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Valencia robertae, a new killifish from southern Greece (Cyprinodontiformes: Valenciidae)

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Valencia robertae, new species, from the lower Pinios in northern Peloponnese and Mornos Rivers in southern mainland of Greece is distinguished from *V. letourneuxi* and *V. hispanica* by having short lateral bars or vertically elongated small blotches along the midlateral body and an almost triangular anal fin in females, prominent lateral bars between the axial blotch and the caudal-fin base and a long anal fin reaching almost or to the first caudal-fin rays in males larger than 27 mm SL. It is also distinguished by 32 fixed, diagnostic nucleotide substitutions in the mtDNA COI barcode region.

Introduction

The killifish family Valenciidae is the only fish family endemic to Europe and the Mediterranean basin. *Valencia* is the only genus in Valenciidae (Myers, 1928; Parenti, 1981), and two species are known: *V. hispanica*, the type species, (Valenciennes, 1846: 214) described from Catalonia, Spain, and *V. letourneuxi* (Sauvage, 1880) described from Kerkyra Island [Corfu], Greece. Gomez Caruana et al. (1984) described an additional species from Spain, *Valencia lozanoi*; Fernández-Delgado et al. (1986) identified it as an alien species of the new-world genus *Fundulus*.

The ranges of the two currently valid *Valencia* are largely disjunct. *Valencia hispanica* is endemic to the central Mediterranean coast of Spain between Tortosa and Cape San Antonio, and *V. letourneuxi* is endemic to Western Greece and Albania between Lake Butrint (Albania) and Alfios River (Peloponnese) drainages (Kottelat & Freyhof, 1997). Both species usually inhabit densely vegetated springs, slowly flowing streams and marshes close to the Mediterranean coast where they are often the victims of habitat modifications (Kalogianni et al., 2010). *Valencia* species are also very sensitive to competition and predation by alien *Gambusia holbrooki*, which has invaded almost all of their habitats (Bianco & Miller, 1989; Planelles & Reyna, 1996; Barbieri et al., 2000; Kalogianni et al. 2010). Therefore, both species are of major conservation concern. Due to their continuing deteriorating conservation status, both have been included (as *V. hispanica*) in the Appendix II of the Bern Convention as endangered and strictly protected species, and have been characterized as priority species for conservation in Annex II of the European Union Habitats Directive 92/43/EEC (Barbieri et al., 2002; Kalogianni et al. 2010). In 1996, both *Valencia* were assessed as Endangered by IUCN, and in 2005 reclassified as Critically Endangered (Crivelli, 2006a–b).

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Valencia letourneuxi is further protected by Presidential Decree No. 67/1981 of the Greek State (Barbieri et al., 2002) and has been recently listed among "The World's 100 most threatened species" (Baillie & Butcher, 2012). Due to the strict conservation legislations, special permissions are needed to collect *Valencia* in the wild. As several populations are still declining (see Perdikaris et al., 2010 for Greek Kalamas population) and many populations seem to be small, collection should be limited to a minimum and only for important reasons.

Within an European initiative to generate DNA barcode data for all freshwater fishes of Europe (www.fredie.eu), we received materials of *V. hispanica* from the tissue collection of the National Museum of Natural Sciences in Madrid (I. Doadrio, pers. comm.). No tissues of *V. letourneuxi* were available in collections, but at least four captive populations of this species exist since before the mid-1990th in Europe (fom Corfu, Pinios, Mornos and Lake Butrint). Furthermore, in 2011, JF, Maria Stoumboudi & Roberta Barbieri caught one female *V. letourneuxi* (Fig. 9) in the lower Acheron, from which a fin could be taken. Sequencing the mitochondrial COI barcode region from the material mentioned above, revealed that the Pinios and Mornos populations are genetically very distinct from the other three populations examined. It had never been stated in any of the above cited studies that two species might be involved in *V. letourneuxi*, as indicated by our molecular data. The aim of this study was to test whether the molecular groups of Albanian and Greek *Valencia* might indeed represent two species.

