Structural Diversity of Nuptial Pads in Phyllomedusinae (Amphibia: Anura: Hylidae)

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ABSTRACT We studied the morphological variation of the nuptial pads using light microscopy and scanning electron microscopy (SEM) in 26 species of phyllomedusines (Anura: Hylidae), representing the five currently recognized genera. All phyllomedusines have single nuptial pads with dark colored epidermal projections (EPs). Spine-shaped EPs occur in *Cruziohyla calcarifer*, *Phrynomedusa appendiculata* and in one species of *Phasmahyla*. The other species have roundish EPs. The density of the EPs on the pad is variable. Species in the *Phyllomedusa hypochondrialis* Group have EPs with a density that varies between 764 ± 58/mm^2 and 923 ± 160/mm^2. In all other studied species (including the *Phyllomedusa burmeisteri* and *Phyllomedusa perinesos* groups, *Phyllomedusa camba*, *Phyllomedusa boliviana*, *Phyllomedusa sauvagii*, *Phyllomedusa bicolor*, and *Phyllomedusa tomopterna*) the density of EPs varies between 108 ± 20/mm^2 and 552 ± 97/mm^2. Pores were observed with SEM in *C. calcarifer*, *Agalychnis lemur*, *Agalychnis moreletii*, but its presence is confirmed through histological sections on several other species. Its visibility using SEM seems to be related with the level of separation between adjacent EPs. The pores in the four studied species of *Agalychnis* are shown with SEM and histological sections to have a characteristic epidermal rim, that is absent in the other phyllomedusines. Unlike most previous reports on breeding glands, those of phyllomedusines are alcian blue positive, indicating the presence of acidic mucosubstances on its secretions. J. Morphol. 273:712–724, 2012.

KEY WORDS: Phyllomedusinae; scanning electron microscopy; secondary sexual characters; histology; Anura

INTRODUCTION

Nuptial pads are secondary sexual characters present in males of several species of anuran amphibians. They are modified epidermal and dermal tissues typically located in the first digit of the hand (Noble, 1931). Comparable structures are known to occur in other places of the body in some species (Liu, 1936; Thomas et al., 1993). Histologically, the pads usually consist of alveolar glands having secretory cells with well-defined cellular boundaries (Fujikura et al., 1988; Thomas et al., 1993; Epstein and Blackburn, 1997). In several species, the epidermis of the pad is distinctively thick, heavily keratinized, and dark colored; in some species, the glands are hypertrophied and form external protrusions of the skin (Fujikura et al., 1988); in other species, the pad is devoid of a thick epidermis and is mostly glandular.

Nuptial pads exhibit different morphological and physiological properties during the reproductive cycle. The extent of their development depends on hormone levels, reaching their maximum size during the breeding season (Inger and Greenberg, 1956; Parakkal and Ellis, 1963; Lofts, 1964; Rastogi et al., 1986; Kao et al., 1994; Kaptan and Murathanoglu, 2008). In several species, the pads remain present throughout the year, whereas in others, they regress completely. It has been suggested that the presence of the nuptial pads in males facilitate the grip of the female during the amplexus (Lataste, 1876; Boulenger, 1897; Noble, 1931; Liu, 1936). It has also been proposed that in some species they may play a role in combat between males (Duellman and Trueb, 1986) and in

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holding to the female when other male tries to dislodge them (Wells, 1977). Another function might be the release of secretions that attract females and/or stimulate the ovulation (Thomas et al., 1993).

Several authors have noticed that nuptial pads in different species are quite diverse macroscopically in terms of their distribution, size, texture, and coloration (e.g., Boulenger, 1897; Liu, 1936; Lavilla and Barrionuevo, 2005; Scott, 2005; Grant et al., 2006). Using scanning electron microscopy (SEM), the surface structure of the nuptial pad of *Xenopus laevis* has been studied by Kurabuchi and Inoue (1981). Zweifel (1983) studied the nuptial pad of two species of *Litoria* (then in the genus *Nyctimystes*; Hylidae: Pelodryadinae). Tyler and Lungershausen (1986) surveyed the structural complexity of nuptial pads in some species of Pelodryadinae and Limnodynastidae. Kurabuchi (1993, 1994) studied nuptial pads of one species of Hylidae, four species of Ranidae, and three species of Rhacophoridae. He described in detail the morphology of the elevations constituting the pad, their density, and height.

Leaf frogs of the hylid subfamily Phyllomedusinae currently comprise 58 species in five genera (Faivovich et al., 2010; Frost, 2011). So far, all known species have heavily keratinized and colored nuptial pads (Cruz, 1990). In this article, we present a survey of nuptial pad diversity in selected species of the five genera of Phyllomedusinae. We described the structure and diversity of nuptial pads using histological sections, light microscopy (LM) and SEM, and discussing these results in the context of the phylogenetic hypothesis for this group proposed by Faivovich et al. (2010).

