Genetic evidence for human-mediated introduction of Anatolian water frogs (*Pelophylax cf. bedriagae*) to Cyprus (Amphibia: Ranidae)

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We found in two water frogs captured in Cyprus, the distribution area of *P. cypriensis*, nuclear alleles and mitochondrial haplotypes that are characteristic of Anatolian water frogs, *Pelophylax cf. bedriagae*. Mitochondrial and nuclear DNA data strongly indicate that the ancestors of these two frogs did not arrive on Cyprus via a land bridge or overseas dispersal but were unintentionally introduced by humans, most probably together with carp fingerlings. Both individuals exhibited recombined nuclear genomes characteristic of backcross hybrids. Further investigations are needed to estimate the amount of introgression of allochthonous alleles into the gene pool of *P. cypriensis*.

**Keywords:** Water frogs; *Pelophylax cypriensis*; Cyprus; genetic pollution; backcross hybrids

Introduction

Western Palaearctic water frogs (genus *Pelophylax*) are distributed from western Europe to Central Asia including northern Africa. Currently, at least 12 species and three hybridogenetic hybrid forms are recognised (see review by Plötner, 2005). Water frogs played and still play an important role as food and delicacies; in the Mediterranean coastal regions, for example, thousands of frogs are collected every year and exported to Western Europe (Bilgin, Akın, & Plötner, unpubl.), especially to Italy and France. Moreover, water frogs are sold for stocking ornamental ponds (Holsbeek et al., 2008; Holsbeek, Maes, De Meester, & Volekaert, 2009); until approximately 30 years ago, they were also used as experimental animals for research and educational purposes. As an undesirable side effect, non-native individuals were released, intentionally or accidentally, into natural habitats resulting in the establishment of stable ‘alien’ populations, often resulting in hybridisation with indigenous individuals. Genetic traces of such events are found in many areas, for example in Belgium (Holsbeek et al., 2008; 2009), France (Pagano, Lodé, & Crochet, 2001; Pagano, Dubois, Lesbarrères, & Lodé, 2003), Switzerland (Grossenbacher, 1988), and southern Germany (Ohst, 2008).

We here present genetic data that provide evidence for an introduction of non-native water frogs from the Anatolian mainland (*P. cf. bedriagae*) to Cyprus as well as hybridisation events between these frogs and *Pelophylax cypriensis* Plötner, Baier, Akın, Maz-
epa, Schreiber, Beerli, Litvinchuk, Bilgin, Borkin & Uzzell, 2012, a frog species restricted to Cyprus (Plötner et al., 2012, Baier, Sparrow, & Wiedl, 2013). Based on mtDNA (ND2, ND3) haplotypes in a single small sample (5 individuals) from northeast of Nicosia (~35°11’N, ~33°34’E) two distinct lineages were distinguished, one unique to Cyprus, the other clearly related to Anatolian frogs (Plötner et al., 2010; Akın et al., 2010b). Nuclear sequences of serum albumin intron 1 and the respiratory gene ubiquinol-cytochrome c reductase confirm the introduction of Anatolian alleles into the gene pool of *P. cypriensis* at this locality. We here discuss putative sources and routes of introduction of non-native frogs to Cyprus.

**Material and Methods**

Mitochondrial (mt) and nuclear (nu) DNA sequences of 82 individuals from Cyprus and the adjacent mainland (Anatolia, Syria, Jordan) were analysed (Figure 1, Supplementary Table 1). The complete mt ND2 and ND3 genes were sequenced as described by Akın et al. (2010b). Sequence analysis of the nu serum albumin intron 1 (SAI-1) followed the method described by Plötner et al. (2009). We also sequenced 441 bp of exon 2 of the respiratory gene ubiquinol-cytochrome c reductase subunit 1 (uqcrfs1, EC: 1.10.2.2) from selected individuals, including the five individuals from North Cyprus northeast of Nicosia (see above). PCR amplifications of this marker were conducted in 25 µl volumes containing 1x PCR buffer, 200 µM each dNTP, 2.0 mM MgCl2, 0.5 µM each primer (uqcrfs1-Exon2_F: TCCGTTTCGGCTTCTTACACGC; uqcrfs1-Exon2_R: CCAAGGTGAGTGCAAGACTCC), 1.25 units of Taq polymerase (New England Biolabs, Ipswich, MA, USA), and approximately 50 ng of DNA. An initial denaturation step of 3 min at 96°C was followed by 35 cycles each at 94°C for 30 s, 60°C for 40 s, and 72°C for 60 s; PCR was terminated by a final extension step at 72°C for 5 min. New sequences have been deposited in the EMBL Nucleotide Sequence Database under accession numbers given in Supplementary Table 2.

Genealogical relationships of mt haplotypes (HTs) and nu SAI-1 alleles were reconstructed with maximum likelihood (ML) as implemented in MEGA 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) using all sites for gaps/missing data treatment and Nearest-Neighbor-Interchange as the heuristic search method. Nodal support was evaluated by bootstrapping (Felsenstein, 1985) with 1,000 replicates.

