in frequencies

	Aba	Afr	GCo	Zar	Shu
Aba	4	1	0	2	3
Afr	0	9	1	0	0
GCo	1	2	2	0	4
Sar	0	0	0	7	2
Shu	0	1	3	3	3

cross-validated classification result in %

	Aba	Afr	GCo	Zar	Shu
Aba	40.000	10.000	0.000	20.000	30.000
Afr	0.000	90.000	10.000	0.000	0.000
GCo	11.111	22.222	22.222	0.000	44.444
Sar	0.000	0.000	0.000	77.778	22.222
Shu	0.000	10.000	30.000	30.000	30.000

Overall classification accuracy: 52.08333%

Kappa statistic: 0.40065

LPJ shape CVA – cross-validation

cross-validated classification results in frequencies

	Aba	Afr	GCo	Zar	Shu
Aba	4	0	0	2	0
Afr	0	9	0	0	0
GCo	0	0	7	0	0
Sar	0	0	1	6	2
Shu	0	0	0	2	8

cross-validated classification result in %

	Aba	Afr	GCo	Zar	Shu
Aba	66.667	0.000	0.000	33.333	0.000
Afr	0.000	100.000	0.000	0.000	0.000
GCo	0.000	0.000	100.000	0.000	0.000
Sar	0.000	0.000	11.111	66.667	22.222
Shu	0.000	0.000	0.000	20.000	80.000

Overall classification accuracy: 82.92683%

Kappa statistic: 0.78421

Table S1. Canonical Variates Analysis (CVA) of groups correct classification using sampling site as a priori known group, with leave-one-out cross-validation. CVA cross-validated correct classification is overall low revealing a certain degree of overlap in shape between sampling sites, but Afr stands out for its very high cross-validated correct classification. Cohen's kappa coefficient measures agreement for categorical items. This statistic takes into account the possibility of the agreement occurring by chance: $k=1$ indicates complete agreement.

CHAPTER 4

STUDIES IN PROGRESS AND IN PERSPECTIVE



A remnant strip of riparian vegetation along the shores of threatened Lake Afrera (Ethiopia).

dd-RAD sequencing: an innovative genetic tool for investigating recent speciation

The presently differentiated *Danakilia* morphospecies of the Danakil Depression are the product of a recently formed environment and successive separation(s) in isolated water-bodies (see Chapter 2). As reported in the Introduction (Chapter 1) and in Chapters 2 and 3, the diversification of the existing morphospecies results evident for comparison of morphometric traits between *D. franchettii* and *D. dinicolai* (Stiassny *et al.*, 2010), for the different coloration of the breeding males in *D. franchettii*, *D. dinicolai* and the three undescribed riverine populations (see Chapter 2) and for the body and lower pharyngeal shapes across the genus' entire range (see Chapter 3). Nevertheless, their genetic diversification, at least at the investigated mitochondrial DNA markers (*COI* and *cytb*), shows poorly evident (see Chapter 2).

A 648 bp portion of the cytochrome c oxidase *subunit I* (*COI*) region of mtDNA is widely used as a marker in the so-called DNA barcoding approach for species recognition (Hebert *et al.*, 2003), its use having been widened also to commercial and forensic aims, especially in consideration of its relatively low cost and rapid utilization. Functioning as a portal to BOLD (Barcode of Life Data Systems; <http://boldsystems.org/>), the Fish Barcode of Life Initiative (FISH-BOL; <http://www.fishbol.org>), launched in 2005 (Ward *et al.*, 2009; Becker *et al.*, 2011), is a global effort to assemble a standardized reference sequence library for all fish species derived from voucher specimens with authoritative taxonomic identifications. The benefits of barcoding fishes include facilitating species identification for all potential users perhaps, most importantly, enabling identifications where traditional methods are not applicable.

