The Comparison of Important *Salamandra infraimmaculata* Populations in Turkey by Means of Morphological, Histological and Karyotypical Characteristics

Türkiye'deki Önemli *Salamandra infraimmaculata* Populasyonlarının Morfolojik, Histolojik ve Karyotipik Özellikleri Açısından Karşılaştırılması

Research Article / Araştırma Makalesi

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ABSTRACT

In this study, the differentiation of *Salamandra infraimmaculata* populations of Turkey had been investigated by means of morphometry, karyology and reproductive organs histology. *Salamandra infraimmaculata* specimens were collected from Erzincan, Malatya, Hatay and Mersin provinces. According to the results of morphometric measurements, while the Malatya and Mersin populations are related, Erzincan and Hatay populations are distinct from the others. The karyotypes and histological structures of the reproductive organs did not have any differences among the provinces.

Key Words
Salamandra infraimmaculata, morphometry, karyotype, histology

ÖZET

Bu çalışmada, Türkiye'nin farklı illerinde dağılım gösteren *Salamandra infraimmaculata* populasyonlarındaki farklılaşma morfolojik, histolojik ve karyotipik yönlerden karşılaştırılmıştır. *Salamandra infraimmaculata* örnekleri, Erzincan, Malatya, Hatay ve Mersin illerinden toplanmıştır. Morfometrik ölçüm sonuçlarına göre, Malatya ve Mersin yörelerine ait populasyonlar birbirlerine yakın, Erzincan ve Hatay yöresine ait populasyonlar ise diğer ikisinden uzak bulunmuştur. Karyotip ve üreme organı histolojileri bakımından, bölgeler arasında fark gözlenmemiştir.

Anahtar Kelimeler
Salamandra infraimmaculata, morfometri, karyotip, histoloji.

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INTRODUCTION

Salamandra infraimmaculata is classified in class Amphibia, order Urodela (Caudata), family Salamandridae and genus *Salamandra*. Populations of the genus *Salamandra* occur in Europe, North Africa and Near East. The species *Salamandra salamandra* is considered to recolonized in Central Europe after the last ice age [1]. Biogeography of many species are deeply affected by the climatic fluctuations which are triggered recurrent glacial periods in Quaterner period (1.7-0.01 million years ago). Since the *Salamandra* populations originated in Anatolia in the glacial periods of Quaterner, owing to the land formation of the area and the limited active and passive distribution abilities of this species, these populations are isolated from each other and distribution of the species is limited in specific refigiums. Therefore, the *Salamandra* populations of Anatolia have discontinuous distribution patterns [2]. According to Baran (2005), *Salamandra salamandra* populations are distributed in Erzincan, Bitlis, Adana, Mersin, Hatay and Southeast Anatolian region of Turkey. This species lives in humid forests and woodlands of mountainous and hilly areas, under the leaves, between the rocks or holes and there have to be a fresh water source close to their habitat [3]. Steinfartz et al. (2000), made mitochondrial D-loop sequence analysis to the specimens that were collected from all over the distribution area of genus *Salamandra*. According to the phylogenetic analysis of these sequences, the genus *Salamandra* consists of six major monophyletic groups (*S. salamandra, S. algira, S. infraimmaculata, S. corsica, S. atra ve S. lanzai*) which split between 5 and 13 million years ago. Considering the recent studies which molecular techniques were used, European populations of the genus *Salamandra* is classified as *Salamandra salamandra*, whereas the Anatolian populations which used to be classified as *Salamandra salamandra* is closest to the *Salamandra infraimmaculata* species [1,4,5]. Therefore, we need to revise the scientific name of this species. Furthermore, Steinfatz et al. (2000), suggested that the subspecific differentiation of *S. infraimmaculata* populations should be reconsidered[1].

Salamanders are interesting animals for naturalists and evolutionary biologists because of their broad range of coloration, life history and ecology [6-9]. Salamanders have a limited capability of movement and have strict ecological requirements. They generally split genetically isolated populations and they are favorable model organisms for phylogeographic studies.

