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Acta Herpetologica 7(1): 81-90, 2012

A scanning electron microscopic study of the surface morphology of nuptial pads in male amphibians (Genus: *Bombina, Pelophylax, Rana*)

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Submitted on: 2011, 6th June; revised on: 2011, 27th November; accepted on: 2012, 21st March.

Abstract. The fine structure of nuptial pad surface of the anuran amphibians *Bombina variegata*, *Pelophylax epeiroticus*, *Pelophylax ridibundus* and *Rana dalmatina*, was examined by scanning electron microscopy. Nuptial pads are cutaneous secondary sexual characters of males that appear during the breeding season and disappear afterwards following an annual cycle. In males of *P. epeiroticus*, *P. ridibundus* and *R. dalmatina,* nuptial pads were observed on the ventrolateral aspect of the first digit (the thumb) as darkish and remarkably keratinized papillae. In males of *B. variegata* nuptial pads were almost black and very visible on the thumb, the second and the third digit of the front legs. They also extended on the ventral surface of the forearms. Under scanning electron microscope numerous small papillae were observed rising above pad's surface. In *P. epeiroticus*, *P. ridibundus* and *R. dalmatina*, these papillae were almost rounded at the base while at the dome shaped top they had many microprocesses organised in groups, thus assuming the shape of a "flower" which differed slightly among these three ranid species. In *B. variegata* the protuberances were conical with heavily keratinized hooks without microprocesses. Our results show that surface morphology of nuptial pads is unique for each species and could be considered as a species-specific character.

Keywords. SEM, nuptial pads, *Bombina variegata*, *Pelophylax epeiroticus*, *Pelophylax ridibundus*, *Rana dalmatina*.

INTRODUCTION

Nuptial pads, also called thumb pads or nuptial excrescences, are cutaneous male secondary sexual characters of anurans. They appear during the breeding season as dark and strongly keratinized protruberances or papillae on the ventrolateral surface of the first dig-

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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it or the first three digits and also on the inside of the forearms and disappear afterward. They consist of thickened epidermis and dermis. The external dark layer of the epidermis is thickened to form a horny covering that may be simply rugose or modified into cones or spines. Clusters of large mucous glands occupy the pad dermis their ducts reaching the pad surface. These patterns are confirmed both under light and transmission electron microscopes (Parakkal and Ellis, 1963; Kurabuchi, 1993, 1994; Brizzi et al., 2003). Nuptial pads are thought to facilitate the male's grip on the female during amplexus. Furthermore, the surface micro-sculptures provide the friction necessary for clasping the smooth body of the female (Lofts, 1974; Duellman and Trueb, 1994; Wells, 2007).

The development and maintenance of these structures is seasonal and regulated by androgenic hormones (Parakkal and Ellis, 1963; Lofts, 1974; Zamachowski and Zysk, 1978; Guarino and Bellini, 1993; Thomas et al., 1993; Epstein and Blackburn, 1997; van Wyk et al., 2003; Kaptan and Murathanoglu, 2008).

Studies on the 3-dimensional structure of the surface architecture of nuptial pads are limited and these mainly concern species from Japan and Italy (Kurabuchi, 1993, 1994; Brizzi et al., 2003). In the present study light and scanning electron microscopy was employed to investigate the fine structure of nuptial pads of some common anurans in Greece. The investigation aim at revealing potential interspecific differences. For this purpose four species were selected: the closely related water frogs *Pelophylax epeiroticus* and *Pelophylax ridibundus* and the brown frog *Rana dalmatina*, from the family Ranidae; the yellow-bellied toad *Bombina variegat*a was also studied which belongs to the distant and more primitive family of Bombinatoridae.

