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Preliminary data on the differentiation of Milos Island water frogs

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The water frog of Milos Island (Southern Cyclades, Greece) has been repetitively reported to differ from the surrounding islands frogs. Geological history of Milos and the occurrence of a rich endemic herpetofauna would justify an endemic frog species on the island. Morphometric analyses and comparison with the closest frog population yield a significant differentiation. Specific phenotypic characters, such as the presence of black hands and the spotted throat, make this morph quite distinct compared to other *Pelophylax kurtmuelleri* populations. Bioacoustic data, besides a slightly different mating call, fell short of supporting distinct acoustical recognition characteristics for the Milos population. Our study provides evidence, through a cluster of particular morphometric features, that Milos water frog merit a thorough taxonomic review. Nevertheless, such a revision requires a robust genetic support and further specialized studies.

Key words: Greek Marsh Frog, speciation, Mediterranean, bioacoustics, *Pelophylax kurtmuelleri*.

INTRODUCTION

In the last 25 years, a wide variety of new tools and techniques has been introduced in systematics and are broadly used together with classical methodologies. The genus *Rana* represents an excellent example of the explosive changes that took place thanks to new techniques. Dubois (1992) recognizing the problematic nature of the Ranidae systematics, proposed a new subgenus, the *Pelophylax* group. At that time, he described 17 species that gradually increased to reach today 22 species (Frost, 2013).

In the case of Greece, Werner (1938) reported that *R. ridibunda* was the only water frog inhabiting the country. However, an impressive among-populations variation was described (Kyriakopoulou-Sklavounou *et al*., 2003) and an increasing number of new species were separated from the *R. ridibunda* cluster: *P. epeiroticus* (Schneider *et al*., 1984), *P. bedriagae* (Schneider & Sinsch, 1992), *P. cerigensis*, *P. cretensis* (Beerli *et al*., 1994) and *P. kurtmuelleri* (Schneider & Sinsch, 1992).

Milos Archipelago, located in the southern Aegean volcanic arc, is notorious for its fascinating geological history (Fyticas *et al*., 1986). The island was separated from the rest of the Cyclades by middle Pleistocene (Dermitzakis, 1990). This long isolation is reflected on the rich herpetofauna of the island: Milos hosts four endemic taxa (the viper *Macrovipera schweizeri*, the wall lizard *Podarcis milensis*, the Balkan green lizard *Lacerta trilineata hansschweizeri* and the grass snake *Natrix natrix schweizeri*). The presence of so many endemic reptiles makes the existence of an endemic water frog expectable. Indeed, this prediction has been phrased repeatedly in the past (Broggi, 2000; Speybroeck, 2006; Van Hecke & Hordies, 2007). Re-

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cent studies have focused on Aegean water frogs providing valuable results (Beerli *et al*., 1996; Lymberakis *et al*., 2007) but none of them included the Milos population. Hence, the identity of Milos frog remains unknown though some variation has been reported (Tunner & Heppich, 1982).

Water frogs' populations in Cyclades are largely classified to *Pelophylax kurtmuelleri* (Lymberakis *et al*., 2007). However the status of these populations remains largely unclear (Broggi, 1996; Valakos *et al*., 2008). This study represents a first attempt to shed light on the status of Milos water frog population, presenting data on the diversity of morphology and call parameters.

MATERIALS AND METHODS

Study site

This investigation was conducted in 2009 on two islands: Serifos (37°15′N, 24°50′E) and Milos (36°41′N, 24°28′E), Southern Cyclades, Greece. Frogs were located at night with the aid of electric torches, or oriented by the location of calling males. One individual was euthanized by means of immersion in Sandoz MS222, fixed in 95% ethanol, and preserved in 75% ethanol while 15 specimens from Milos and 45 from Serifos were used for morphometric measurements.