The aim of this study is to examine the differences between distinct populations of *Salamandra infraimmaculata* in Turkey which do not have any intersections and to compare the differentiation of subspecies by means of morphological, histological and karyological studies. In a previous study related to the same populations in these areas, problems about subspecies was tried to find out by comparing the morphometric measurements and the colour patterns of individuals[10]. However, there is still a continuing debate on the subspecies of *Salamandra infraimmaculata*, as many other species. In this study, we examined the differantiation of these populations by using morphological characters along with histological and karyotypical traits, and discussed the reasons underlying this differentiation.

The genus *Salamandra* (order Urodela) is a widespread monophyletic group in Western Palaearctic. Considering the morphological, biological and physiological traits, it was suggested that this group had a complicated evolutionary process, under the effects of the geographic and climatic changes of last few millions of years [1, 11, 12].

Postmetamorphic coloration of this group is generally characterized with yellow patches determined by epidermal xanthophores on dermal iridophores. These patches strewed on a black surface, where only epidermal and dermal melanophores are present [13]. Distinct lineages differ from each other especially by patterns, lengths, and color tones of these patches [11, 14].

Salamandra infraimmaculata, which can be reached to 324 mm, is the largest species of this genus. Females are generally larger than males. The ventral integument of this species is uniformly black and do not have any patches.

There are some morphological differences between the subspecies of *S. infraimmaculata* [15-17].

MATERIALS AND METHODS

In this study, thirty-eigth *Salamandra infraimmaculata* specimens were collected from four different provinces. During the field studies on April and May of 2006 and 2007, salamanders were collected from Yuva and Salihli Villages of Kemaliye, Erzincan (n=10); from Yayladağ and

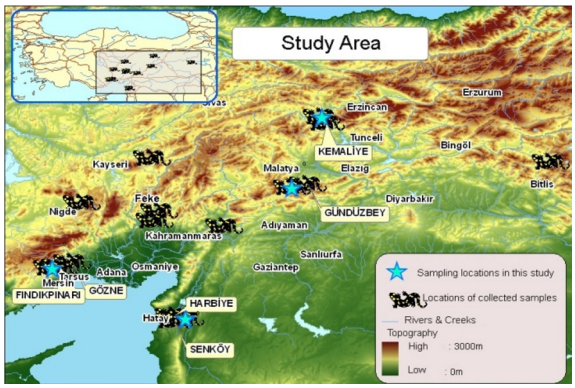

Figure 1. Sampling locations [18].

Harbiye, Hatay (n=10); from Gündüzbey Village, Yeşilyurt, Malatya (n=10) and from Fındıkpınarı and Gözne villages, Mersin (n=8) (Figure 1). Both sexes were collected in equal numbers from each of the locations.

Morphometric Measurements

The measurements which was used to make morphometric comparisons of the specimens from different areas are; total body length (distance from nose to the end of tail); head and body length (distance from nose to the end of cloaca); body length (distance from nose to the beginning of cloaca); body width (measurement of the widest part of the body); body depth (the length of the highest point of the body height); body circumference (the perimeter of the body in the widest part); length between limbs (the closest distance between basis of limbs); tail length (distance from the end of cloaca to the end of tail); tail width (the width of the base of tail); tail height (the highest point of the tail length); forelimb length (distance from the tip of third finger to the base of forelimb); hindlimb length (distance from the tip of forth toe to the base of hindlimb); finger length (the length of third finger); toe length (the length of forth toe); head length (the distance between nose tip to gular fold); head width (measurement of the widest part of the head); head depth (the distance between jaws); distance between nostrils; inner distance between the eyes (the closest distance between eyes); outer distance between the eyes (the furthest distance between eyes); eye distance (the widest distance of eye); cloaca length; parotid length; parotid width; the distance between parotids. Also for analyzing the coloration patterns of different populations, all specimens were photographed. The statictical analyses were done using SPSS 13.0 software.