MATERIALS AND METHODS

Mature males of four anuran species were used in this study. All specimens were collected during the breeding season March and April of 2006-2007. Two males of *Bombina variegata* with a snout-vent length 56.7 mm and 42.1 mm, two males of *Rana dalmatina* with a snout-vent length 57.2 mm and 62.7 mm were collected from Pertouli (Altitude 900 meters, Longitude 21°27'51" E, Latitude 39°32'19" N). Two males *Pelophylax epeiroticus* (formerly known as *Rana epeirotica*), with a snout-vent length 76 mm and 84.2 mm were collected from Lake of Ioannina (Altitude 480 meters, Longitude 20°50'56" E, Latitude 39°40'3" N). Two males of *Pelophylax ridibundus* (formerly *Rana ridibunda*) with a snout-vent length 82.5 mm and 64.2 mm were collected from Lake Kerkini (Altitude 52 meters, Longitude 23°15'4" E, Latitude 41°15'42" N). Also one or two female individuals from each species were collected in order to point out sex-linked differences.

Preparation of tissues for scanning electron microscope (SEM)

Frogs were transported alive to laboratory and within few hours were prepared for examination. After being sacrificed in 0.1-0.5 % solutions of MS 222 the first digits (the thumbs) or the entire hands with well-developed nuptial pads were excised and fixed in glutaraldehylde 3 % for 24 hours. After they were dehydrated in serial ethanol, they were dried using the critical point method with liquid CO_2 and coated with gold in a sputtering system. Also the first digits of females were prepared on the same manner. Finally, materials were examined with a scanning electron microscope JEOL (JSM-840A).

Preparation of tissues for light microscope (LM)

For the histological examination nuptial pads were removed and fixed in formaldehyde solution 4%. After dehydration with ethanol solutions (70%, 90%) and clearing with xylene, the thumb pads were embedded in paraffin, serially sectioned at 7 μm and stained with Harris haematoxylin-eosin (HE). Sections were examined in a ZEIS AXIOSKOP and photographed with a digital camera OLYMPUS C-5060 adjusted to Microscope.

RESULTS

Gross anatomy observation (not shown) of the three ranid species *P. epeiroticus P. ridibundus* and *R. dalmatina* revealed that they are cutaneous papillae located on the first digits (thumbs) of the forelimbs and are extended from the ventrolateral base of the thumb to near the digital tip. In *B. variegata*, nuptial pads are visible on the 1st, 2nd and 3rd digits of the forelimbs, also extending on the ventral surface of the forearms. The pad regions were darkish in *R. dalmatina*, dark grey in *P. epeiroticus*, *P. ridibundus* and almost black in *B. variegata*.

Light microscopy (LM)

Sections of the nuptial pads of the four studied species observed under LM revealed the basic structure of the skin of anuran amphibians (Fig. 1A-F). They consisted of a thickened epidermis with several layers of cells and a dermis that contained linear clusters (rows) of large mucous glands opening on the pad surface. However, several differences were detectable among the four studied species. In *R. dalmatina* the outer skin surface shows conical hill-like protruberances with obvious keratinised layers (Fig. 1A-B) whereas in *P. epeiroticus* and *P. ridibundus* the protruberances are slightly rounded (Fig. 1C-D). In *B. variegata* the epidermis is modified into spines with remarkable amounts of melanin (Fig. 1 E-F). Differences also appeared in the size and shape of the mucous glands that were large and elongated orthogonal to the skin surface in the three species of ranids (Fig. 1A, D). They were ellipsoidal in *B. variegata* the major axis parallel to the skin surface (Fig. 1E).

Scanning electron microscopy (SEM)

Under SEM, the region of nuptial pads in the three species of ranids, the region of nuptial pads was easily distinguishable, even at low magnification, from the ordinary epidermis by the occurrence of numerous protuberances or papillae (Fig. 2, 1A-3A). In contrast to males the epidermis on the digits of females was smooth.