Material and methods

All fish were preserved in 5 % formaldehyde and stored in 70 % ethanol. Measurements were made with dial caliper and recorded to 0.1 mm. All measurements are made point to point, never by projections. Methods for counts and measurements follow Kottelat & Freyhof (2007). Standard length (SL) is measured from the tip of the upper lip to the end of the hypural complex. The length of the caudal peduncle is measured from behind the base of the last anal-fin ray to the end of the hypural complex, at mid-height of the caudal-fin base. The last two branched rays articulating on a single pterygiophore in the dorsal and anal fins are noted as "1½". Numbers in parentheses after a given count are numbers of individuals examined with that count. The holotype is included in the calculation of means and SD.

Abbreviations used: bp, base-pairs; SL, standard length; HL, lateral head length; CMK, Collection of Maurice Kottelat, Cornol; FSJF, Fischsammlung J. Freyhof, Berlin; MNCN, National Museum of Natural Sciences, Madrid; ZFMK, Zoologisches Forschungsmuseum Alexander Koenig, Bonn.

DNA extraction and PCR. Genomic DNA was extracted using Machery & Nagel NucleoSpin® Tissue kits following the manufacturer's protocol on an Eppendorf EpMotion® pipetting-roboter with vacuum manifold. The standard vertebrate DNA barcode region of COI (cytochrome c oxidase subunit 1) was amplified using a M13 tailed primer cocktail including FishF2_t1 (5'TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC), FishR2_t1 (5'CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA), VF2_t1 (5'TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC) and FR1d_t1 (5'CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA) (Ivanova et al. 2007). Sequencing of the ExoSAP-IT (USB) purified PCR product in both directions was conducted at Macrogen Europe Laboratories with forward sequencing primer M13F (5'GTAAAACGACGGCCAGT) and reverse sequencing primer M13R-pUC (5'CAGGAAACAGCTATGAC).

Table 1. List of the 32 diagnostic nucleotide substitutions found in the 610 base pairs long mtDNA COI barcode region. Nucleotide position is given with reference to the complete mitochondrial genome of *Oryzias latipes* (GenBank accession number AP004421).

	5596	5634	5637	5667	5679	5682	5718	5739	5745	5754	5769	5805	5808	5817	5847	5850	5883	5901	5908	5958
V. hispanica (5)	T	C	C	A	T	G	G	T	A	G	G	T	A	G	C	A	A	A	C	T
V. letourneuxi (7)	T	C	C	A	T	G	C	T	A	G	G	T	A	G	C	A	A	A	C	T
V. robertae (8)	C	T	T	G	C	A	T	C	G	A	A	C	C	A	T	G	T	T	T	C

Molecular data analysis. Data processing and sequence assembly was done in Geneious Pro (Biomatters 2013) and the Muscle algorithm (Edgar, 2004) was used to create a DNA sequence alignment. SpeciesIdentifier (Meier et al. 2006) was used to compute Kimura2-corrected distances (K2P), and screening for diagnostic nucleotide substitutions was performed manually from the resulting sequence alignment.

Valencia robertae, new species
(Figs. 1–5)

Holotype. ZFMK 59197, 38.6 mm SL; Greece: River Pinios south of Kavasilas, 37°52'16.4"N 21°15'53.6"E; from a captive population.

Paratype. ZFMK 59198–59206, 9, 28.6–42.5 mm SL; same data as holotype.

Additional material. FSJF 3448, 2, 32.6–33.5 mm SL; Greece: Central prov: Chiliadou Springs in Mornos delta, 38°24'N 21°55'E; from a captive population.

Material used in the molecular genetic analysis: ZFMK 53006–53007 (GenBank accession numbers: KF767519, KF767509); ZFMK 53077–53080 (GenBank accession numbers: KF767514, KF767521, KF767513, KF767512); same data as holotype. – FSJF-DNA 2460; Greece: Central prov: Chiliadou Springs in Mornos delta, 38°24'N 21°55'E; from a captive population (GenBank accession numbers: KF767516, KF767524).