Models that best describe the substitution patterns of sequences used in this study were selected on the basis of the Bayesian Information Criterion (BIC) (Schwarz, 1978) as implemented in MEGA. Model selection was based on ML. The Tamura-Nei model (Tamura & Nei, 1993) with gamma-shaped rate variation (G=0.15) was recommended for the mtDNA data set (BIC=7208.6); for the SAI-1 data set the Tamura 3-parameter model (Tamura, 1992) of sequence evolution with gamma-shaped rate variation (G=0.08) was selected (BIC=6966.3).

**Results**

In the mt genealogy (Figure 2) three main clades can be distinguished: The first clade comprises Cypriot frogs (*P. cypriensis*), the second frogs from Jordan and one individual from Syria that are considered to represent *P. bedriagae*, and the third consists of individuals from Turkey (*P. cf. bedriagae*) including two individuals from Cyprus (19410, 19416). The mt HTs of these individuals (CA1.CAR12) were identical to HTs detected in two Anatolian individuals (CA1451 and CA1459) from Lake Beyşehir and Seydişehir, respectively (Supplementary Table 1).

The topology of the ML tree calculated on the basis of SAI-1 sequences reveals four main clades, each supported by bootstrap values of ≥99% (Figure 3). Again, one clade is characteristic of *P. cypriensis* and a second comprises alleles specific for Anatolian *P. cf. bedriagae*. Unlike the mt genealogy, *bedriagae*-specific SAI-1 alleles form two main clades (3 and 4). In clade 3 alleles characteristic of frogs from Syria and Jordan cluster
Figure 1. Sampling localities of water frogs on Cyprus (red circles) and the adjacent mainland (Turkey: triangles, Syria/Jordan: squares). The approximate locality in northern Cyprus where frogs with allochthonous genes were found is identified by a white asterisk. Supplementary Table 1 provides detailed information.

together with alleles specific for individuals from the Cilician plain of Anatolia, while clade 4 comprises only bedriagae-specific alleles.

A single small sample of frogs from one unspecified locality in northern Cyprus contains individuals with either typical P. cypriensis SA1-1 alleles (cyp2, cyp3) in homozygous or heterozygous combinations (specimens 19411, 19412, 19413) or with both a cypriensis- and an Anatolian-specific allele (19410: ant5-6/cyp2, 19416: ant5-5/cyp2) (Figure 3, Supplementary Table 1); ant5-5 and ant5-6 are characteristic of frogs from coastal populations of southwestern Turkey. In the uqcrfs1 gene, however, both 19410 and 19416 were homozygous for an allele that was found in Anatolia but not in P. cypriensis (Supplementary Table 1, Supplementary Figure 1).

Discussion

Based on mt and nuDNA we found clear evidence that water frogs from Anatolia were introduced to Cyprus where they hybridized with the indigenous water frog species P. cypriensis (Plötner et al., 2012). Two of five frogs, both females, exhibited a mt HT (CA1.CAR12) characteristic of the Anatolian “caralitanus” lineage (Plötner et al., 2010). Because this particular haplotype has previously been found only at two localities (Beyşehir and Seydişehir), which are 75 or more km inland from the Anatolian shore opposite Cyprus, the arrival of this haplotype to Cyprus seems almost certain to have been human-mediated.
Figure 2. Genealogical relationships of mitochondrial haplotypes (ND2+ND3 sequences, 1378 bp) obtained from eastern Mediterranean water frogs (Cyprus, Anatolia, Syria, Jordan) inferred by maximum likelihood (lnL = -3115.29) based on the Tamura-Nei model of sequence evolution (Tamura & Nei, 1993). Differences in evolutionary rates among sites were modulated by using a discrete Gamma distribution with a shape parameter of G = 0.15. Bootstrap values >50% are given at nodes. Labels of terminal units: Population No. [Country] sample ID_haplotype (see Supplementary Table 1). CY: Cyprus, JO: Jordan, SY: Syria, TR: Turkey. Individuals from the locality in Cyprus where frogs with Anatolian genes were found are are marked by an asterisk (*). The unit of the scale bar is equivalent to the expected mutations per site.
Figure 3. Genealogical relationships of SAI-1 alleles (1054 bp) obtained from eastern Mediterranean water frogs (Cyprus, Anatolia, Syria, Jordan) inferred by maximum likelihood (ln\(L=-2211.95\)) based on the Tamura-3-parameter model of sequence evolution (Tamura, 1992). Differences in evolutionary rates among sites were modulated by using a discrete Gamma distribution with a shape parameter of \(G=0.08\). Bootstrap values \(>50\%\) are given at nodes. Labels of terminal units: Population No. [Country] sample ID_allele (see Supplementary Table 1). CY: Cyprus, JO: Jordan, SY: Syria, TR: Turkey. Individuals from the locality in Cyprus where frogs with Anatolian genes were found are marked by an asterisk (*). The unit of the scale bar is equivalent to the expected mutations per site.
The fact that two Cypriot individuals with Anatolian specific mtDNA exhibited both an Anatolian allele and a cypriensis allele at the SAI-1 locus but were homozygous for Anatolian alleles at the uqcrfs-1 locus is evidence that they possessed recombined genomes as is typical for backcross hybrids. Because the Anatolian SAI-1 alleles ant5-5 and ant5-6 are different from those found in the “caralitanus” lineage, it seems possible that different water frog lineages were introduced to Cyprus; alternatively the introduced frogs may have belonged to a hybrid lineage, as is often the case in Anatolia. Our data do not allow us, however, to estimate the amount of hybridization and introgression in Cyprus.