Rana dalmatina

Observation from above of papillae of nuptial pads in *R. dalmatina* showed that they were cylinder-shaped at base with a dome-like at top or apex (Fig.2, 1B-1D). More specifi-

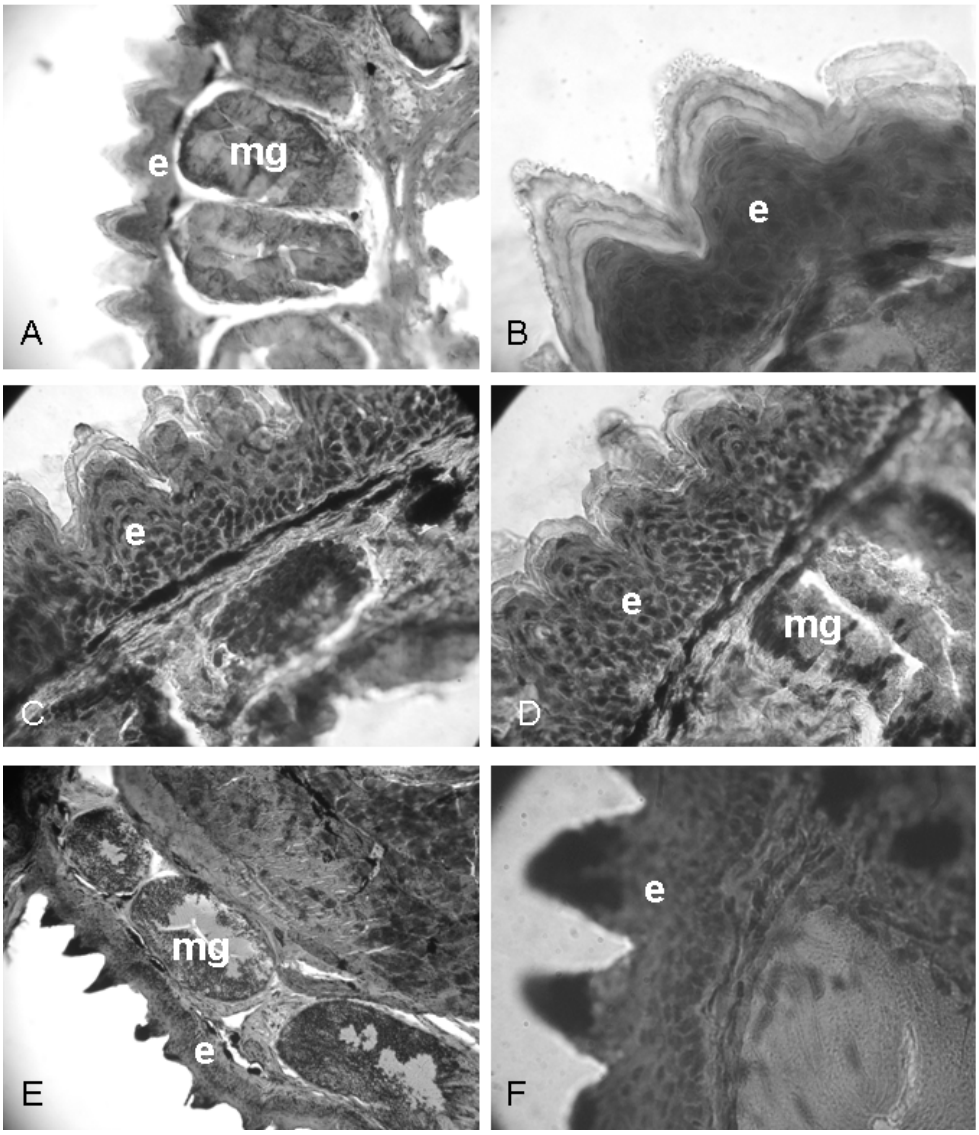

Fig. 1. Cross-sections of nuptial pads observed under LM. A-B. *Rana dalmatina* A. Epidermis and underlying elongated mucous glands. ×100. B. Epidermis with obvious keratinised layers ×400. C-D. Epidermis and underlying mucous glands of *Pelophylax epeiroticus* and *Pelophylax ridibundus*. ×400. E-F. *Bombina variegata*. E. Epidermis and underlying ellipsoid mucous glands. ×100. F. Epidermis with dark pigmented spines. ×400.

cally several groups of microprocesses were detected on the top of the papilla, whereas the lower microprocesses were arranged in concentric circles round the apex.