Diagnosis. *Valencia robertae* is distinguished from *V. letourneuxi* (Figs. 8–11) by the presence in females of short lateral bars or small vertically elongated blotches along flanks (vs. no lateral bars or blotches) and an almost triangular anal fin with straight posterior margin (vs. anal fin almost rounded, posterior margin convex) and anal fin depth, measured from anal-fin origin to tip of third branched ray, 1.4–1.5 times in caudal peduncle depth (vs. 1.2–1.3). Males *V. robertae* are distinguished by having prominent bars on flank between the axial blotch and the base of the caudal fin (vs. bars absent or very faint anterior to vertical of pelvic-fin origin); neck and back bluish brown (vs. yellowish) and a long anal fin, reaching to or almost to the base of the caudal fin in individuals longer than 30 mm SL (vs. to middle of caudal peduncle).

Valencia robertae is distinguished from *V. hispanica* by having a hyaline to bluish caudal fin (vs. yellow to orange) with a bold black posterior margin in males (vs. reddish-brown).

5982	6000	6001	6030	6033	6042	6048	6069	6075	6084	6126	6129
C	C	C	T	T	T	C	R	T	C	G	A
C	C	C	T	T	C	C	G	T	T	T	A
T	T	T	C	C	A	T	C	C	A	A	G

Description. See Figures 1–4 for general appearance and Table 2 for morphometric data of holotype and nine paratypes. Dorsal and ventral profiles straight or gently convex from jaws to dorsal- and anal-fin origins; concave or straight along caudal peduncle. Body slender, deeper than wide, compressed posteriorly. Body deepest at about anal-fin origin. Greatest body width at pectoral-fin base. Jaws short, snout slightly pointed. Caudal peduncle compressed laterally, 1.1–1.4 (males), 1.4–1.7 (females) times longer than deep. A very small axillary lobe at base of pelvic fin.

Pectoral fin rounded, posterior margin reaching anterior to vertical of pelvic-fin base. Pelvic-fin origin distinctly in front of dorsal-fin origin. Pelvic fin short, tip reaching to anus in males, not reaching to anus in females. Pelvic-fin bases very closely together, almost fused. Anus situated directly in front of anal-fin origin. Urogenital papillae slightly prolonged in females. In females, anal fin almost triangular with almost straight posterior margin, last ray reaching to slightly behind middle of caudal peduncle. In males, anal fin elongated, reaching to or almost to base of caudal fin in individuals larger than 30 mm SL. Dorsal-fin origin above 2nd–4th branched anal-fin ray. Dorsal fin roundish in females, reaching to middle of caudal peduncle, elongated in males, almost reaching to hypural complex in individuals larger than 30 mm SL. Extremity of dorsal fin rounded in both sexes. Caudal fin rounded to slightly spatulate. Largest male examined 38.6 mm SL; largest female examined 42.5 mm SL.

Dorsal fin with 7½ (2), 8½ (7) or 9½ (1) branched rays. Anal fin with 10½ (4) 11½ (4), 12½ (1) or 13½ (1) branched rays. Caudal fin with 8+8 (8) or 9+8 (2) branched rays. Pectoral fin with 13 and pelvic fin with 4–5 rays.

Scales large, cycloid. Trunk and head entirely scaled. One scale row on upper part of opercle. Body with 28 (1) 29 (6) or 30 (3) scales along lateral series. Three additional small scales on anterior caudal-fin base. Lateral line incomplete, with 18–29 pores, scales pored mostly behind vertical of pelvic-fin origin. Teeth unicuspid, pointed.

Fig. 1. *Valencia robertae*, ZFMK 59197, holotype, 38.6 mm SL; Greece: River Pinios.

Fig. 2. *Valencia robertae*, ZFMK 59198–59203, left from top: males 28.6, 31.2 and 28.6 mm SL; right from top: females 34.9, 37.9 and 42.5 mm SL; Greece: River Pinios.

Fig. 3. *Valencia robertae*, ZFMK 59197, holotype, male, 38.6 mm SL; Greece: River Pinios.

