Introduction

The Balkan Peninsula is considered to be a major hotspot for European biodiversity (Griffiths et al., 2004; Strong et al., 2008). Balkan low-altitude lakes have been intensively studied in terms of biodiversity, as many of them are among the most ancient European lakes and thus old enough to feature endemic faunas and floras (Frogley et al., 2001; Frogley and Preece, 2007; Albrecht and Wilke, 2008; Vareli et al., 2009; Wilke et al., 2010; Albrecht et al., 2012). In contrast, Balkan alpine lakes are less well studied, due to their tiny size and difficult access. Originated in the end of the last glacial period, only a few thousand years ago, their species assemblages probably derive from a process of recent colonization from neighbouring, geologically older habitats (Shehu et al., 2009). Greek alpine lakes are small, often fishless and located at high-altitude areas above 1900m, near the summit of mountains. They are called “dragon-lakes” (Drakolimmes in Greek) because their main vertebrate inhabitant, the alpine newt (Ichthyosaura alpestris), is reminiscent of the shape and appearance of a small dragon.

Ichthyosaura alpestris (initially referred to as Triturus alpestris and more recently as Mesotriton alpestris) is a small animal with a total length of about 80 to 100 mm for males and up to 120mm for females (Sotiropoulos, et al., 2008; Vukov et al., 2011). Phylogenetic analysis revealed the existence of a putative ancestor relict

Abstract. Large dorsal dark spots were observed in a number of alpine newts Ichthyosaura alpestris inhabiting Lake Gistova, the highest altitude alpine lake in the Balkans ever studied. The trait is mainly exhibited by female paedomorphs. Histopathological examination excluded any pathological cause of pigmented skin and revealed that even in paedomorphic skin sections, the altered pigmentation pattern was located only in regions with metamorphic characteristics. Phylogenetic analysis of the previously unstudied Gistova’s newt population based on mtDNA sequences revealed that it belongs to the D2 subclade, comprising only two other newt populations of the wider geographic region that interestingly, also exhibit the trait. The putative genetic or environmental basis for dark spot appearance in these populations is discussed.

Keywords: alpine lakes, Balkan Peninsula, black spots, 16S mitochondrial rRNA, mitochondrial cytb gene.
lineage (Clade A) originating from south-eastern Serbia and two other lineages designated Western and Eastern further divided in Clades B, C and D, E respectively (Sotiropoulos et al., 2007). Clades D and E representing southern and central-northern Balkan populations were further subdivided, suggesting greater isolation into multiple refugia (Sotiropoulos et al., 2007). More recently, Recuero and colleagues, by using a combined analysis of mtDNA, nuclear (nDNA) and allozyme data tried to resolve the evolutionary history of the species *I. alpestris* (Recuero et al., 2014). The phylogenetic hypotheses based on mtDNA from this study recovered the same lineages and phylogenetic structure as suggested earlier (Sotiropoulos et al., 2007), but nDNA and allozyme analyses revealed more complex phylogenetic relationships. Nevertheless it is of interest that both nDNA and allozyme data support also that southern Balkan populations (clade D) is a discrete phylogenetic lineage (Recuero et al., 2014).

Paedomorphosis is common in a vast number of newt populations living in the south margin of the species geographical range, mainly in the Italian and Balkan Peninsulas (Denoël et al., 2001). It is a polymorphism widespread in newts and salamanders and by which larval characters, such as gills and gill slits, are retained in adults (paedomorphs) (Whiteman, 1994). The adults characterized by absence of gill slits are called metamorphs. In the five Greek paedomorphic populations examined so far, the percentage of paedomorphs in the total population ranged between 23% and 77% (Denoël et al., 2001). Paedomorphosis is under genetic control in several species of salamanders (Voss and Shaffer, 1997). A genetic basis favouring paedomorphosis in *I. alpestris* is also suspected (Denoël et al., 2001). Moreover, resource partitioning between morphs is in favour of the maintenance of polymorphism in this species (Denoël and Joly, 2001a; Denoël and Joly, 2001b).

The Greek alpine newt was classified as a subspecies named *Ichthyosaura alpestris veluchiensis* (Breuil and Parent, 1988; Denoël, 2004). Although *I. alpestris* specimens in northwestern Greece are found in various water bodies above 1190m such as ponds, drinking troughs and watering basins (Denoël, 2004), large populations inhabit only the alpine lakes of the region (Denoël and Schabetsberger, 2003).