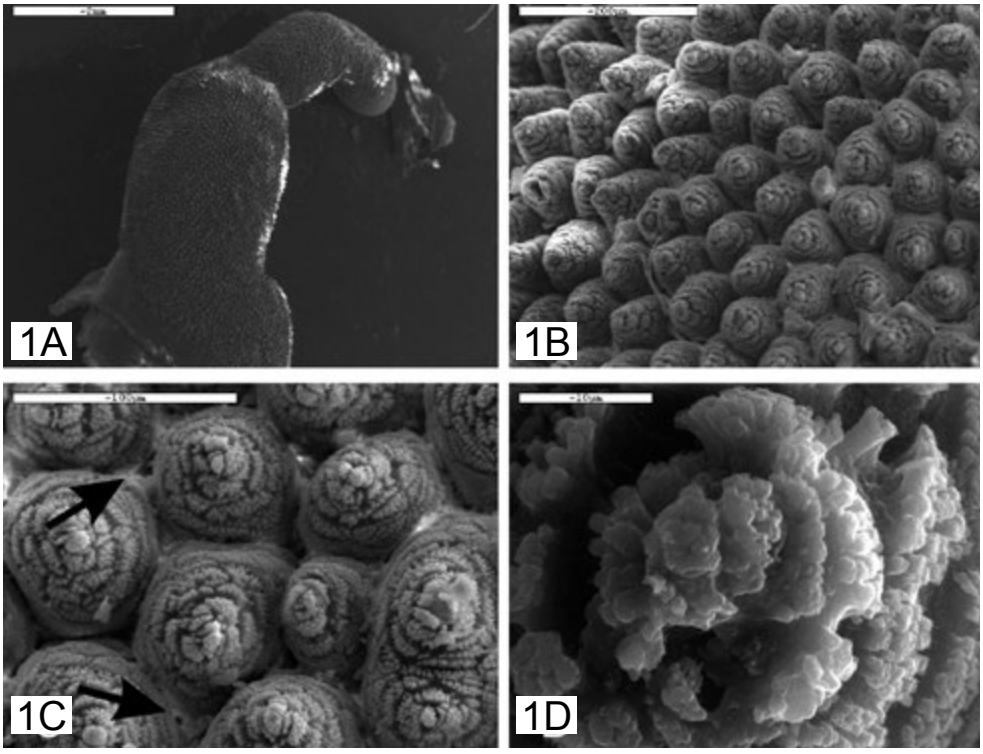

Fig. 2. SEM micrographs of nuptial pads of the ranid species *Rana dalmatina* (1A-1D), *Pelophylax epeiroticus* (2A-2D), and *Pelophylax ridibundus* (3A-3D) at four different magnifications. 1A-3A: First digit of a male with numerous papillae (scale bar = 2 mm). 1B-3B: Numerous papillae cylinder-shaped at base with a dome-like top (scale bar = 200 μm). 1C-3C: The pattern of papillae and the arrangement of microprocesses (scale bar = 100 μm). Arrows show the gland pores of the skin distributed among the papillae. 1D-3D: The flattened tips of the microprocesses (scale bar = 10 μm, 1D and 5 μm 2D, 3D).

Pelophylax epeiroticus.

In *P. epeiroticus* the papillae were cylinder-shaped with a rounded or semi-spherical top (Fig. 2, 2B-2D). The arrangement of groups of microprocesses was different from that of *R. dalmatina*. In *P. epeiroticus* two main groups were seen on the top of the papilla whereas the contiguous groups exhibited a spiral course (Fig. 2, 2C).

Pelophylax ridibundus

In *P. ridibundus* the shape of papillae was cylindrical at base and spherical at the top (Fig. 2, 3B-3D). Specifically, four or five smaller groups of microprocesses that are arranged in a cycle surround the central large group (Fig. 2, 3C). Higher magnification of the top showed the numerous microprocesses with flattened tips that differed from those of *P. epeiroticus* and *R. dalmatina* (Fig. 2, 1D).