Morphometrics

Terminology and measurements mainly follow Bossuyt & Dubois (2001). The following external measurements were taken with electronic calliper (CD-20PP Mitutoyo Corporation, Japan, precision 0.1 mm). The following morphometric parameters were measured: snout-vent length (SVL), head width at the angle of the jaws (HW), head height at the highest point on the head (HH), head length from the rear of mandible to the tip of the snout (HL), distance from rear of mandible to nostril (MN), distance from rear of mandible to anterior orbital border of eye (MFE), distance from rear of mandible to posterior orbital border of eye (MBE), snout length from tip of the snout to anterior orbital border of the eye (SL), eye length – the horizontal distance between bony orbital borders of the eye (EL), inter upper eyelid width – the shortest distance between the upper eyelids (IUE), maxi-

TABLE 1. Morphological measurements (in mm) and samples sizes. Abbreviations are explained in the text (see Materials and Methods)

Trait	*Milos island* (N = 15)			*Serifos island* (N = 45)		
	Range	Mean	SD	Range	Mean	SD
SVL	39-53.77	46.22	7.1	40.45-73.52	51	9.92
HW	14.03-19	16.38	2.46	14.51-26.87	18.1	3.81
HH	7.58-9.89	8.73	1.2	7.64-11.87	9.16	1.34
HL	15.98-19.77	17.79	1.73	16.41-31.8	20.7	4.85
MN	13.82-16.55	15.2	1.42	13.97-29.32	17.65	4.9
MFE	12.03-13.86	12.88	0.85	11.34-23.27	13.92	3.89
MBE	7.83-8.15	7.98	0.14	6.8-15.82	8.8	2.9
SL	6.13-8.14	7.15	1	6.54-9.87	7.86	1.13
EL	5.37-6.23	5.8	0.41	4.97-8.16	5.98	0.95
IUE	2.74-3.78	3.27	0.5	3.02-4.73	3.9	0.61
UEW	6.11-6.9	6.49	0.36	3.92-6.63	5.72	0.7
IN	2.39-3.11	2.86	0.27	2.43-4.75	3.27	0.68
IFE	6.37-7.33	6.86	0.47	6.55-8.92	7.44	0.8
IBE	10-12.69	11.55	1.17	9.95-15.57	11.58	1.76
NS	3.17-4.49	3.81	0.63	2.95-4.51	3.74	0.47
EN	2.54-3.39	2.94	0.43	2.78-4.9	3.65	0.74
TYD	3.24-4.03	3.63	0.41	3.47-6.21	4.34	0.83
FW	9.02-14.05	11.56	2.61	8.01-16.77	10.1	2.81
FOL	25.3-27.9	26.62	1.34	21.51-42.92	29.03	3.76
FTL	18.01-25.7	21.88	4	20.98-35.27	26.16	4.22
IML	1.67-2.29	1.98	0.27	2.41-4.41	3.14	0.63
ITL	9.81-14.38	12.1	2.37	11.74-19.85	14.4	2.48
FiTL	6.39-9.16	7.8	1.38	7.79-13.26	9.82	1.6

mum upper eyelid width (UEW), distance between internal borders of the nostrils (IN), internal front of eyes – shortest distance between anterior orbital border of the eyes (IFE), internal back of eyes – the shortest distance between posterior orbital border of the eyes (IBE), distance from nostril to tip of snout (NS), distance from nostril to anterior orbital border of the eye (EN), largest tympanum diameter (TYD), maximum femur width (FW), foot length – from base of inner metatarsal tubercle to tip of fourth toe (FOL), fourth toe length – from base of first subarticular tubercle to tip of fourth toe (FTL), inner metatarsal length (IML), first toe length – from inner metatarsus to tip of first toe (FiTL), and inner toe length (ITL). Analysis of (co-) variance was used to examine the effects of location on SVL, extremity morphology and head shape. We regressed the log-transformed morphological data against log-SVL and entered the residuals into a principal component analysis (correlation matrix, varimax rotation). Morphological measurements and samples sizes are summarized in Table 1.

Sonograms

We recorded and analyzed mating, territorial and defence calls of male frogs. Calls were taped using a Philips Voice Tracer model 7655 (Philips Electronics, Philips Speech Processing, 2006; Frequency response 500-3500 Hz). The audio file was processed using the Raven Lite 1.0 Software (2003-2007 Cornell Lab of Ornithology, Cornell University).