Although there is no evidence of a direct shipment of water frogs from the Beyşehir or Seydişehir region to Cyprus, there were a number of occasions in 1993 when south Anatolian water frogs might have been introduced to Cyprus. As confirmed by Yılmaz Emre, a fisheries biologist and currently director of the Antalya Fisheries Institute, about 15 tons of water holding fingerlings of the Carp (Cyprinus carpio) were transported to North Cyprus. The reservoirs stocked are not recorded, but he reported that at this time, “a fish net dipped into breeding ponds would have more tadpoles than fingerlings” (pers. comm., 2013). The water and fingerlings appear to have originated from district Kepez (Antalya) where, to our knowledge, the CA1.CAR12 HT detected in the Cyprus frogs does not occur, although in this region frogs largely possess mitogenomes falling within the “caralitanus” clade (Akın, unpubl.). The putative source of stocked fish in Kepez, however, was originally Lake Eğirdir (just 35 km west of Lake Beyşehir), where “caralitanus”-like frogs are abundant.

There is another possible source of introduction of water frogs from Anatolia to Cyprus: Between 1998 and 2008, seven million tons of water were transported annually from Aydınçık (Mersin) to Girne (Cyprus) (E. Sinay, pers. comm., 2014). We do not know, however, whether the water (and any adults or spawn it may have contained) were pumped into reservoirs for further use, or instead directly provided to households, with no opportunity for included individuals to escape into natural habitats. A third but rather unlikely possibility is the direct release of frogs by humans captured either for culinary purposes or as pets (e.g. Holsbeek et al., 2008).

Despite the fact that all these possibilities are equally parsimonious from a scientific point of view, there are enough factual data (a transfer of aquatic biota that links the Beyşehir region to northern Cyprus, where the specimens were collected; a haplotype identical to that of the putative source) to state that the ancestors of these two frogs did not reach Cyprus in a natural way from the Anatolian mainland, for example by overseas dispersal. Certainly, if “caralitanus”-like frogs had actually arrived via such processes in the distant past, one would expect to find caralitanus-specific HTs on the Anatolian mainland opposite Cyprus, but today we find there only frogs with mitogenomes of the relatively distinct Cilician clade (Akın et al., 2010b). Our molecular data and clock estimates (Akın et al., 2010b; Plötner et al., 2012) remain convincing to us that frogs giving rise to P. cypriensis arrived on Cyprus during the Messinian rather than the Pleistocene as suggested by Poulakakis et al. (2013) and that the “caralitanus” lineage has been introduced by humans.

The introduction of allochthonous genes always poses a serious threat to the integrity of the indigenous gene pool, particularly for populations with small sizes such as relict or island populations. It can be assumed that hybrids between the island endemic P. cypriensis and Anatolian P. cf. bedriagae are still fertile because our comprehensive data indicate that genomic compatibility among Mediterranean water frog lineages is still high and antihybridization mechanisms, if any, are only weakly developed (Akın et al., 2010a; Plötner et al., 2010).
Hybridization among distant lineages exposed to different selective pressures may result in new combinations between nuclear genes, but also between nuclear and cytoplasmic genes (cytonuclear interactions; e.g. Plötzner et al., 2008), resulting in the emergence of adaptive evolutionary traits in hybrids that are not expressed in the parental lineages. On the other hand, an admixture of genomes coming from regions experiencing different selective pressures may result in reduced fitness because of the breakdown of co-adaptations among genes (local adaptations) and subsequent outbreeding depression.

It is not known what impact on Cyprus water frog populations Anatolian water frog lineages have already had or may have in the future, but the spread of allochthonous alleles should to the greatest extent possible be prevented to protect the authenticity of P. cypriensis. Our present data (Plötzner et al., 2012, Supplementary Table 1) suggest that the introduction of allochthonous frogs is restricted to the northern part of the island (and probably to only a few populations), but introgressed alleles may easily spread to other areas as a result of overland dispersal of juveniles and adults. Thus, more attention should be paid to this potential threat to the indigenous gene pool.

Supplementary Material
The Supplementary Tables 1 and 2 and Supplementary Figure 1 are available via the “Supplementary” tab on the articles online page (http://dx.doi.org/10.1080/09397140.2015.1027495).

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No potential conflict of interest was reported by the authors.

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