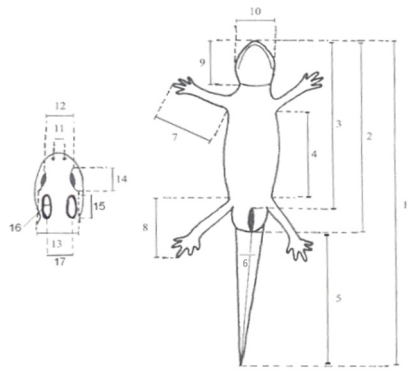

Figure 2. Morphometric measurements; 1. Total body length; 2. Head and body length; 3. Body length 4. Length between limbs 5. Tail length 6. Tail width 7. Forelimb length ; 8. Hindlimb length 9. Head length ; 10. Head width; 11. Length between nostrils; 12. İnner length between the eyes; 13. Outer length between the eyes; 14. Eye length; 15. Parotid length 16. Parotid width; 17. The length between parotids [19].

Chromosome Analyses:

For chromosome analyses, animals were intraperitoneally (IP) injected with 0,2 cc colchicine, 6-8 hours before the operation. By intramuscular injection of 10-20 mg/g ketamine and diazepam, the animals were first anesthetized and then euthanized. Chromosome analyses from blood were done according to Zhu et al. [20]. Chromosome slides were stained with Giemsa solution [21]. The best areas from these preparations were photographed. The chromosomes were ordered from these photographs according to their sizes and karyotypes were obtained.

Histological Analyses

For histological analyses the reproductive organs of euthanized animals were taken, routin histological techniques were applied and the slides of oviducts, ovaries and testicles were stained with haematoxylen and eosin [22].

RESULTS

In the present study, the measurements of the specimens, that were collected from Malatya, Hatay, Erzincan and Mersin provinces, statistically analysed considering both the provinces and sexes. Canonical variate analyses were performed to examine the differences between populations.

According to the results of canonical variate analysis, Mersin and Malatya specimens were found to be closely related, in other words, these specimens are presumably belong to the same subspecies. However, Erzincan and Hatay specimens probably occur in different subspecies (Figure 3).

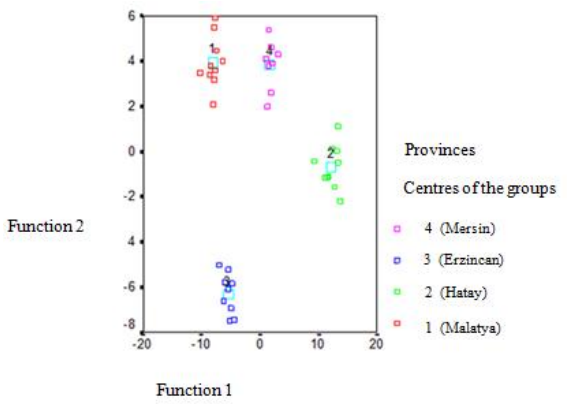

Figure 3. Population positions with respect to canonical variance functions.

With reference to the coloration pattern analyses, Mersin specimens have compound spot/stripe patterns on their dorsal side, and there are no spots on their ventral side. Except some little spots on the lower jaw, the ventral side is completely black. Erzincan specimens have medium to large yellow spots which are flower shaped. The centres of almost all of these yellow spots are black. In the ventral side, there are small yellow spots which are thicken under the jaws. Specimens from Hatay province have both large and small patches and some stripes which are formed by the combination of the spots on their dorsal side. On the contrary, ventral side is uniformly black and has no spots. Malatya specimens also have large or small, different shaped spots or stripes on the dorsal side. In addition to this dorsal spots, serial spots which are located on the lateral side of their bodies are remarkable. The ventral side of these specimens are almost black except some small yellow spots under the jaw (Figure 4).

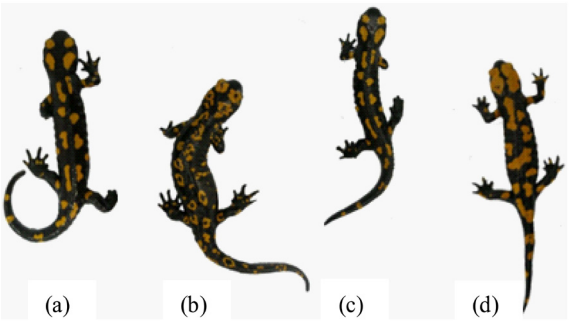

Figure 4: The dorsal coloration patterns of *S. infraimmaculata specimens* (a) Malatya, (b) Erzincan, (c) Hatay, (d) Mersin.