It is a common erroneous misconception to intend DNA barcoding as a flawless tool for species description (which is not) in spite of considering it mainly as a valid instrument for species identification. In this case, DNA sequences are used as markers for *a priori* established species, whose morphological identification should be based on sound taxonomic authority. DNA barcoding does not rely on an established species concept, but is consistent with any species concept that a taxonomist used to name a species (Rach *et al.*, 2008). Conversely, species delimitation requires a species concept to be adopted by the taxonomist and no single instrument, whether it is DNA, morphology, ecology, reproductive isolation or behavior, can be in itself used to discover (and then describe) species (De Salle *et al.*, 2005; De Salle, 2006).

However, DNA sequences can be used to tag potential new species units. This is particularly important in the framework of conservation biology, when the management of global diversity often requires rapid instruments for the assessment and identification of lineages in need of protection, also in consideration that understudied taxa are disproportionately vulnerable to extinction to the respect of described ones (Rubinoff, 2006).

One of the critical issues with DNA barcoding is the threshold at which consider two specimens belonging to separate species or not (Collins and Cruickshank, 2012). The use of a distance-based threshold technique has been a major point of contention since the beginning of DNA barcoding (Moritz and Cicero, 2004; Hickerson *et al.*, 2006; Hebert *et al.*, 2004). In particular, Hebert *et al.* (2004) suggested that, because patterns of interspecific and intraspecific variation in COI appear similar in various distant animal groups, specimens showing >10 times the average intraspecific distance for the group under study should be flagged as “provisional (new) species”. Soon after its publication, this approach encountered criticisms and even if certainly helping to flag situations worth a deeper taxonomic study (Baker *et al.*, 2009), it will necessarily fail to recognize recently radiated or strictly related species (Ward *et al.*, 2009 and references therein). Threshold is not a wrong approach *a priori*, but it is not universally applicable and it works well when calibrated on the group of organisms under investigation. In DNA barcoding analyses, taxonomic inference better rely on a molecular threshold based on the comparison between intraspecific and interspecific distribution of nucleotide divergence. This approach can lead to two kinds of error: type I error (false positive: when specimens belonging to the same species show a genetic distance greater than the threshold value) and type II error (false negative: when specimens belonging to different species show a genetic distance minor than the threshold value). The Optimum Threshold (OT) is defined as the threshold value relative to the minor rate of cumulative error (error rates generated by Type I and Type II on different threshold values). When a range of threshold values is relative to the same minimum cumulative error, the OT is calculated as the average value of the range (Casiraghi *et al.*, 2010; Ferri *et al.*, 2009). Recently, in order to standardize taxonomic definitions, Galimberti *et al.* (2012) introduced the concept of Integrated Operational Taxonomic Unit (IOTU) that incorporates both molecular and taxonomic characters to define biological entities: IOTU comes from the Operational Taxonomic Units (OTU: Sokal and Sneath, 1963) and parallels the Molecular Operational Taxonomic Unit (MOTU: Floyd *et al.*, 2002).

The cytochrome *b* (*cyt b*) region of mt-DNA (approximately 1140 bp), has frequently been used in identification of genetic discontinuity among distinct intra-specific lineages resulting from alternative colonization patterns, and the recognition of spatially distinct haplogroups in vertebrates (e.g. Johns and Avise, 1998; Zardoya and Doadrio, 1999; Galván-Quesada *et al.*, 2016).

In this operational framework, the results emerged from my analyses (see Chapter 2), even if highlighting a weakly supported clustering of the *Danakilia* genus across the Danakil Depression that is also convincingly supported by morphometric analyses (see Chapter 3), appear rather inconclusive in defining the limits of the various *Danakilia* morphospecies, most probably young lineages early in the process of speciation that became separated only quite recently (7300 BP).

In fact, rather than using phylogenetic inference methods and detect gene flow more efficiently (Martin *et al.*, 2015), recent speciations are better studied using alternative genetic techniques (Next Generation Techniques). These techniques employ thousands of single-nucleotide polymorphisms (SNPs) as genetic markers across the entire genome of sizable numbers of specimens belonging to congeneric species or different populations of the same species, even in non-model organisms (e.g. Shaffer and Thomson, 2007; Hohenlohe *et al.*, 2010).