The mating, territorial and defence calls of Milos water frog were recorded at water temperature 21.3°C and air temperature 19.5°C from a distance of 3 meters.

RESULTS

Morphology and morphometric analysis

Pelophylax kurtmuelleri from Milos island has the typical phenotype for water frogs: eyes are set closely together, hind legs are long, and paired vocal sacs exist at the corners of the males' mouth. Vomerine teeth are present. Posterior part of the tongue is free and forked. Toes webbed. Pupil of the eye is horizontal. Omosternum and sternum ossified. Snout moderately blunt, belly and throat white, with small dark grey spots in the periphery of the throat. Milos water frog is a medium-sized, yet robust, water frog with a SVL of up to 57.33 mm. No temporal spot. The dorsal coloration is greyish-brown reticulated, but can also be

FIG. 1. *Pelophylax kurtmuelleri* in its natural habitat (Milos Island, Greece).

partially or entirely brown-grey. The underside is white and the zone between back and lower part is dark and marbled. The dorsolateral skin folds are poorly developed, tympana are bronze uniform in colour and mani are black (Fig. 1).

Specimens were found in a wide variety of flowing and stagnant water, ranging from shallow puddles and ponds to small, temporary rivers. Milos water frog was also often found in halophyte vegetation, near the sea and in the vicinity of damp coastal marshes. Conductivity and pH of the water were measured using a portable device (WTW pH/Cond 340). Frogs were found in relatively high salinity water (5.28 mS cm^{-1} at 21.7°C) and lightly basic water (pH 7.55 at 21.5°C).

PCA on the morphological measures of the frogs in the total data set resulted in six composite variables that together explained 93.97% of the total variation. The first principal component (eigenvalue 11.95, explaining 42.69%) was associated with residual limb dimensions (factor loadings for FL: 0.928; for FTL: 0.951; for FiTL: 0.882; for TL: 0.86). The second axis (eigenvalue 6.92, explaining 24.72%) correlated most strongly with MN (factor loading: 0.921), UEW (factor loading: 0.923) and NS (0.854). The third axis (eigenvalue 2.441, explaining 8.72%) was associated primarily with the TYM (factor loading: 0.875) and IN (factor loading: 7.88). The fourth axis (eigenvalue 2.181, explaining 7.79%) was associated primarily with the IML (factor loading: 0.673). The fifth axis (eigenvalue 1.743, explaining 6.23%) was associated primarily with the SL (factor loading: 0.865) and HH (factor loading: –0.550). The sixth axis (eigenvalue 1.070, explaining 3.82%) was associated primarily with the TL (factor loading: 0.477) and IUE (factor loading: 0.401). There was considerable among-population variation in all composite variables (MANOVA, Pillai's Trace = 0.922, $F_{6,18} = 35.417$, $p < 0.0001$; ANOVA on first