In our survey conducted in October 2012, large dorsal dark spots were observed in a number of newts from Lake Gistova, the highest altitude lake of Greece (2360m a.s.l) and one of the highest in the Balkans (Sehu et al., 2009). Interestingly, in a 1988 study (Breuil and Parent, 1988) newt populations from lakes of the northern Pindus area were characterized as potentially endemic and it has been reported that newts from lakes Tymphi and Smolikas exhibit colour variants (e.g. specimens with large black spots). However, no effort was made in this study, either to examine histopathologically these colour variants or to reveal any correlation to population’s demographics.

Thermoregulation, UV protection and avoiding detection from predators have been proposed as putative adaptive functions of colour patterns in amphibians (Rudh and Qvarnström, 2013). In common frogs the degree of melanism increases as a function of latitude and altitude (Alho et al., 2010; Vences et al., 2002). Dark markings in montane common frogs were found to be valuable to attain faster heating rates of higher body temperatures, and thereby performance benefits (Vences et al., 2002). In larvae of newts and salamanders, UV induces skin darkening implying that melanization may have a protective role against UV radiation. However, direct experimental evidence for such a role is missing (Rudh and Qvarnström, 2013).

On the other hand, in the spotted salamander *Ambystoma maculatum*, UV induces DNA damage despite the increased melanin production in this species (Lesser et al., 2001). Furthermore, dark spots in some Urodele amphibian species (e.g. *Calotriton arnoldi, Triturus cristatus*) were found to be of cancerous origin (Koussoulakos, et al., 1994; Martinez-Silvestre et al., 2011).

Based on these facts, we set the following specific objectives for our study: (a) to investigate the histological nature of the dark spots, (b) to study the distribution of the trait by sex and life history type, (c) to investigate the presence/absence of the trait in other geographically neighbouring populations and (d) to resolve the phylogenetic relationship of Gistova’s unstudied newt population to other newt populations exhibiting the same colour trait.

**Materials and Methods**

**Study Area**

Our study area comprises three alpine lakes (Figure 1). Lake Gistova, located on Mt. Grammos (40°21’N, 20°47’E), is the highest lake in Greece (2360m a.s.l) (Figure 1). It is a fishless elliptical lake (150m x 70m), with a maximum depth of about 4m, characterized by a muddy bottom, rocky shores, surrounded by alpine pasture. Aquatic vegetation is absent along the shoreline and in the lake.
Two other lakes in the wider region are also included (Figure 1). The first one, located on Mt. Smolikas (40°05’N, 20°54’E, 2140 m a.s.l.), has a rectangular shape (122 m long, 61 m wide) and a maximum depth of 3.7 m. Vegetation is limited to small shallow patches. The bottom is muddy and rocks are rare (Denoël and Schabetsberger, 2003). The other one is located on Mt. Tymphi (39°59’N, 20°47’E, 2000 m a.s.l), has a somewhat quadratic appearance (max. diameter: 100 m) and a maximum depth of 4.95 m. It is characterized by rich vegetation along the shoreline, consisting mostly of Carex sp. (Denoël and Schabetsberger, 2003).

Lake Gistova differs from the other two lakes in being more homogeneous (lacking of shelters) with Lake Tymphi being the most heterogeneous compared to the other two. Gistova Lake lays 41.51 km away from Lake Tymphi and 32.46 km away from Lake Smolikas. The distance between Lake Tymphi and Lake Smolikas is 14.85 km. No exchange exists between newt populations inhabiting the three lakes due to impassable deep valleys that separate them (Figure 1).
Sampling, gender ratios and proportion of life history phases—preservation of samples

We conducted three visits to sample newt populations, during 14 and 15 October of 2012 at Gistova Lake, on 23 October 2013 at Tymphi Dragon Lake and on 26 October 2013 at Smolikas Dragon Lake.

Newt specimens (250 from each lake) were caught by dip-netting from the shores of each lake. Gender ratios and proportion of life history phases were measured in the field.

Two larval, two paedomorphic, two metamorphic and seven spotted (both paedomorphic and metamorphic) newts from each lake were anaesthetized (0.5% phenoxyethanol solution) and then fixed in 10% buffered formalin to be histologically examined.

Histological evaluation of skin sections

Dorsal skin sections from both spotted and unspotted areas were taken from formalin fixed specimens and embedded in paraffin blocks. Tissue sections 5 μm thick were cut by using a microtome and applied on microscope slides. Subsequently, they were deparaffinized, hydrated and exposed to Mayer’s haematoxylin for 15 min, washed in tap water for 20 minutes and counterstained with eosin for 1 min. Finally, sections were dehydrated with alcohol/xylene baths and stabilized with mounting medium.