According to the chromosome analysis, this species has a karyotype consisting of 2n= 24. First six chromosome pairs are metacentric, seventh and eigth chromosome pairs are submetacentric and the last four pairs are shorter than the others. Respecting the last four pairs, the first two of these are metacentric, but since the last two are excessively small, the centromere of them could not be seen. Because we could analyze only the karyotypes, we could not find any structural differences in chromosomes between these populations (Figure 5).

Female Reproductive System

In oviduct slides, there was simple cuboidal epithelium in the internal side which was directed towards the lumen. There was an intensive

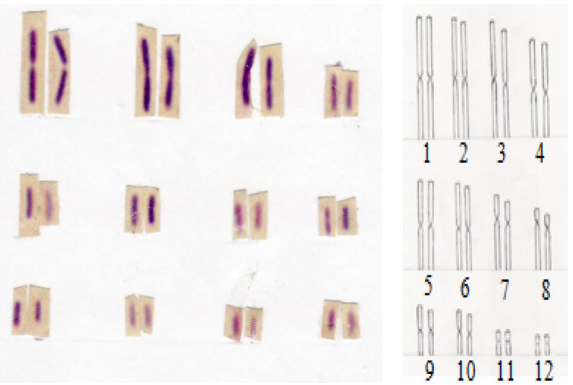

Figure 5. Karyotype of *Salamandra infraimmaculata* (X1000).

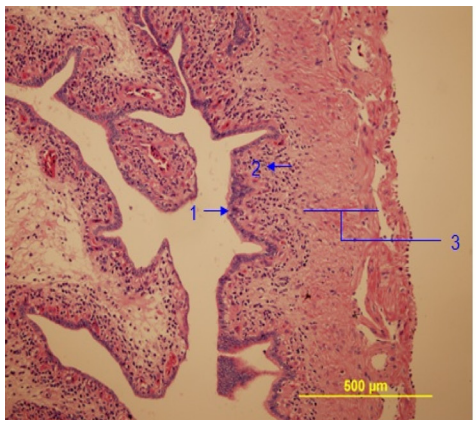

Figure 6. Oviduct structure of *Salamandra infraimmaculata* (Sampling site: Mersin)
1. Simple cuboidal epithelium,
2. Connective tissue layer,
3. Smooth muscle layer
(X100, Haematoxylin and Eosin)

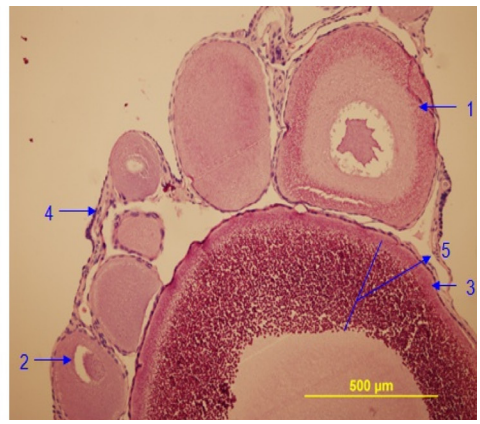

Figure 7. Ovary structure of *S. infraimmaculata* and the oocytes (Sampling site: Malatya).
1, 2 and 3. Vitellogenic oocyte,
4. Germinal epithelium,
5. Vitellus granules
(X100, Haematoxylin and Eosin)

vascular connective tissue under the epithelium, and beneath this there was a smooth muscle layer (Figure 6).

Ovaries were covered by a thin, transparent sheat and oocytes were attached to the internal side of the ovaries. The sheat which encloses the ovaries were formed from collagen fibers and connective tissue. Between the collagen fibers, there were broad and round granular cells, which were formed the germinal epithelium (Figure 7).

In the ovaries of female *Salamandra* specimens, the oocytes which were in various developmental phases (previtellogenic, vitellogenic and postvitellogenic) exist simultaneously. Along with the oocyte development, an increase in the amount of vitellus granule content was observed (Figure 7).