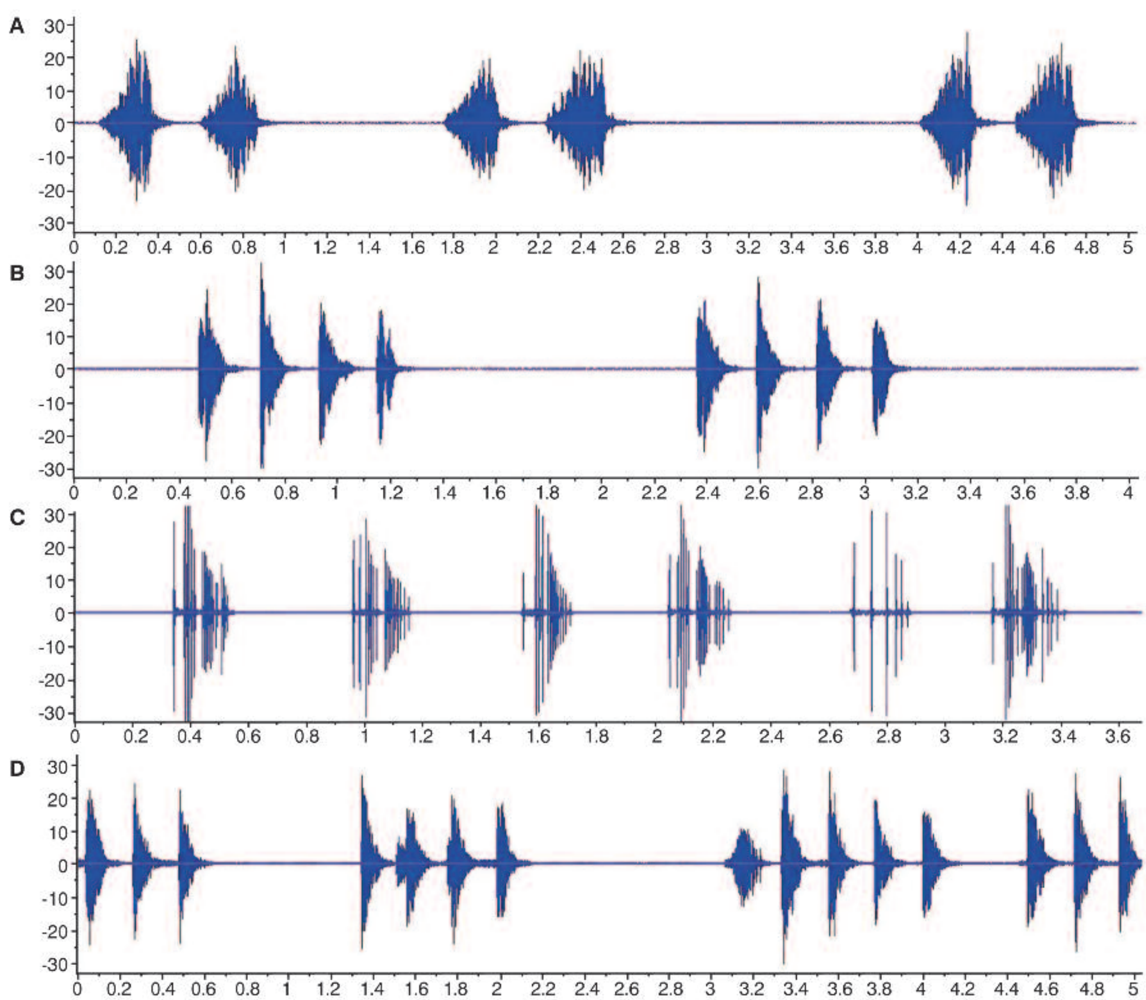

FIG. 2. Sonograms of the territorial call (A), mating call (B) and defense call (C) of the Milos water frog and the mating call of *Pelophylax kurtmuelleri* from Serifos (D).

axis: $F_{1,23} = 55.338$, $p < 0.0001$; on second axis: $F_{1,23} = 2.553$, $p = 0.124$; on third axis: $F_{1,23} = 0.538$, $p = 0.471$; on fourth axis: $F_{1,23} = 5.134$, $p = 0.033$; on fifth axis: $F_{1,23} = 0.073$, $p = 0.790$ and on sixth axis: $F_{1,23} = 0.181$, $p = 0.674$).

Bioacoustic analysis

The territorial call (Fig. 2A) consists of a large number of relatively short call series with short intervals. The call series (609 ± 195 msec; mean ± SE) are altered by intervals (1347 ± 264 msec). Each call series is composed of a relatively low number of calls (1.65 ± 0.35), with durations of 314 ± 20 msec and intercall intervals of 155 ± 18 msec. The mating call (Fig. 2B) produces another call type with call series of 774 ± 31 msec (mean ± SE), altered by intervals of 1831 ± 288 msec. Each call series is composed of a relatively low number of calls (3.85 ± 0.14), with durations of 146 ± 3 msec and intercall intervals of 73 ± 4 msec. This call highly resembles the one from *P. kurtmuelleri* on Serifos (Fig. 2D), which consists of call series of 644 ± 73 msec (mean ± SE), altered by intervals of 530 ± 110 msec. Each call series is composed of a relatively low number of calls (3.18 ± 0.35), with durations of 144 ± 5 msec and intercall intervals of 85 ± 5 msec. The main difference between the Serifos *P. kurtmuelleri* and the Milos water frog calls is the duration of the intervals between the call series, which is substantially longer at the latter one. The defense call of the Milos water frog (Fig. 2C) differs from the previous given calls. The call series have a mean duration of 2145 ± 714 msec (mean ± SE) and are altered by intervals with a respective duration of 967 ± 342 msec. Each call series is composed of a relatively low number of calls (4.7 ± 1.5), with durations of 228 ± 37 msec and intercall intervals of 338 ± 62 msec.