Molecular analysis

At Lake Gistova tail tips were obtained from five alpine newts both spotted and unspotted (metamorphs and paedomorphs) and homogenized in lysis buffer after adding three stainless steel beads per sample (Qiagen 5 mm diameter) on a Mini Beat-beater (Biospec products, Bartlesville, OK, USA). Subsequently, genomic DNA was extracted with the nucleospin tissue kit (Macherey-Nagel) according to the manufacturer’s protocol.

For molecular phylogenetic analysis two target genes were used: a partial sequence (309bp) of the mitochondrial protein-coding cytochrome b gene (cytb), and a partial sequence (596bp) of the mitochondrial gene coding for the 16S rRNA (16S). Primers and PCR conditions have been described earlier (Sotiropoulos et al., 2007; Korcher et al., 1989)

PCR products were purified with a Macherey-Nagel DNA clean-up kit (Nucleospin Extract), and subsequently cloned with a TOPO TA cloning Kit (Invitrogen) according to the manufacturer’s instructions. Inserts were fully determined by sequencing both strands. Sequencing was performed by VBC-BIOTECH Service GmbH.

The cytb and mt16S rDNA sequences from Lake Gistova’s alpine newts were deposited in GenBank with accession numbers KF941301 and KF941302 respectively.

Phylogenetic analysis

Cloned sequences KF941301 and KF941302 were compared to GenBank entries using BLAST in order to obtain a preliminary phylogenetic affiliation.

Cytb and mt16S rDNA sequences of Icthyosaura alpestris specimens already characterized as members of the clades D, E and A (Sotiropoulos et al., 2007) were manually concatenated and along with the KF941301/KF941302 concatenated sequence were imported into MEGA 5.1. All sequences were automatically aligned using the integrated aligner tool.
Phylogenetic analyses were performed with MEGA5.1 (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2011). Trees were constructed using either the Neighbour-Joining (NJ) method with Jukes–Cantor distance correction or the Maximum Likelihood (ML) method. Both methods gave rise to identical phylogenetic trees (Data not shown). Haplotype networks were built using the minimum spanning algorithm (Bandelt et al., 1999) in PopART (Population Analysis with Reticulate Trees) software (http://popart.otago.ac.nz).

Results

Dorsal dark spot pattern of appearance in lake Gistova’s alpine newts

A total of 250 individuals (Ichthyosaura alpestris) were collected from Lake Gistova and large dorsal dark spots were found on 35 of them (14%) (Figure 2).

In half of the spotted newts, the spots were located on the head. The spots were circular or slightly elliptical (0.4-1cm in diameter). Both paedomorphic and metamorphic newts were found with dark spots but this trait was more prevalent in the paedomorphs as they accounted for the 66% of the spotted newts (Table 1). The percentage of paedomorphs in the total population was found to be 44%. The majority of spotted newts were females (95%) while females accounted only for the 61% of the total population (Table 1).

Histological evaluation of spotted and unspotted skin sections

Four spotted paedomorphic, three spotted metamorphic, two larval, two unspotted paedomorphic and two unspotted metamorphic newts were histologically examined. In all cases only dorsal skin sections, either spotted or unspotted, were studied.

In larval skin the epidermis consists of an inner layer of basal cells, intermediate layers of Leydig cells and a top layer of pavement cells (Figure 3A) (Brunelli et al., 2007). Small pale dark granules were dispersed throughout the cytoplasm of the top layer of epidermal cells giving the dark body appearance in these specimens. Epidermis in metamorphic newts consisted of stratified and flattened epidermal cells (Brunelli et al., 2007). Dark granules were absent (Figure 3B). The skin of paedomorphic individuals exhibited some features of the larvae (such as Leydig cells) and some of the metamorphic stage (such as stratified and flattened epidermal differentiating cells) (Figure 3C1, C2, C3).

In spotted areas, numerous large dark granules were noted in the upper part of the cytoplasm in the top cellular layer, as well as between epithelial cells throughout the epidermis (Figure 3D). It is noteworthy that in paedomorphic individuals the altered pigmentation pattern was observed only in skin regions with metamorphic characteristics. As proliferation abnormalities, ulcerations or necrotizing lesions were not detected, the possibility of a pathological causative agent for black spot appearance was ruled out (Koussoulakos et al., 1994; Martínez-Silvestre et al., 2011; Duffus, and Cunningham 2010).