In particular, the recently developed restriction site-associated DNA sequencing (RADseq: Baird *et al.*, 2008), while allowing for genotyping of multiple individuals with substantially reduced sequencing investment, shows some limitations, including the possibility to precisely tune the fraction of genome sampled adapting it to the experiment, and poor efficiency while analyzing results with existing computational tools in the absence of a reference genome (Peterson *et al.*, 2012 and references therein).

Recently, the double digest restriction-site associated DNA sequencing (dd-RADseq) was proposed by Peterson *et al.*, (2012) as an effective improvement of the RADseq laboratory protocol. While allowing for genotyping across large numbers (hundreds or more) of individuals for a range of markers (hundreds to hundreds of thousands), dd-RADseq does not require prior genomic knowledge of the studied organism(s) and achieves lower costs than “SNP chip” microarray-based technology usually employed for the detection of polymorphisms.

In the framework of the collaborative research project between the Earth and Environmental Science Department of the University of Pavia and the Department of Ichthyology of the American Museum of Natural History (AMNH), during a one-month period spent at the AMNH (May-June 2016), I carried out the preliminary phases for the application of the dd-RADseq technique for SNPs detection to a sample of 96 cichlid specimens. These included *Danakilia franchettii* from two creeks flowing to Lake Afrera (Ethiopia), *D. dinicolai* from Lake Abaeded (Eritrea), three populations of *Danakilia* sp. from the temporary rivers Shukoray, Zariga and Gali Colluli (Eritrea). Outgroup included: *Iranocichla hormuzensis* (3 tissue samples, River Merhan, Iran) and *I. persa* (1 tissue sample, River Rudan, Iran), courtesy Ole Seehausen (EAWAG); *Oreochromis niloticus*, 2 tissue samples, River Mara (Tanzania) and Lake Ziway (Ethiopia) and *Sarotherodon galilaeus*, 3 tissue samples (Cameroon, Bas Congo and middle Congo river) from the collections of the AMNH.

The dd-RAD library preparation was carried out at the facilities of the Sackler Institute for Comparative Genomics of the AMNH, following the protocols indicated in Peterson *et al.*, 2012.

Genome sequencing on a single lane of an Illumina HiSeq 4000 System were carried out at the Genomic Sequencing and Analysis Facility (GSAF) of The University of Texas at Austin

(<https://sites.cns.utexas.edu/cbrs/genomics>).

To perform successive analyses, still in progress, we used the Ipyrad pipeline, without a reference genome (<http://ipyrad.readthedocs.io/>). The analyses are aimed at elucidate the phylogenetic relationship among the different *Danakilia* morphospecies (Fig. 9) and to form the basis for future taxonomic speculations at the population, species, genus and family levels. Restriction site-associated DNA sequencing (RADseq and derived methods, such as dd-RADseq) can be used to answer a wide variety of ecological, evolutionary and conservation-related aims (Andrews *et al.*, 2016), opening a vast array of research opportunities for future studies on *Danakilia*. In general, and in fish-related studies in particular, these aims include genomics of adaptation (e.g. Hohenlohe *et al.*, 2010), inbreeding and genomic diversity (e.g. Perrier *et al.*, 2017), effective population size (e.g. Candy *et al.*, 2015; Larson *et al.*, 2014), population structure, phylogeography and conservation units (e.g. Larson *et al.*, 2014; Gaither *et al.*, 2015), introgression (e.g. Ford *et al.*, 2015; Hohenlohe *et al.*, 2013) and phylogenomics (Wagner *et al.*, 2013).