DISCUSSION

Our data suggest that there is a phenotypic distinctiveness of the Milos population. Indeed, this population deviates in many distinct features from the typical pattern of the species. On the other hand the results of bioacoustic analysis yield little difference from the close conspecific population of Serifos.

Many Greek islands have been isolated for a long time and members of the genus *Pelophylax* could be found on some of them (Lymberakis *et al*., 2007), despite the general scarcity of amphibian species due to the severe xerothermic conditions (Kyriakopoulou-Sklavounou, 2000). Fresh water is a rare commodity on Cyclades and this fact shaped the scattered distribution of water frogs: the presence of *P. kurtmuelleri* has been verified in Andros, Tinos and Mykonos at the north, and in Amorgos, Naxos, Paros, Kithnos, Serifos and Sifnos at the centre (Broggi, 2000, 2007, 2011; Valakos *et al*., 2008). The only frog-hosting island at the south is Milos.

Morphological comparisons between the Milos and Serifos populations revealed differences in body size and shape, coloration of the throat and the frequency of dark mani (Fig. 1). Furthermore the flats of the hands are whiter compared to the neighbouring Serifos population, which are typically greyish (±15% of examined specimens, i.e. seven out of 45 specimens). Also, PCA analysis revealed significant morphological differences between the two populations (Table 1). Some of these quantitative differences in pattern have been previously reported as exclusive of the Milos population and have been considered indicative of a distinct form (Broggi, 2000; Speybroeck, 2006; Van Hecke & Hordies, 2007).

Bioacoustic analyses, that provide an accurate discrimination tool (Schneider & Steinwarz, 1990), revealed slight differences in mating call, which is the primary vocalisation in anurans. The main difference between the Serifos and Milos *P. kurtmuelleri* calls is the duration of the intervals between the call series, which is substantially longer at the latter case. Loftus-Hills & Littlejohn (1971) and Straughan (1975) showed that pulse rate is one of the main component used for the species-discrimination. Our findings suggest that the two islands populations' calls were identical. However, we have to stress out that there is no direct proof of the meaning of the observed difference in the duration of call interval. Investigating female's phonotactic behaviour would be worthwhile in the future to determine the possible functionality of this difference.

The observed resemblance in the phenotypic parameters could be attributed to the historical relationships between Milos and Serifos that were in close connection during antiquity. Milos has been settled and exploited for millennia thanks to obsidian trade that attracted numerous boats (Renfrew & Wagstaff, 1982). On the other hand, people in ancient Serifos worshiped the local mythical hero Perseus and frogs were associated to this cult (Pausanias, 2nd century A.D.). Thus it should come as no surprise if travellers from Serifos transferred frogs to the close Milos. Founding individuals might have gone through a severe bottleneck, resulting in the observed monovariation. Genetic drift, following founder events, may play a role in agonistic signal divergence, while allopatry may be responsible for courtship divergence.

Recent work (Lymberakis *et al*., 2007) highlighted the need for detailed studies of the phenotypic variation within the range of *Pelophylax*. The peculiar nature of Palearctic water frogs' systematics demands a multidimensional approach since single method analysis may bias diagnostic conclusions (Plötner, 1998). We believe that our findings should be bound with studies based on nuclear DNA in order to evaluate the taxonomic status of the Milos water frog distinct form.

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