Salamandra infraimmaculata males have multiple lobed testes. Each of these lobes is structurally and functionally independent of each other. In testis slides, we observed that, there were spermatocyte groups and the pericystic cells of the surrounding seminal sacs and Sertoli cells, between the lobular structures of testis (Figure 8). The primordial germ cells in the immature lobes were distinguished by their light and lobulated nuclei. In this lobe, no spermatid or mature spermatozoa were encountered (Figure 9). The primordial germ cells which were located through the cylindrical cord that seperate the mature and immature lobes were enclosed with fibroblast-like cells (pericyctic cells) which were propagated with mitotic division and converted to cycts. There was long and polygonal Sertoli cells in the immature lobe of testes. On the cyct walls, flat pericyctic cells were seen. Between the mature and immature zones, a significant connective tissue boundary was refined. There were many spermatozoa on the mature lobe of the testes. Between spermatozoa, Sertoli cells and on the borders pericyctic cells were seen (Figure 10).

DISCUSSION

There are some differences between the localities for the measurements of total body length, tail length, tail width, forelimb length, hindlimb length, head length, head width, distance between the eyes, parotid length, parotid width and distance

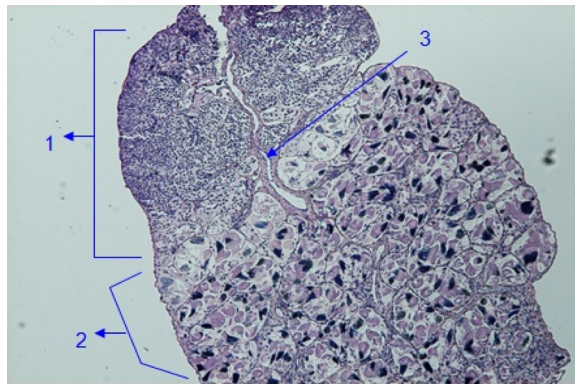

Figure 8. Transversal section of the testis of *S. infraimmaculata* (Sampling area: Malatya). 1. Immature lobe, 2. Mature lobe, 3. Connective cord formed from simple cylindrical epithelium (X40, Haematoxylin and Eosin).

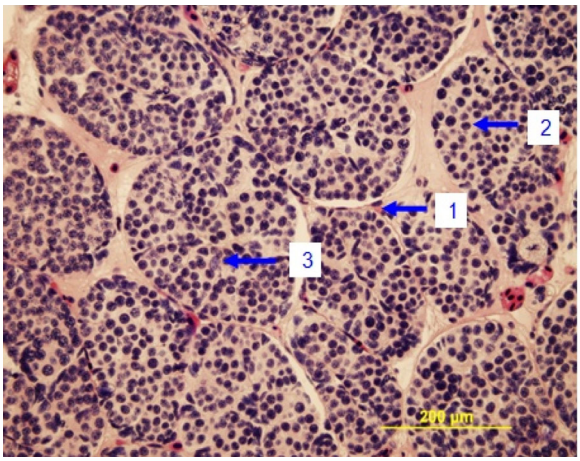

Figure 9. Immature lobe of testis (Sampling area: Hatay)
1. Pericyctic cells,
2. Primer spermatogonia,
3. Sertoli cell
(X200, Haematoxylin and Eosin)

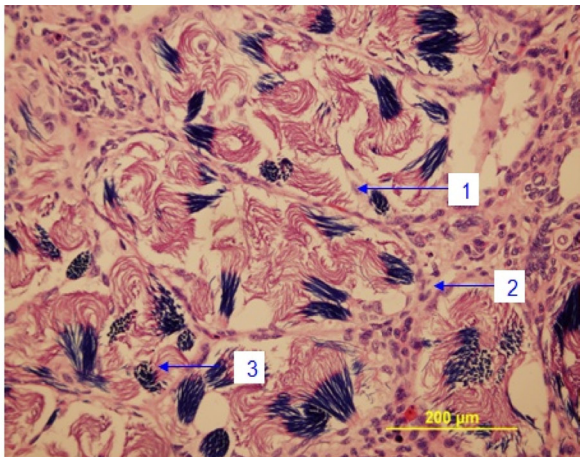

Figure 10. Mature lobe of testis (Sampling area: Mersin)
1. Sertoli cell,
2. Pericyctic cell,
3. Mature spermatozoa
(X200, Haematoxylin and Eosin)

between the parotids. Besides there is no difference between the provinces for the other measurements.