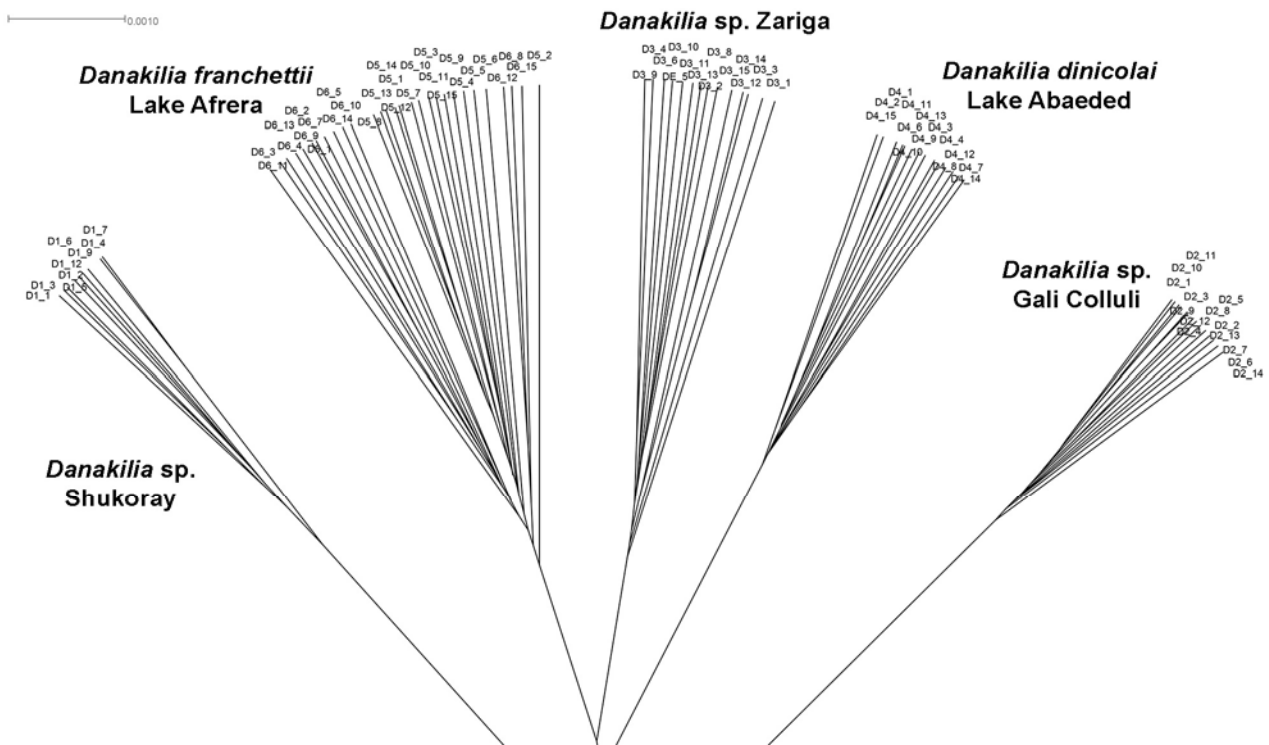


Fig. 9. Partial rooted phylogram of the sampled *Danakilia* populations of the Danakil Depression, an example output obtained through the preliminary Ipyrad analyses. The different populations, in a genome-wide framework, appear well differentiated. Notice how the *D. franchettii* cluster branches in conformity with the two sampling brackish springs flowing to hypersaline Lake Afrera, a sign that populations D5 and D6 are ecologically separated (cf. Chapter 2).

How many fish species do live in the Danakil Depression? Taxonomy of *Danakilia* and *Aphanius*

The investigation of the water-bodies of the Danakil Depression of Eritrea and Ethiopia has led to the discovery of new populations in the genera *Danakilia* and *Aphanius*. Successive genetic analyses based on a barcoding approach (Chapter 2) showed inconclusive for *Danakilia* despite the morphological differences between *D. franchettii* and *D. dinicolai* (Stiassny *et al.*, 2010), the colour variation for nuptial colouration of males among the sampled populations (Chapter 2) and body and lower pharyngeal jaw shapes in a geometric morphometric approach (Chapter 3). However, the same genetic analyses suggested the existence of an unexpected array of putative new taxa in the apparently morphologically uniform populations of *Aphanius* cf. *dispar* (Chapter 2). Overall, the investigations provided interesting insights into the speciation process of both *Danakilia* and *Aphanius*, revealing a pattern of diversification in young lineages and clues for recent speciation.