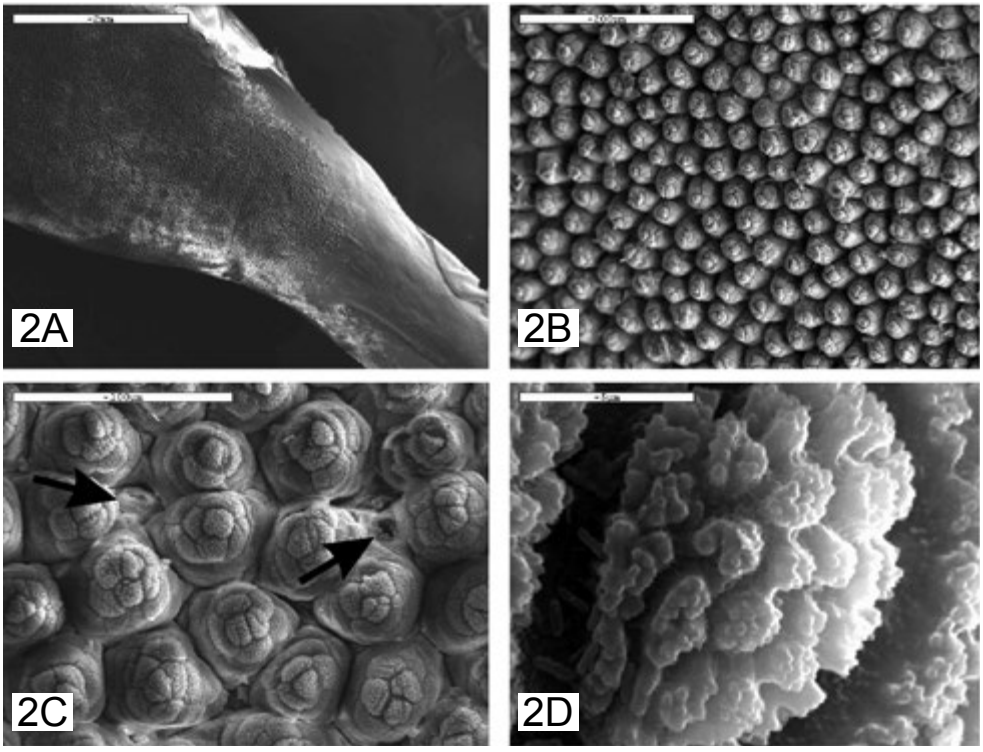

Fig. 2. Continued.

Bombina variegata

Distribution and shape of papillae in the nuptial pads of *B. variegata* were different. In this species we observed numerous hook-like or rose-spiny projections with a remarkably keratinization not only at the first finger but also in the second and partially the third digits and the ventral surface of the forearms (Fig. 3A). The papillae were cylindrical at the base and tapered upward to a point forming a hook or spine strongly keratinized (Fig. 3, B, C, D).

DISCUSSION

As expected, the results of this study showed that scanning electron microscopy is a powerful tool that provides important details of the microstructure of the surface morphology of nuptial pads of frogs that could not be detected under light microscope. Cross-sections of thumb pads observed under LM are in good agreement with previous histological studies concerning the species *Rana dalmatina, Rana esculenta* and *Rana perezi* (Guarino and Bellini, 1993; Brizzi et al., 2003), but they revealed the inner texture of the

