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Lost in the Cyclades: genetic affiliation of the Yellow-bellied Toad, *Bombina variegata* (Anura: Bombinatoridae), from Paros Island, Greece

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The genus *Bombina* (OKEN, 1816) comprises seven recognized species of small-sized aquatic toads widely distributed in Eurasia (FROST 2021). Two of them, *Bombina bombina* (LINNAEUS, 1761) and *B. variegata* (LINNAEUS, 1758), occur throughout Central and Eastern Europe, where they commonly occupy temporary or permanent water bodies. These include puddles, ponds, slow-flowing streams and canals, all situated in wetlands, open grasslands, deciduous and coniferous forested areas and ranging from low to high altitudes (SPEYBROECK et al. 2016). For both taxa, the Balkans have played an important role in their evolutionary history (e.g., FIJARCZYK et al. 2011, DUFRESNES et al. 2021). Both species occur on the Greek mainland, but *B. bombina* is limited to a few places at the Evros River (northeastern Greece). In contrast, *B. variegata* is widespread in the Greek north, whereas farther south, it becomes restricted mostly to upland sites along the Pindos Mountains in the west, the Kaimaktsalan-Vermio-Olympus Mountain Chain in the centre, and the Rhodopes along the Greek–Bulgarian border in the northeast (HELMER et al. 1988, VALAKOS et al. 2008). Across its distribution, *B. variegata* has an altitudinal range from 100 to 2,100 m a.s.l. (KUZMIN et al. 2009), although in Greece, this species is present mostly above 600 m (VALAKOS et al. 2008). It has been also reported from the inland of Euboea (Evia) Island in the Aegean Sea, which is geographically very close to the mainland (GASC et al. 1997).

Recently, a record of *B. variegata* was published in the online magazine L@certidae (Eidechsen online), documenting it from Paros Island in the Cyclades Archipelago

in the Aegean Sea (TROIDL & TROIDL 2019). This record is unexpected due to the xeric character of this island that does not conform to the habitat in which this species typically lives. In the southern Balkans, *B. variegata* usually occurs in hilly or mountainous areas with relatively high humidity far away from coastal areas (VALAKOS et al. 2008). The find by TROIDL & TROIDL (2019) revealed that a small population has established itself in the centre of the island at Levkes village. Given that the occurrence of a primarily upland toad in this arid Mediterranean island is suspicious, we decided to investigate the genetic affiliations of this population to determine its origin.

During a herpetological survey of central Paros Island on 1 June 2018, we observed *B. variegata* along a series of rock pools at the village of Levkes (37.054° N, 25.208° E; 200 m a. s. l.). The observed population comprised at least 30 adult individuals, as well as several dozens of tadpoles at various stages of development. During a second visit on 26 February 2021 (Levkes village; 37.052° N, 25.205° E; 246 m elev.), approx. 400 metres southwest of the first point of observation, we also came across more than fifty *B. variegata* within a linear distance of approx. 25 metres along the Levkes stream. Again, various life stages were observed, suggestive of an established population that has adapted successfully to the dry insular habitat. Both sites are located at the southern margins of the village along a steep east-facing gully carved out by a seasonal stream (Fig. 1A). This locality is furthermore inhabited by a representative of the genus *Pelophylax* (Fig. 1B), most prob-

ably *P. kurtmuelleri* (GAYDA, 1940), the most common species of water frogs in continental Greece, found also in the close-by island of Euboea (LYMBERAKIS et al. 2007), which is considered conspecific with *P. ridibundus* (PALLAS, 1771) by some authors (e.g., SPEYBROECK et al. 2010, 2020).