Alpha taxonomy (Turill, 1938), especially in the case of understudied taxa living in remote and largely unexplored areas, such as the fish of the Danakil Depression, is particularly important in order to provide a fundamental instrument for conservation (Dubois, 2003; Andreone *et al.*, 2014) before any further action is taken on the environment. For this reason, the assessment of the taxonomic status of the fishes of the Danakil Depression is of fundamental importance.

In the framework of a classical, alpha taxonomic approach, we started measuring morphometric and meristic characters traditionally employed in fish taxonomy (Barel, 1977; Holčík, 1989) of both *Danakilia* and *Aphanius*. We obtained a set of 25 measurements and 24 counts for *Danakilia* species and morphospecies. For *Aphanius*, we obtained 26 measurements and 11 counts.

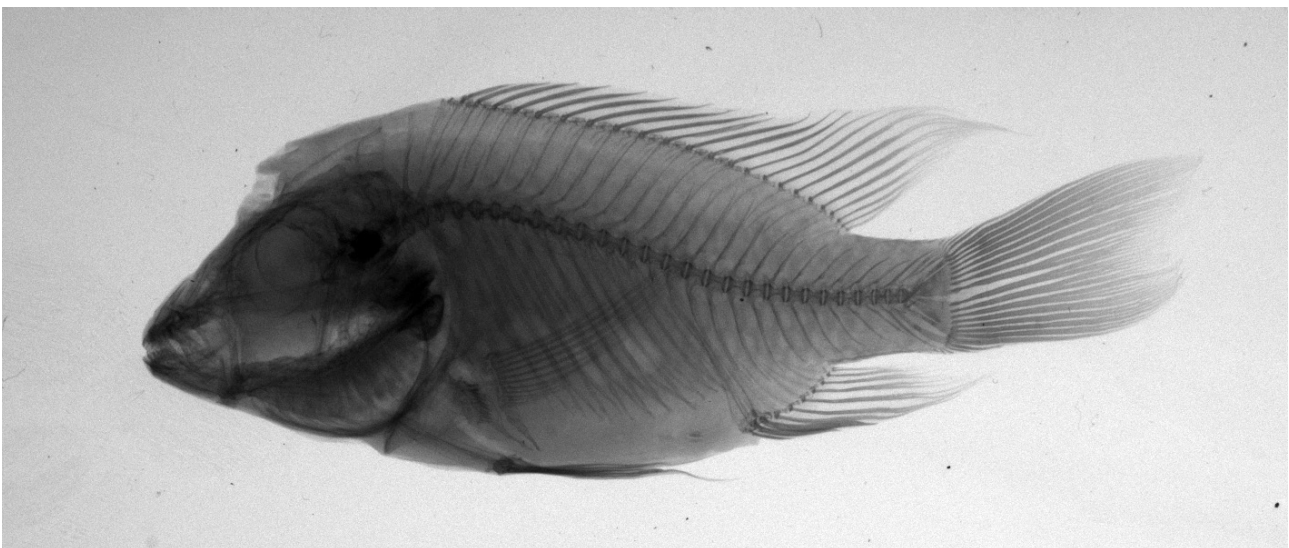


Fig. 10. An x-ray image of a male *Danakilia dinicolai* specimen obtained at the facilities of the AMNH.

Meristic characters (vertebral and fin-ray counts, in particular) were studied by employing x-ray

images (Fig. 10) and clearing and staining of specimens (Cailliet *et al.*, 1986).

Species descriptions require the scrutiny of broad series of specimens (especially type specimens kept in natural history collections) in order to compare not only the intraspecific, but also the interspecific variability within the same genus and in the closer genera (Winston, 1999). Moreover, the array of sampled populations should be as complete as possible.

The study is still in progress with the collaboration of expert fish taxonomists of the Department of Ichthyology of the American Museum of Natural History (M. L. J. Stiassny), the Department of Integrative Zoology of the University of Vienna (A. Lamboj) and the Associazione Italiana Killifish (S. Valdesalici).

The nuchal hump of *Danakilia*: is it really that strange and why is it present?