Retention of the dorsal trait in other newt populations

We studied the possible appearance of the same trait in two other newt populations inhabiting lakes in the wider region. The same colour variant was also observed in

<table>
<thead>
<tr>
<th>Lake</th>
<th>Unspotted Population</th>
<th>Spotted Population</th>
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<tbody>
<tr>
<td>Gistova</td>
<td>215/250 (86%)</td>
<td>35/250 (14%)</td>
</tr>
<tr>
<td>Smolikas</td>
<td>195/250 (78%)</td>
<td>55/250 (22%)</td>
</tr>
<tr>
<td>Tymphi</td>
<td>225/250 (90%)</td>
<td>25/250 (10%)</td>
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Table 1. Comparison of gender and life history phases proportions in both unspotted and spotted alpine newt populations in the three alpine lakes.
Figure 3. Histological examination of Haematoxylin-Eosin (H-E) stained skin sections of Ichthyosaura alpestris specimens from Lake Gistova. A: skin section from a larval specimen (400X). Small pale dark granules are indicated by arrows, B: metamorph’s skin section (400X), C: paedomorph’s skin section (40X) revealed its dual nature with either metamorphic (C2, 400X) or larval (C3, 400X) characteristics, D: skin section of a spotted area taken from a paedomorphic female (400X). Note the presence of numerous large dark granules in the upper part of the cytoplasm in the top cellular layer, as well as between epithelial cells throughout the epidermis indicated by arrows.
these geographically neighbouring populations. The frequency of appearance of these black spots was similar in Lake Tymphi (10%) but higher in Lake Smolikas (22%) compared to Lake Gistova (14%) (Table 1). The majority of affected newts were also females (92% in Lake Tymphi, 98% in Lake Smolikas) (Table 1). Both paedomorphs and metamorphs were found to be affected with the paedomorphs being more susceptible than metamorphs (63% in Lake Tymphi and 80% in Lake Smolikas) (Table 1). Paedomorphs accounted only for 38% of the total population in Lake Tymphi while in Lake Smolikas they accounted for 70% (Table 1).

Genetic relationship of Gistova's newt population to other newt populations

Cytochrome b and 16S rDNA gene fragments amplified from five specimens (three spotted and two unspotted) were used to reveal the genetic relationship of Lake Gistova’s newt population to other previously studied newt populations. An haplotype network was constructed based on previously published phylogenetic results (Sotiropoulos K, et al., 2007) on Ichthyosaura alpestris (Figure 4). Based on the constructed haplotype network it is clear that Gistova’s newt population is a phylogenetically distinct population of the D2 subclade (Figure 4).
Discussion

The *Ichthyosaura alpestris* population from Lake Gistova represents the highest altitude population ever studied in the Balkans (Figure 1). In this remote population, dorsal dark spots were found to be present in 14% of the specimens collected along the shoreline. Moreover, the observed colour variant was detected mainly on paedomorphic females. Histopathologic examination ruled out the possibility that the pigmented skin of our samples is of cancerous origin as it has been suggested earlier for other Urodèle amphibian species (Koussoulakos, et al., 1994; Martínez-Silvestre, et al., 2011).

Most interestingly, it revealed that even in paedomorphs, the altered pigmentation pattern was located only in skin regions with metamorphic characteristics and not in larval-like skin regions (Figure 3).

In a previous study (Breuil and Parent, 1988) newt populations from lakes of the northern Pindus area were characterized as potentially endemic. The same study mentioned that newts from lakes Tymphi and Smolikas exhibit colour variants (e.g. specimens with large black spots). However, no effort was made, in that study either to examine those colour variants histopathologically, or with respect to any correlations with gender and life history type. We studied also those geographically neighbouring populations of the Northern Pindus lakes to confirm the presence and the histological nature of the trait. Indeed, in Lake Smolikas 22% of the newts were found to be affected while in Lake Tymphi only 10%. The histological appearance was the same and once again, paedomorphic females were found to prevail in the total spotted population as was the case with Lake Gistova newts.

Based on our observations, a number of new questions arise. Since this trait has only been observed in the above three populations, is this indicative that it is under genetic control? In other words, is this a colour variant characterizing potential endemic populations in this area?

By using mtDNA phylogenies, Gistova’s newt population found to be a member of the D2 subclade (Figure 4). Moreover, Gistova’s newt population is distinct from the other two populations of the same subclade described thus far and has to be referred to as D2-3. In conclusion, the appearance of dorsal dark spots is a common trait in populations of the D2 subclade, suggesting a possible genetic basis for its origin.