Tissue samples for DNA analysis were obtained from five adult individuals that were captured by hand at the second investigated site and had a small piece of finger clipped off. These samples were stored in 96% ethanol for subsequent DNA extraction. To identify the genetic affiliations of this population, we used a fragment of the cytochrome *b* marker (cyt *b*; mtDNA) that was also used in previous studies on the phylogeography of this species (HOFMAN et al. 2007, FIJARCZYK et al. 2011, DUFRESNES et al. 2021). Total genomic DNA was extracted from our tissue samples using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. We used the primers Bomb-F1/Bomb-R2 (DUFRESNES et al. 2021) to amplify a fragment of cyt *b* of ~500 bp in length. The PCR strategy for extracting mtDNA followed the information described in detail by DUFRESNES et al. (2021). For genetic comparisons, we used 13 sequences of *B. variegata* from nine localities in Greece available from the GenBank database (EF212796-8, EF212800-3, JF898405, JF898416,

JF898419, JF898426, JF898429, JX893176; HOFMAN et al. 2007, FIJARCZYK et al. 2011, PABIJAN et al. 2013; Fig. 2). The final dataset of 19 sequences was analyzed, reconstructed and mapped using PopArt (<http://popart.otago.ac.nz>) and the implemented parsimony network algorithm of TSC (CLEMENT et al. 2000), with a 95% connection limit.

The haplotype network divided the analyzed sequences into three groups: “green” from northeastern Greece, “orange” from the northeast and central parts, and “yellow” from the southern parts. The Paros dataset corresponds to the “yellow” group and is separated from the “orange” group by eight mutation steps (Fig. 2). This group is formed by one haplotype and includes sequence JF898426 from Livadies in the Oete (Oiti) Mountains (38.829° N, 22.272° E, FIJARCZYK et al. 2011) in the southern Pindos range (Hellenides). The “yellow” and “orange” haplotype groups belong to the so-called Balkans lineage of *B. variegata*, whereas the “green” group represents the Rhodope lineage sensu FIJARCZYK et al. (2011) and DUFRESNES et al. (2021). Our data indicates that the Paros population is most closely related to populations in southern Greece rather than any of the other analyzed Greek populations.

Although the Cyclades Archipelago once formed a continuous landmass connected to the Balkan mainland, this connection was interrupted over 3.5 Mya ago (ANASTASAKIS et al. 2006). As a consequence, today's local biota of the Cyclades is made up of unique evolutionary lineages that are endemic to the archipelago in addition to more recent trans-marine arrivals resulting from natural or human-mediated introductions. The latter is what we suggest for the Paros population of *B. variegata* described here.

Paros Island is situated in the central Cyclades and was first visited by herpetologically minded naturalists in the mid-19th century (FIEDLER 1840, ERHARD 1858). Increasingly detailed surveys were then conducted in the 20th century, resulting in a reasonably good knowledge of the local reptiles and amphibians (BIRD 1935, WETTSTEIN 1953, BUCHHOLZ 1955, GRUBER & FUCHS 1977). However, these reports did not mention the presence of *B. variegata* on the island. According to local people, the population discussed here has been present at the investigated site for at least eight years based on the advertising calls that are heard during extended periods in spring. Although our data clearly support a genetic affiliation of the Paros population with southern Greek populations (Fig. 2), it is impossible to deduce how exactly *B. variegata* arrived on the island. Given the location of the site at the margins of a human settlement, the lack of any other records of this species on other Aegean islands or even in similar habitats, we suggest it to represent a recent anthropogenic introduction. The most likely explanation for such an event is a release of adult individuals or tadpoles, which could have been collected at an unknown locality in southern Greece and later released at the Levkes stream on Paros. Being an attractive, colourful species, these may have initially been kept in a terrarium, brought across to populate a garden pond, and escaped or intentionally released, or deliberately introduced here because people liked its pleasant advertising calls. In this con-



Figure 1. (A) The habitat of *Bombina variegata* with the seasonal stream at Levkes village, Paros Island. (B) The introduced population of *B. variegata* on Paros Island in sympatry with the Balkan Water Frog (*Pelophylax kurtmuelleri*).