The most striking feature distinguishing the fishes of the genus *Danakilia* is without doubt the impressive nuchal hump that develops in sexually mature males (Fig. 11) in each one of the observed morphospecies.

The French ichthyologist Jacques Pellegrin in his classic study on the family Cichlidae (Pellegrin, 1904) dedicated an important part of his paper to describe this anatomical feature in fish, in general, and in cichlids, in particular. At histological examination, Pellegrin (1904) found that the hump of *G. brasiliensis* is formed by connective tissue and, to a minor extent, of fat. Alternatively, according to Bleick (1975), the nuchal hump of the Midas Cichlid *Cichlasoma citrinellum* is not a lipoma, but a local edema, with fluids flooding the tissues in a day or two.



Fig. 11. A male *Danakilia dinicolai* in nuptial colouration while defending its bower in Lake Abaeded (Eritrea).

Episodic field and aquarium observations carried out during the present research, even if not corroborated by an adequate sample size and standardized measurements or observations, seem to indicate a correlation between the appearance of this bulbous swelling on the forehead of male *Danakilia* to their physiological condition. In fact, a rise of water temperature in aquaria in the range of 28-32 °C induces females to spawn and males to enter in a reproductive condition that, apart from being shown with the dig of a bower, aggressive behaviour towards conspecifics of both sexes and courtship behaviour, it is signalled by the growth of the nuchal hump

It is also possible that the development of a big hump is also partly conditioned by the age of the male, as it was observed by Pellegrin (1904) for *Geophagus brasiliensis*. In fact, male *Danakilia* of two years can grow bigger humps than younger males that start showing nuchal hump growth and courtship behaviour when only about 5 cm SL (age about 0.5-1 a). Conversely, two years old subordinate males sharing the same aquarium do not show such big humps. Evidence based on episodic observations on aquarium bred *Danakilia* suggests that reduction of the hump can take only a few days (<5 d) and corresponds to a visible decline of the aggressive behaviour. For all these reasons, a hormonal control on hump development and reduction can be hypothesized; in fact, growth was experimentally induced in males of the Midas Cichlid *Cichlasoma citrinellum* with the administration of mammalian gonadotropins, and reduction via the administration of fish prolactin and, to a minor extent, of sheep prolactin (Bleick, 1975).

Nuchal hump in monogamous Midas cichlids (Barlow and Siri, 1995; Barlow, 1998; Barlow, 2000) represents a male sexually selected trait that shows like a flag for sex recognition during pair formation, but during spawning, it subsides. In a fashion, the lack of a nuchal hump becomes a clue to male femaleness for the paired female, thus reducing intra-pair aggressiveness.

This is probably not the case with *Danakilia* that likely exhibits a polygynous or polygynandric mating system (cf. Chapter 1).

In order to find a pattern for the presence of this trait in Cichlidae, I carried out a complete search of the 1770 species at present included in the family. The search was made on bibliographic and web-based information, in the latter case employing only websites reporting accredited facts compiled by trustworthy authors (e.g. FishBase, The Cichlid Room Companion). The search produced a list of 97 cichlid species (5.5%) showing a more or less pronounced nuchal hump.

The following information for each species was collected for now:

- *Taxonomy*: Genus, Species and Subfamily, this last after Van der Laan *et al.*, (2014);
- *Biogeography*: Geographic Subregion (range) and Freshwater Ecoregion (Abell *et al.*, 2008);
- *Nuchal hump*: presence of a nuchal hump in males only or in both males and females;
- *Mating system*: after Barlow (2000) (cf. Chapter 1 for a description);
- *Parental care*: type of parental behaviour (Barlow, 2000);
- *Colour dimorphism*: present or absent;
- *Spawning site*: type of substrate for spawning;
- *Habitat*: lotic or lentic.

The hypothesis of the existence of a direct causal link among the different variables will be tested in a model. Path analysis is a statistical method used to test multivariate hypotheses about the cause–effect links between variables and allows to quantify these links and to separate the causal effects (Shipley, 2004). To evaluate the role of the phylogenetic signal, also the taxonomic information will be included in the model (von Hardenberg & Gonzales-Voyer, 2013).