MATERIALS AND METHODS

We examined adult males of 26 species of the five genera of Phyllomedusinae (see Supporting Information for a list of studied specimens). Furthermore, we analyzed three specimens each of *Phyllomedusa azurea* and *P. tetraploidea* from different localities to understand levels of intraspecific variation in the structure of the nuptial pads. All specimens were collected during reproductive activity or were selected for having indirect evidence of its active reproductive condition (partially dilated vocal sac, personal communication from the collectors, etc.). The specimens were fixed in a 10% solution of formaldehyde and preserved in 70% ethanol.

Morphological and structural analyses of nuptial pads were done using a dissection microscope. We estimated the density of protuberances on each pad by counting them on 8–10 squares of 250 × 250 μm^2 randomly placed on photographs of the pad using Micrometrics® SE Premium 4 software (ACCU-SCOPE INC., Commack, New York, USA) and expressed it as number of EPs per mm^2. The term "base of the digit" is used throughout the article as a landmark of the area covered by the nuptial pad in reference to the dorsal and medial surface of metacarpal II and prepollical elements.

The nuptial pads of some of the species examined were removed, dehydrated through an ascending series of ethanol through 100% ethanol, dried using an EMS 850 critical point dryer (Electron Microscopy Sciences, Fort Washington, New York, EEUU), and coated with gold:palladium (40:60) using a SC 7620 Mini Sputter Coater Termo VG Scientific (Quorum Technologies, East Grinstead, West Sussex, UK), and submitted to SEM using a Philips XL30 TMP New Look microscope (Eindhoven, The Netherlands). In one of the specimens of *P. tetraploidea* we removed only the stratum corneum of the pad, mounted this piece backward and submitted it to SEM (see "Results" section).

For histological studies, tissues were dehydrated in an ascending series of ethanol, cleared in toluene, and embedded in Paraplast. All samples were sectioned at 5 μm and stained with hematoxilin and eosin and modified Masson's Trichrome to describe general morphology of the pads. Histochemical studies were conducted by standard staining techniques, including, alcian blue (AB), pH 2.5 (Bancroft, 1975) for acid mucosubstances, periodic acid-Schiff (PAS) for neutral mucosubstances, with hematoxilin used as a counterstain, and performic acid-AB (PAAB) for protein-bound sulfur (Bancroft, 1975). The histochemical properties of glands were only recorded as positive (+), negative (−) or equivocal (±), to avoid confounding effects of fixation histories of the exemplars.

Throughout this article, we use the term EP/EPs to refer to each of the individual epidermal and dermal elevations that constitute the nuptial pad, which are evident using stereomicroscope, LM, and SEM. The term papilla seems to be adequate to describe each projection, because it includes not only an epidermal component but also a dermal stratum papilla in the core of the structure. However, we retained the term EP, because there is evidence that at least in one species (*Hyla japonica*), there are some projections of the epidermis devoid of a dermal component (Kurabuchi, 1994).

Institutional codes follow Leviton et al. (1985) with the exceptions of CENAI: Collection of the Centro Nacional de Estudios Iológicos (housed in MACN), and CFBH: Coleção Célio F. B. Haddad, Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil. All materials examined are listed in Supporting Information.

RESULTS

The nuptial pads in all studied phyllomedusines occur only on the first manual digit. The surface of the nuptial pad is easily distinguishable from the rest of the skin by the presence of numerous colored EPs. Its coloration varies from light to dark brown. The space between the EPs is not colored. There is interspecific variation in density of EPs (Table 1). In almost all analyzed species, the nuptial pad covers the base of the digit reaching the proximal half of the basal phalanx or the basal phalanx completely. The pad usually tapers off toward the basal phalanx and rarely reaches the ventral surface of the digit (Fig. 1A).The most different morphology of the pad is seen in *Phyllomedusa perinesos,* where it is a circular patch that extends from the distal half of the base of the digit to the beginning of the basal phalanx (Fig. 1C).