Moreover, the sexual differences are statistically significant ($P<0.001$) for almost all of the characters as we expected for this species. Females have larger measurements than males for most of the morphometrical characters, which is approved by the general situation that females are larger than males in amphibians [23]. Similarly, it was reported for *Salamandra salamandra*, *Chioglossa lusitanica* and *Mertensiella luschani* that females have bigger sizes than males [6, 24, 25].

The specimens from each province were compared among localities and sexes in respect to the morphometric characters. Considering the body lengths and cloacal view, all of the specimens were adults; but because of the long life cycles of this species exact ages could not be determined. Therefore, the probability of being in different ages of the specimens which collected from different provinces, were considered. For this reason, allometric sizes were used instead of arithmetic sizes and qualification of these data were done by using the indices of the arithmetic measurements. For understanding whether there are any difference between these populations of different localities or not and whether the differences are adequate for classifying these populations in different species/subspecies, we used Canonical Variate Analysis test. According to the results of this test, the populations in these provinces were not overlapped. But as seen in Figure 3, Malatya and Mersin populations are closely related of each other. On the other hand, Erzincan population are located further and Hatay population is located between Mersin and Erzincan populations. In a previous study, it was suggested that Malatya population should be classified as *Salamandra salamandra semenovi*, Erzincan and Mersin-Adana populations should be considered as *Salamandra salamandra salamandra* and Hatay population should be remained typically *Salamandra salamandra infraimmaculata* [10]. Considering the molecular analyses of Steinfartz et al. (2000), *S. infraimmaculata* was separated from *Salamandra* group approximately 13 million years ago and the subspecies of this species should be reviewed [1].

The coloration patterns were also investigated during this study. Similar to the fingerprints of human, these dorsal patterns are individual specific, therefore specimens can be identified by only examining the dorsal pattern, without using any marking technique [26]. With respect to the dorsal coloration models, Mersin, Hatay and Malatya specimens generally have both patches and stripes, while Erzincan specimens have flower like patterns. There are significant differences between provinces according to the dorsal coloration patterns. While Malatya specimens have numerous small patches, Mersin and Hatay specimens have larger patches and Erzincan specimens have flower like patterns.

In this study, no difference could be found

between different populations by means of karyotypes. Because karyotypes are specific for species, this result complies with our estimates. However, banding patterns may represent some differences between populations. Since enough magnification could not be done for detailed examination of the chromosomes, band staining procedures could not be performed. For this reason, the chromosomal differences between different populations could not be determined.

The number of diploid chromosomes of this species was found to be 24 in karyotype analysis. The first six pairs are metacentric, next two pairs are submetacentric, ninth and tenth pairs are small and metacentric. But eleventh and twelfth pairs are too small, so the centromere of these chromosomes could not be found, but probably these are submetacentric chromosomes. In a previous study, Kessous et al. (1968) indicates that, in *Salamandra salamandra* first 5 pairs of chromosomes are metacentric, 6-12th chromosome pairs are submetacentric [27]. Findings of Mancino et al. (1969) are also compatible with this study [28].

In histological analysis of female reproductive tissues, numerous oocytes were seen, bounded inside the ovary, and these oocytes were in different developmental phases. Same histological structures were found in specimens collected from all provinces. In oviduct slides, simple cuboidal epithelium, highly vascular connective tissue and smooth muscle layer were seen respectively. Various researchers reported same structures in oviducts [29-31].

Multiple lobular structure of testes was found to be remarkable. While some of these lobes comprise immature cells, the others have mature cells. Spermatocyte groups, which are surrounded by pericyctic cells and Sertoli cells between these spermatocyte groups were also remarkable. Imai and Tanaka (1978) reported similar findings [32].

As a result of the histological investigations, no difference could be found between populations. Results are complied with previous studies about this subjects [29-32].

As a result of all assessments, according to the indices and dorsal color patterns, three different populations could be distinguished, so Malatya-Mersin, Erzincan and Hatay populations could be considered as different subspecies.

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