Conservation of the Danakil Depression fish assemblages: challenges and possible solutions.

At present, the conservation assessment of the fish of the Danakil Depression is partially limited by the lack of a complete knowledge of the taxonomy of the two genera inhabiting the area. Strong signals coming from the present research (Chapter 2 and 3) may indicate the presence of more species than the four today recognized for this desert region.

Today, the conservation effort placed on the Danakil Depression is still weak. The Government of Eritrea, through the establishment of a National Board, recently indicated the Buri-Irrori area as pre-eminent for the conservation of the IUCN Critically Endangered African Wild Ass (*Equus africanus somalicus*), a species which is object of in-depth research carried out by Eritrean experts in collaboration with foreign research and conservation agencies. This proposed Nature Reserve virtually encompasses only a part of the northern Danakil Depression. On the Ethiopian side of this desert region, no protected areas exist at the moment or are seemingly planned for the future and this results in a major gap in the protected areas network in the Afrotropical Realm (Stuart *et al.*, 1990).

The most important action undertaken for conservation of the endemic fish of the Danakil Depression until now was directed at assessing the conservation status of two species. In particular, even if the endemic genus *Danakilia* as a whole is immediately recognizable as worth the attention of conservation specialists for its fragmentary and limited range in the Danakil Depression (in itself a reason for concern: Fagan *et al.*, 2005), only one species (*D. franchettii*) was given a conservation assessment, that resulted in a IUCN Endangered classification (Getahun, 2010a) and formal protection by the Ethiopian law. Conversely, the genus *Aphanius*, with its broader range in coastal East Africa and the Near East, counts only one endemic species (*A. stiassnyae*) in the region, that was assessed as IUCN Endangered (Getahun, 2010b) and was put under legal protection.

Many threats can potentially jeopardise the survival of the fish taxa of the Danakil Depression.

- *Seismic and volcanic hazards.* Afar (with the Danakil Depression) is a dynamic geological hotspot (Mohr, 1978) and earthquakes and volcanic activity can dramatically and sometimes rapidly impact on the environment: for example, in 2005 the largest volcanic vent ever observed on land (400×80 m) rapidly opened not far from Lake Afrera, in the Dabbahu volcano area, when an impressive earthquake swarm ruptured a 60 km long rift segment (Yrgu *et al.*, 2006). Volcanic eruptions can potentially affect the Danakil Depression water-bodies with the emission in the atmosphere and successive fallout of ashes, causing turbidity, and strong mineral acids, such as H₂SO₄ and HCl, causing acidification of water (Stewart *et al.*, 2006).

- *Climate change.* In a scenario of global climate change and increasing desertification in the Sahel (Mitchell, 2013), it is not treacherous to think that also the delicate water ecosystem of the Danakil Depression may suffer from heavy repercussions, as it already happened in other desert areas (Trape, 2009; Hillyard *et al.*, 2015) and is expected to happen in the next decades in many drainage basins of the World (Xenopolous *et al.*, 2005).



Fig. 12. Salt extraction in the area of Lake Afrera (December 2015-January 2016).

- *Salt extraction.* The west coastal area of Lake Afrera, now reachable by tens of trucks through a newly paved road (G. Chiozzi and G. De Marchi, pers. obs.), is menaced by industrial salt mining started about 20 years ago (Fig. 12). Intensive pumping of water likely affects the lake's level and its salinity that dropped from 158 g/l in 1968 (Martini, 1969) to 130 g/l in 2016 (Chiozzi *et al.*, submitted), while the salt pan coverage over most of the area is possibly the cause of the disappearance of many thermal springs and, possibly, of some fish populations (Getahun, 2001). The loss of most of the riparian vegetation belt is likely attributable to its exploitation for timber, firewood and fodder and is certainly linked to the dramatic demographic increase in an area once underpopulated (G. Chiozzi and G. De Marchi, pers. obs.).
- *Potash mining.* An important potash mining project was recently inaugurated (2015) in the Danakil Depression of Eritrea (Colluli area): development plans will potentially consume

huge amounts of land at the mouth of rivers hosting *Danakilia* populations for the stocking of ore; a truck road, with predictable important impact on the terrestrial mega-fauna, will connect the mining area to the 75 km distant Red Sea coast (Bay of Anfile) where a port for cargo boats is going to be dig. Besides, large amounts of groundwater will be employed in the mining process with foreseeable impact on the whole desert ecosystem.