Fig. 4. *Valencia robertae*, ZFMK 59204, paratype, female, 37.8 mm SL; Greece: River Pinios.

Fig. 5. *Valencia robertae*, not preserved, male collected in 1994, about 30 mm SL; Greece: River Pinios (right side reversed).

Fig. 6. *Valencia robertae*, not preserved, males, about 30 mm SL; Greece: River Mornos. Photograph by Paul Eckstein.

Coloration. See Figures 1–7 for general appearance. Live males: Faint or prominent dark brown or black roundish axial blotch at shoulder above pectoral fin base. Body with 12 (1), 15 (2), 17 (1) or 18 (1) dark brown or black lateral bars as wide as interspaces above lateral midline and narrower than interspaces on ventral part of body. Number of bars increasing with size. In examined specimens, 7 (2), 9 (2) or 10 (2) bars anterior to dorsal-fin origin, 2 (2), 3 (2) and 4 (2) bars below dorsal-fin base, and 3 (4), 4 (1) and 5 (1) bars behind dorsal-fin base. In front of dorsal fin base, bars not reaching ventral midline and back, reaching to back behind dorsal-fin base. Back brown in front of dorsal-fin base, above lateral midline with some iridescent blue scales in spaces between bars. Below lateral midline and behind dorsal-fin origin, spaces between bars bluish or white. Cheek, breast and belly yellow. In large males, body above anal-fin base also yellow. Top of head pale brown. Opercle bluish iridescent in upper part, yellow in lower part. Pectoral fin hyaline or yellow. Pelvic fin yellow with narrow black margin. Anal fin yellow with bold black margin. Dorsal fin blue with a bold black margin in all mature males larger than 25 mm SL. In anal and dorsal fins, membranes between last 2–3 anal-fin rays with a dark brown marbled pattern or dark brown blotches forming concentric rows. Caudal fin blue with a bold black margin and 3–5 vertical rows of small dark brown spots, most prominent on fin membrane.

Live females: Body dark or pale brown. Ventral part of head and belly white. A prominent midlateral stripe reaching from posterior margin of head to caudal peduncle, wide on anterior body and narrow on posterior body, reaching caudal-fin base in some individuals and not reaching in others. Body with 5 (2), 6 (1), 7 (1) and 8 (1) dark brown or black lateral bars narrower then interspaces, not reaching dorsal and ventral midlines. Bars most prominent where crossing midlateral stripe. In one individual, bars very short, reduced to small vertically elongated blotches. In examined specimens, 1 (2), 2 (2) and 5 (1) bars anterior to dorsal-fin origin, 2 (2) and 3 (3) bars below dorsal-fin base, and 1 (4) and 3 (1) bars behind dorsal-fin base. Scale margins slightly darker brown than centre of scales. All fins hyaline or with a brown hue on fin rays.

Table 2. Morphometric data of *Valencia robertae* (holotype ZFMK 59197, paratypes ZFMK 59198–59206; 10). Means, min, max and SD include holotype. H, holotype.

	H	males			females		
		min–max	mean	SD	min–max	mean	SD
Standard length (mm)	38.6	28.6–31.3			34.8–42.5		
In percent of standard length							
Head length	30.0	30.0–32.5	30.7	1.3	27.9–30.5	29.0	0.9
Body depth at anal-fin origin	27.0	24.9–27.1	25.9	1.1	21.3–23.6	22.5	0.7
Predorsal length	70.9	70.8–73.9	71.8	1.5	71.1–74.3	72.9	1.1
Preanal length	67.6	65.2–67.6	66.7	1.2	67.8–68.9	68.4	0.4
Prepelvic length	51.8	50.7–52.6	51.6	1.0	51.4–52.9	52.0	0.6
Distance between pectoral and pelvic-fin origins	23.6	21.0–24.2	22.8	1.6	21.7–24.2	22.7	0.9
Distance between pelvic and anal-fin origins	16.2	14.4–16.2	15.3	0.9	15.5–17.3	16.4	0.7
Depth of caudal peduncle	15.4	15.1–15.7	15.3	0.3	13.1–14.4	13.6	0.4
Length of caudal peduncle	18.9	17.3–20.8	18.9	1.7	19.2–22.0	20.5	1.0
Dorsal-fin base length	14.8	13.3–14.8	14.2	0.8	9.7–11.4	10.7	0.7
Anal-fin base length	17.6	17.4–17.9	17.7	0.3	13.9–15.6	14.6	0.7
Pectoral-fin length	20.8	18.2–21.8	20.3	1.8	15.7–19.1	16.9	1.3
Pelvic-fin length	15.3	12.6–15.3	13.7	1.4	11.1–12.3	11.8	0.4
In percent of head length							
Head depth at eye	56	47–56	50	5	48–52	50	1
Snout length	27	24–31	28	3	24–30	28	2
Eye diameter	32	30–33	32	1	27–34	31	2
Postorbital distance	46	41–46	44	2	43–47	45	1
Maximum head width	64	54–64	58	5	58–65	62	3
Interorbital width	46	39–46	43	4	43–48	46	2