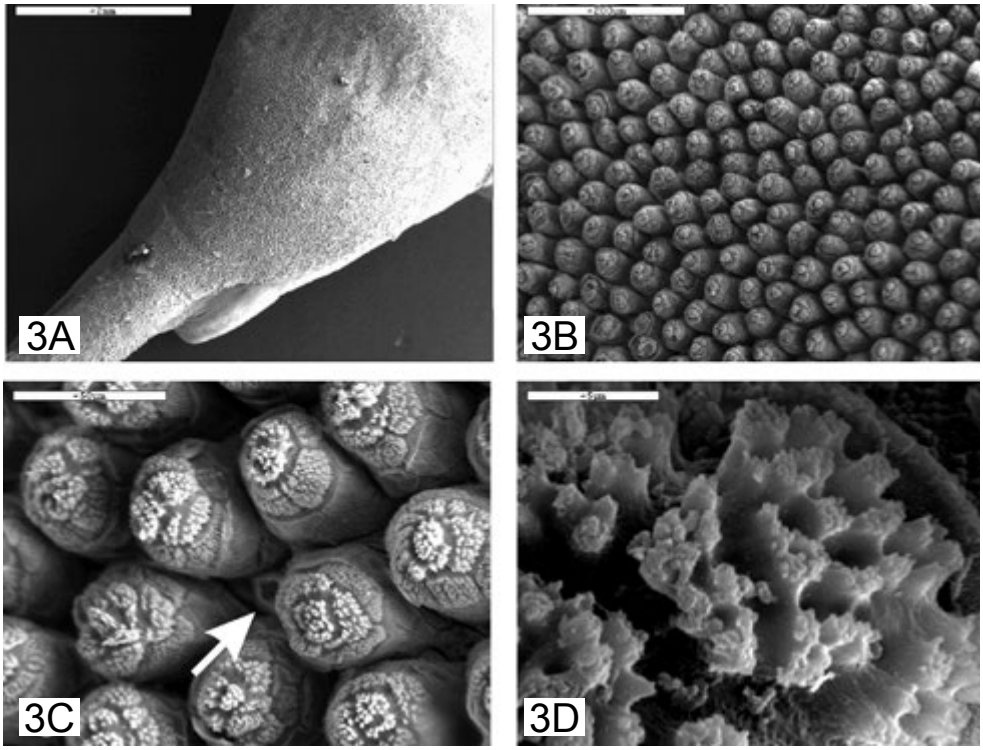

Fig. 2. Continued.

skin (including mucous glands in the spongy dermis) without many details of the external surface.

The results of SEM revealed remarkable differences in the surface morphology of the nuptial pads in the four species. Furthermore, slight differences existed between the water habit frogs *P. epeiroticus* and *P. ridibundus*, which are considered as very closely related by Schneider et al. (1984). It should be noticed that among the three ranid species, the observed differences between the surfaces of the nuptial pads mainly consisted of the arrangement and the number of groups of microprocesses on top of the papillae: *Pelophylax epeiroticus* shows two central groups of microprocesses and a spiral arrangement, *P. ridibundus* has a clear central group surrounded by four to five smaller clusters, whereas in *Rana dalmatina*, there is no clear central grouping but several small groups of microprocesses in concentric circles. On the other hand, a completely different pattern, with epidermal keratin hooks, was found in *B. variegata*. These results suggest that surface morphology of nuptial pads may be species-specific in anurans.

There are not many SEM studies on the surface morphology of nuptial pads of anurans for comparisons. Moreover, the few existing concern some hylid, ranid and rhacophorid frog species of Japan (Kurabuchi 1993, 1994). More recently Brizzi et al. (2003) published a SEM study for the European species *Rana perezi* (now *Pelophylax perezi*). In this species, there are five to six groups of microprocesses arranged spirally around the top