Urgent action is needed for the conservation of the water-bodies of the Danakil Depression and this shall be achieved through strict regulation of the mining activity, the implementation of recovery plans for the exploited areas and the planning of protected areas in the still untouched parts of the territory following interdisciplinary surveys aimed at assessing their ecological value. Simultaneously, alternative forms of economy can be implemented in order to limit the impact of intensive industrial activities, among which ecotourism is one of the most promising, given the spectacular natural sceneries and fascinating anthropological perspectives offered by the area (Erta Ale and Dallol volcanoes, Lake Afrera, Lake Abaeded, Afar pastoralist culture, etc.).



Fig. 13. A female *Danakilia* sp. mouthbrooding in aquarium conditions.

In order to provide immediate relief to the threatened fishes of the Danakil Depression, a captive breeding program (Fig. 13) was partly achieved in the framework of the present research for the three

riverine populations of *Danakilia* and one riverine population of *Aphanius* (Shukoray) with the involvement the fish department of the Vienna Zoo and of expert fish hobbyists in Europe and in in North America. In fact, the participation of public institutions (zoos and aquaria) and, secondarily, of passionate and expert fish hobbyists to captive breeding programmes has revealed a good strategy for *ex situ* conservation of some endangered fish species (Reid *et al.*, 2013; Maceda-Veiga *et al.*, 2016).

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COURSES AND CONFERENCES

Courses

Imparare a progettare in Europa (2 CFU). Aula Giulotto, Dipartimento di Fisica, Università degli Studi di Pavia, 10-11-18 febbraio 2015

Linguaggi, problemi e metodi della comunicazione della ricerca scientifica (8 CFU). Alessandro Bacchetta, Gianluca Mainino, Marco Cagnotti. Collegio Nuovo – Fondazione Sandra e Enea Mattei, 3 marzo-19 maggio 2015

Causal inference in ecology (3 CFU). Achaz von Hardenberg. DSTA, Università degli Studi di Pavia, 22-23-24 aprile 2015

Rappresentazione ed analisi dei dati (6 CFU). Roberto Sacchi. DSTA, Università degli studi di Pavia, 1 ottobre 2015-14 gennaio 2016

How to write a scientific paper (1 CFU). B. Jiménez-Alfaro, A. Mondoni. DSTA, Università degli Studi di Pavia, 5 febbraio 2016

Cambiamenti globali ed impatto sugli ecosistemi (3 CFU). A. Occhipinti, A. Mondoni, G. Rossi. DSTA, Università degli Studi di Pavia, 2-3-7-23 marzo 2016

Genetica della conservazione (6 CFU). Antonio Torroni, Anna Olivieri. Università degli Studi di Pavia, 4 ottobre-15 dicembre 2016

Conferences

XXIII Congresso Nazionale Associazione Italiana Ciclidofili. Congress, Museo di Storia Naturale della Certosa di Calci, Calci (PI), 19-20 settembre 2015

Akvariehelg mit Ciklidstämna. Congress, Oslo Akvarieklubb. Oslo (Norvegia), 8-10 aprile 2016.

XXIV Congresso Nazionale Associazione Italiana Ciclidofili. Congress, Hotel Holiday Inn, Torino, (TO), 17-18 settembre 2016

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- De Marchi G., **Chiozzi G.** & Fasola M. 2017. Predatory risk favours intermittent incubation of a colonial burrowing shorebird, the Crab Plover *Dromas ardeola*. *Journal of Ornithology*, 158(4): 1081-1090.
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