Preserved males: Body yellowish brown, darker on back. A dark brown middorsal stripe from nape to dorsal-fin origin. Lateral bars dark brown. Fins hyaline, pelvic and unpaired fins with bold dark grey margin. Unpaired fins with a brown hue on fin rays and marbled pattern or dark brown spots described in live males. Paired fins hyaline.

Preserved females: Body yellowish brown, darker on back. A dark brown middorsal stripe from nape to dorsal-fin origin. Lateral stripe and dark brown bars or elongated spots prominent. All fins hyaline or with a brown hue on fin rays.

Distribution and conservation. *Valencia robertae* is known from the lower Pinios River in Peloponnese and the lower Mornos River in mainland Greece. P. Eckstein (pers. comm.) provided a picture showing two males and one female *Valencia* caught in the lower Mornos River on mainland Greece (Figs. 6–7). These fishes clearly show the diagnostic colour pattern of *V. robertae*. It can be speculated, that at least the extirpated population from the Alfios (Kalogianni et al., 2010), which is south of the Pinios, also belonged to *V. robertae*. One female from Louros drainage available to us (CMK 16952) lacks vertical bars and has the typical rounded anal fin of *V. letourneuxi*. Furthermore, *Valencia* from the Acheloos are expected to belong to *V. robertae*, as all freshwater fish species known from the Pinios occur also in the Acheloos. Populations from the Dimitrios as well as from the Astakos springs north of the Acheloos might belong to *V. robertae* but populations further north probably belong to *V. letourneuxi*.

In the Pinios, Kalogianni et al. (2010) found *V. robertae* occurring in very low abundance in 2005. In 2011, E. Kalogianni (pers. comm.) suggested that the population might now be extirpated and we were not able to find it during our visit to Pinios River in 2011. Kalogianni et al. (2010) noted that the status of the Pinios population is deteriorating rapidly while Bianco & Miller (1989) reported that it was still abundant. HK (unpubl. data) observed that the species was abundant in September 1995, when the broodstock for the captive population was collected. The Mornos River population is reportedly the only one to be relatively safe (Kalogianni et al., 2010). The other populations possibly belonging to *V. robertae* are in a more or less critical conservation status (Kalogianni et al., 2010).

Valencia letourneuxi has already been assessed as Critically Endangered following the IUCN criteria (Crivelli, 2006b) and the description of *V. robertae* further restricts the distribution area and number of populations of *V. letourneuxi*. There is an urgent need to examine at least the southern populations previously identified as *V. letourneuxi* and to survey again the Pinios River in order to better assess the conservation status of *V. robertae*.

Etymology. The species is named for Roberta Barbieri (Athens), who studied the Greek *Valencia* species for many years and is engaged in the conservation of the two species.

Remarks. *Valencia robertae* is distinguished from the other two *Valencia* species by 32 diagnostic nucleotide substitutions (Table 1) and a COI-based K2P minimum distance of 16.9 % to *V. hispanica* and 8.2 % to *V. letourneuxi*.