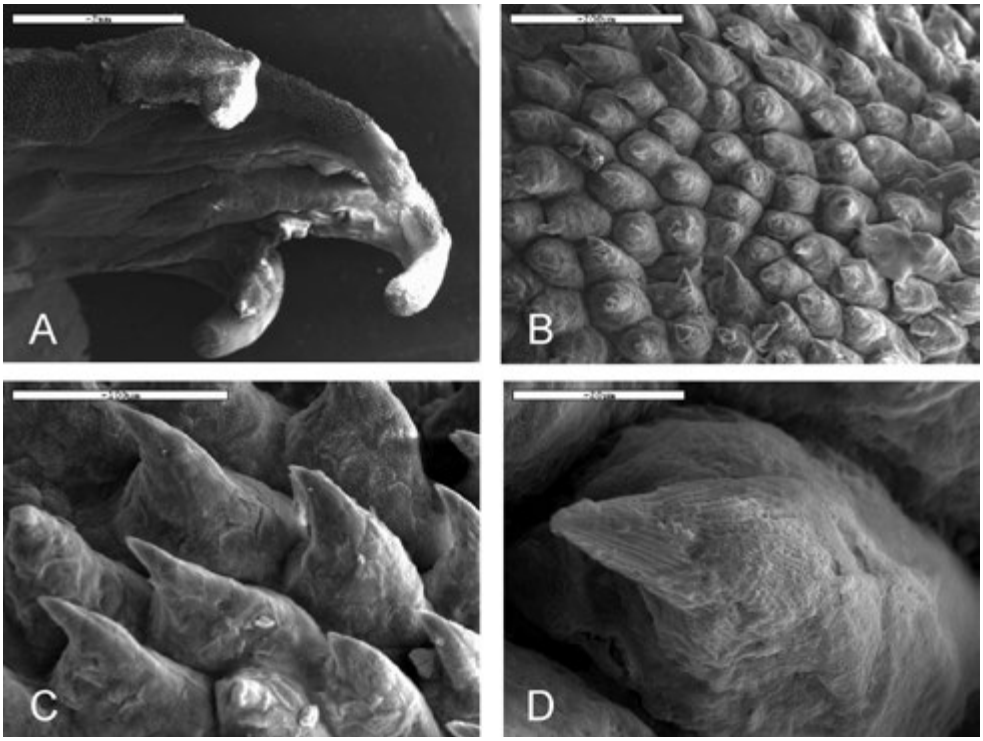

Fig. 3. SEM micrographs of nuptial pads of *Bombina variegata*. A: Forelimb of a male showing the nuptial pads at the first three digits (scale bar = 2 mm). B-C: The form of the spines at different magnifications (scale bar = 200 μm and 100 μm). D: The form of the top of a spine (scale bar = 20 μm).

of the papillae. This morphology appears more similar to that of *P. epeiroticus* and differs considerably from those of *P. ridibundus* and *Rana dalmatina*.

Regarding the Japanese species, Kurabuchi (1993) reported that *R. nigromaculata* (now *Pelophylax nigromaculatus*) and *R. brevipoda porosa* (now *Pelophylax porosus*), are similar in shape, size and colour. They have overlapping ranges and produce frequent natural hybrids, whereas they differ only on the morphology of the nuptial pads. This observation suggests that these species are actually different and stress the taxonomic value of the pattern of external morphology of nuptial pads.

Nuptial pads are associated with amplexus, helping the male hold onto the female, and preventing the female from escaping, while they may play a role in male-male combat (Duellman and Trueb, 1994; Wells, 2007). The correlation between the type of amplexus (axillary or inguinal) and the 3-dimensional architecture of the nuptial pad surface has been also discussed by Kurabuchi (1993). Axillary amplexus is common in ranids, while inguinal amplexus is encountered in *Xenopus laevis*. The nuptial pads of the latter species show numerous hook-like or rose-spiny projections with heavy keratinisation of surface cells. These differences in nuptial pad morphology were supported by our findings. The nuptial pads of the three ranid species were similar to that of the Japanese ranids, which

all use an axillary amplexus. In contrast, the pattern of *B. variegata* was similar to that of *X. laevis*, which both use an inguinal amplexus.

Moreover, it has been reported that well-developed nuptial pads are associated with breeding in water, while less-developed pads are found in terrestrial or land breeders (Wells, 2007). All four species of the present study breed in water and had accordingly well-developed nuptial pads.

The results of the present study support previous inferences of taxonomic and functional importance of the nuptial pads. Given their significance in anuran behaviour and evolution, we believe that further studies on more species may contribute to elucidate such aspects.

ACKNOWLEDGEMENTS

The authors thank Prof. Sklavounos S.A., and Assist. Prof. Pavlidou E. from the SEM Laboratory of Aristotle University of Thessaloniki, for their valuable contribution to this investigation.

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