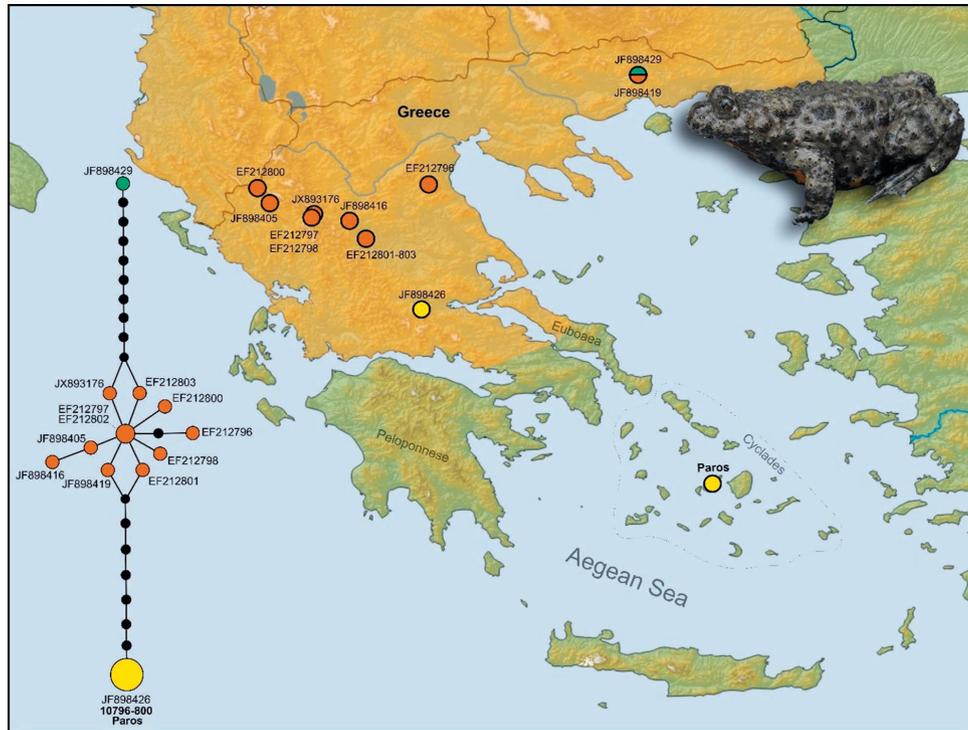


Figure 2. The haplotype network of *Bombina variegata*, showing relationships between haplogroups (green, orange and yellow) and their geographic distributions in Greece. The distribution range of *Bombina variegata* in the Balkans is marked in light orange. The pictured individual originates from Paros.

text, we found an anecdotal record from the Ionian Island of Lefkada, where local people had translocated *Pelophylax* sp. from the mainland to enjoy their choruses around their houses (TZORAS, pers. data). Amphibian translocations to outside their natural distributional ranges have previously been reported from elsewhere in Greece (e.g., *Pelophylax epiroticus* to farther east of its natural geographic range, STRACHINIS 2021). Similarly, in other parts of Europe, different amphibian taxa, including *Bombina* spp., have been subject to human-mediated dispersal, either directly or indirectly (e.g., through imported vegetables) that could have relevance to local allele composition and adaptation (see SZYMURA 1998, CABELA & GRILLITSCH 2005, RECUERO et al. 2007, PINYA & CARRETERO 2011, DOMENEGHETTI et al. 2013, MEILINK et al. 2015, DUFRESNES et al. 2015, 2016, DUFRESNES & BÉRÉNICE 2020, DE CAHSAN et al. 2021).

We believe that the only basis for the existence of this species in this isolated extralimital location is the availability of year-round moisture in an otherwise warm and arid environment. Until now, it has only been found in this single stream, even though it is conceivable that it may eventually spread to nearby, equally suitable sites. The general habitat in the area consists of a mosaic of open thermo-Mediterranean vegetation interspersed with partially abandoned terraced fields. The watercourse consists of a series of rock pools with clear water and is flanked by dense riparian vegetation dominated by *Arundo donax* L., *Nerium oleander* L. and *Ficus carica* L. The vegetation beyond the immediate

vicinity of the stream at both sites is very dry and differs strikingly from this species' habitat requirements, i.e., well-vegetated, often wooded sites (VALAKOS et al. 2008). Hence, this record is noteworthy because it highlights the ability of *B. variegata* to survive in a region that would conventionally be considered to be outside its thermal envelope (FOUFOPOULOS et al. 2011). It also provides an unexpected natural laboratory for studying genetic consequences arising from the given environmental conditions on the island of Paros.

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