All individuals of *V. robertae* examined are captive bred fishes originating from a founder stock collected in the 1990s. The K2P COI sequence divergence of 8.2 % in the DNA barcode region between *V. robertae* and *V. letourneuxi* cannot be attributed to bottlenecks or selective breeding in captivity, and strongly supports the view that they are two distinct species. The captive stock of *V. letourneuxi* from Pinios is maintained for 19 years, encompassing between 6 and 10 generations (HK, own data). There is a need to exclude the possibility that morphological differences and differences in colour pattern result from selective breeding of both stocks. Figure 5 shows a wild caught male *V. robertae* from the lower Pinios River. Furthermore, P. Eckstein (pers. comm.) provided two pictures of wild caught *Valencia* from Mornos (Figs. 6–7). They clearly show the diagnostic characters of *V. robertae*: the long anal fin and the bluish back of males (Fig. 6). There are no dark brown bars but vertically elongated blotches visible in the female from Mornos River (Fig. 7). In the type series, there is one female (ZFMK 59202) that also has vertically elongated blotches instead of prominent bars along the lateral midline. These pictures do not suggest that there are considerable differences between the wild populations and the captive stocks available for this study.

Fig. 7. *Valencia robertae*, not preserved, female, about 30 mm SL; Greece: River Mornos. Photograph by Paul Eckstein.

Fig. 8. *Valencia letourneuxi*, not preserved, male, about 40 mm SL; Greece: Corfu Island. Photograph by Andreas Hartl.

Fig. 9. *Valencia letourneuxi*, not preserved; about 50 mm SL; Greece: lower Acheron drainage.

Comparative material. *Valencia letourneuxi*: MNHN A-2342, 3, 27.7–56.6 mm SL; MNHN A-2343, 3, 27.5–35.5 mm SL, MNHN A-2344, 3, 25.2–47.4 mm SL; syntypes, Greece: Corfu Island: Cressida. – FSJF 3426, 8, 31.6–45.6 mm SL; Greece: Corfu Island: stream about 1.5 km south-east of Sidhari, on road from Sidari to Antiperni, 39°46'36" N 19°43'02" E; from a captive population. – CMK 16941, 1; Greece: Epiros prov: canal

Fig. 10. *Valencia letourneuxi*, FSJF 3426, left from top: males 34.8, 31.6, 35.2 mm SL; right from top: females 43.9, 41.9, 45.6 mm SL; Greece: Corfu Island.

Fig. 11. *Valencia letourneuxi*, not preserved, male, about 30 mm SL; Albania: Lake Burint; from a captive stock.

on road to Krestinu, at turnoff from road from Igoumenitsa to Smertos, 39°32'53" N 20°14'6" E. – CMK 16946, 14; Greece: Epiros prov: Acheron River estuary, canal in swamp west of Mesopotamo, 39°14'54" N 20°30'52" E. – CMK 16952, 1; Greece: Epiros prov: Barbanakos spring at Stefani, about 5 km northeast of Louros, 39°10'28" N 20°48'29" E.

Material used in the molecular genetic analysis: *V. hispanica*: MNCN AT19409, AT19413; Spain: Rio Bullent: El Calapatar Alicante España, 38°52'55" N 0°5'01" W; (GenBank accession numbers: KF767510, KF767517). – ZFMK 53076, 53086, 53087; Spain: Peniscola II, region L'Ametlla, 40°21'40" N 0°23'7" E; from a captive population (GenBank accession numbers: KF767528, KF767523, KF767525).

V. letourneuxi: FSJF 2459 Albania: Lake Butrint; from a captive population (GenBank accession numbers: KF767511, KF767527). – ZFMK 59009; Greece: Epiros prov: spring stream at Kipseli springs west of Koroni, 39°16'56" N 20°32'36" E (GenBank accession number: KF767515). – ZFMK 53081–53084; Greece: Corfu Island: stream about 1.5 km south-east of Sidhari, on road from Sidari to Antiperni, 39°46'36" N 19°43'02" E; from a captive population (GenBank accession numbers: KF767522, KF767518, KF767526, KF767520).

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