According to a previously published study, eastern European *Ichthyosaura alpestris* paedomorphic lineages (such as subclade D2) seem to have evolved at the early Pleistocene around 1.4-1.2 Mya as a consequence of the oscillating glacial cycles (Sotiropoulos et al., 2007). Moreover, the almost complete fixation of different haplotypes in many single populations within each of the phylogroups identified, suggests that the relevant populations have survived the adverse climatic conditions of the Pleistocene in altopatry with limited postglacial expansion and contact (Sotiropoulos et al., 2007). In contrast, according to a more recent study (Recuero et al., 2014), the split of the major eastern lineages (clades D-E) took place during the Miocene, in the Tortonian (7.3 Mya).

It would be of interest to study the dorsal dark spot trait in populations from other phylogenetically related subclades (such as subclade D1) (Figure. 4) as well as in other already known newt populations from the broader region of Northern Pindus, inhabiting ponds or swamps at lower altitudes for which systematic phylogenetic studies are currently missing (Figure 1). To date, *I. alpestris* populations of Northern Pindus have been found at 15 sites all above 1,190m a.s.l. (14 sites described in previous studies (Denoël, 2004) and one described in this study). Only three of these 15 already known populations, have been studied phylogenetically mainly due to the fact that these populations are large compared to the others which inhabiting ponds or swamps. The glacial sequence in the Pindus Mountains, especially for Mt.Tymphi and Mt. Smolikas constitutes the best-dated glacial record in the Mediterranean area (Hughes, et al., 2006). Based on this glacial sequence, three separate glacial cycles have been identified (Hughes, et al., 2007). The oldest one dates back to > 350,000 years BP with an ELA (Equilibrium Line Altitude - "snowline") of 1741m and 1680m respectively, the middle one dates to 127,000 years BP with an ELA of 1862m and 1997m and the most recent one occurred during the last cold stage with an ELA of 2174m and 2241m (Hughes, et al., 2006; Hughes, et al., 2007). Thus, it is obvious that all three newt populations in our study have migrated to these high altitude lakes after the last glacial period. Given that ELAs for the oldest and the middle glacial periods are well above the altitude of the numerous small lakes, ponds or swamps where other newt populations inhabit nowadays, further research efforts might be important to focus on the lower altitude populations in search of a putative parental population.
Nevertheless, the already studied populations of the D2 subclade are the highest altitude populations examined thus far (Figure 4) and it is tempting to speculate that their dorsal dark spots could be related to adaptations to high altitudes. Melanism is known to occur in amphibians from high altitudes and life history patterns are known to vary along altitudinal and latitudinal gradients (Alho et al., 2010; Vences et al., 2002). For instance, comparison of Rana temporaria and I. alpestris populations showed that both species share similar life history trait responses to increasing altitude and latitude (Miaud and Merilä, 2001). Interestingly, Rana temporaria alpine populations consist of individuals with extended black dorsal pattern and individuals with few dark spots (Vences et al., 2002). A potential thermoregulating function has been suspected but no significant difference was found in the body temperature between dark and light individuals in the field (Vences et al., 2002). An experimental procedure led to the conclusion that the potential benefit of the dark colouration may be that it allows a shift in time allocation due to faster heating, an effect that is negligible under the conditions prevailing in the environment (Vences et al., 2002). In our case, we did not perform experiments that could provide evidence for the primary function of the dark spots in I. alpestris specimens from the Greek alpine lakes but it seems unlikely that such spots serve thermoregulatory purposes in view of the fact that we did not find individuals with extended dark pattern but only with one or two black spots.

In larvae of newts and salamanders, UV induces skin darkening, implying that melanization may have a protective role against UV radiation. However, direct tests of the fitness significance of colouration in protecting amphibians from UV-radiation are limited (Rudh, and Qvarnström 2013). With respect to a putative protective function of the black spots from UV-radiation, the higher frequency of the black spotted newts observed in Lake Smolikas population compared to Lake Tymphi could be attributed to the fact that Lake Smolikas is higher in altitude and more homogeneous - with lack of shelters - than Lake Tymphi. Similarly, the expected frequencies in Lake Gistova might be higher than those measured, since Gistova is higher in altitude and more homogeneous than the other two.

A possible genetic basis is also not to be excluded because in all cases the trait seems to be prevalent in female paedomorphs. Paedomorphosis is under genetic control in several species of salamanders (Voss and Shaffer 1997) and a genetic underpinning is probably necessary to allow a paedomorphic ontogenetic pathway in I. alpestris (Denoël et al., 2001). Based on our demographic measurements the high percentage of affected newts in Lake Smolikas compared to the other two lakes is related to the fact that 74% of the lake’s total population are females and paedomorphs account for 70% of the total population. Moreover, although paedomorphic populations are comparable in Lakes Tymphi and Gistova, the higher frequency of black spot appearance in the latter is probably related to the higher percentage of females in the total population (Table 1).

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References


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