Phyllomedusa

We analyzed 16 of the 30 species of the genus. In all specimens, the pads are constituted by numerous roundish EPs. Two different arrangements of density are seen among its species. One arrangement is found in the *Phyllomedusa hypochondrialis* Group, which has EPs with a density that varies between 764 ± 58/mm^2 and 923 ± 160/mm^2 (Fig. 1A,B). The other arrangement is found in the exemplars of the *P. burmeisteri* and

TABLE 1. Estimated densities of EPs of the nuptial pads of phyllomedusines

Species	Density	*n*
Agalychnis hulli	346 ± 48	1
A. aspera	678 ± 62	1
A. lemur	438 ± 95	1
A. dacnicolor	429 ± 69	2
A. callidryas	552 ± 130	1
A. moreletii	338 ± 67	2
Cruziohyla calcarifer	294 ± 47	2
Phrynomedusa appendiculata	108 ± 20	1
Phasmahyla jandaia	245 ± 71	2
P. guttata	386 ± 111	1
Phyllomedusa bicolor	365 ± 42	1
P. camba	536 ± 33	1
P. boliviana	430 ± 48	6
P. sauvagii	430 ± 59	6
P. bahiana	405 ± 69	1
P. burmeisteri	409 ± 94	3
P. distincta	525 ± 59	2
P. tetraploidea	461 ± 62	5
P. tomopterna	499 ± 40	2
P. perinesos	552 ± 97	1
P. hypochondrialis	923 ± 160	3
P. nordestina	837 ± 137	5
P. azurea	848 ± 113	5
P. rohdei	832 ± 125	4
P. megacephala	815 ± 111	1
P. ayeaye	764 ± 58	1

Values are presented as mean density and standard deviation expressed as number of EPs per mm^2. Densities estimated on each pad by counting 8–10 randomly picked squares of 250 × 250 μm^2 on the pad(s) of one or more specimens (*n*).

P. perinesos groups, and in *P. camba*, *P. boliviana*, *P. sauvagii*, *P. bicolor*, and *P. Tomopterna*, which present EPs with a density that varies between 365 ± 42/mm^2 and 552 ± 97/mm^2 (Fig. 1C,D).

Phrynomedusa

Only one of the five species of this genus was available for study. The nuptial pad of *Phrynomedusa appendiculata* is constituted by few and conspicuous EPs that are conically shaped with spiny ends (Fig. 2A), which we call spine-shaped EPs. The density of the EPs is 108 ± 20/mm^2.

Cruziohyla

One of the two species of the genus was available for this study. The nuptial pad of *Cruziohyla calcarifer* is constituted by numerous spine-shaped EPs. The spiny ends of each EP are only evident under higher stereoscopic magnification (Fig. 2B). The density of the EPs is 294 ± 47/mm^2.

Agalychnis

We analyzed six of the 14 species of the genus. The nuptial pads of all studied species are constituted by numerous EPs with roundish shape (Fig. 2C). The density of EPs varies among species, from 338 ± 67/mm^2 to 678 ± 62/mm^2.

Phasmahyla

Two of the seven species of the genus were available for study. In *Phasmahyla guttata* the pad is constituted by numerous roundish EPs. The density of EPs is 386 ± 111/mm^2. The nuptial pad in *P. jandaia* is constituted by numerous spine-shaped EPs (Fig. 2D). The density of the EPs is 245 ± 71/mm^2.

SEM and Histological observations

The SEM observations show epidermal surface specializations on the nuptial pad area. Near the margins of the pads, the EPs are gradually depressed and less conspicuous. For that reason, we do not consider that area for the description of the shape of the EPs. The structure of the EPs can be generally described as hemispherical or spine shaped (Fig. 3A,B). They are in general irregularly sized, although in one of the studied species, *P. camba*, the EPs are uniformly sized (Fig. 3C,D). It is frequent to find EPs partially fused in several areas of the pad (Fig. 3E). Ornamentations are seen covering the surface of each EP. These ornamentations are constituted by multiple processes usually concentrated on the top of the EP that gradually shorten toward the sides. The disposition of the processes on the top of the EPs is variable as well. In species of *Agalychnis, Phyllomedusa,* and *Phasmahyla,* the processes are seen as central radiating clusters of irregular shape (Fig. 4A), projected in small bundles (Fig. 4B) or projected as single bundles on the top of the EP. In *Cruziohyla, Phrynomedusa*, and one species of *Phasmahyla*, which have spine-shaped EPs, the ornamentations consist of flattened cells covering the EP (Fig. 4C). In all studied species, multiple structures resembling microridges are seen using higher magnification of the ornamentations (Fig. 4D). Pores, when visible, are seen in the spaces between adjacent EPs.

The stratum corneum of some EPs is missing in our sample of *A. lemur*, so the stratum granulosum is seen through the space left by them (Fig. 4E). The superficial layer of this stratum is constituted by multiple radial projections (Fig. 4F). The dispositions of these projections closely resemble the ornamentations of the stratum corneum.

The analysis of intraspecific variation in pads of *Phyllomedusa azurea* and *P. tetraploidea* indicates the same structure in different specimens from different localities. On scanning electron micrographs of the nuptial pads of *P. azurea*, we find that all EPs are less prominent and with poorly defined limits, although they have similar ornamentations to those of other specimens. The same is seen on scanning electron micrographs of one of the nuptial pads of *P. tomopterna*.

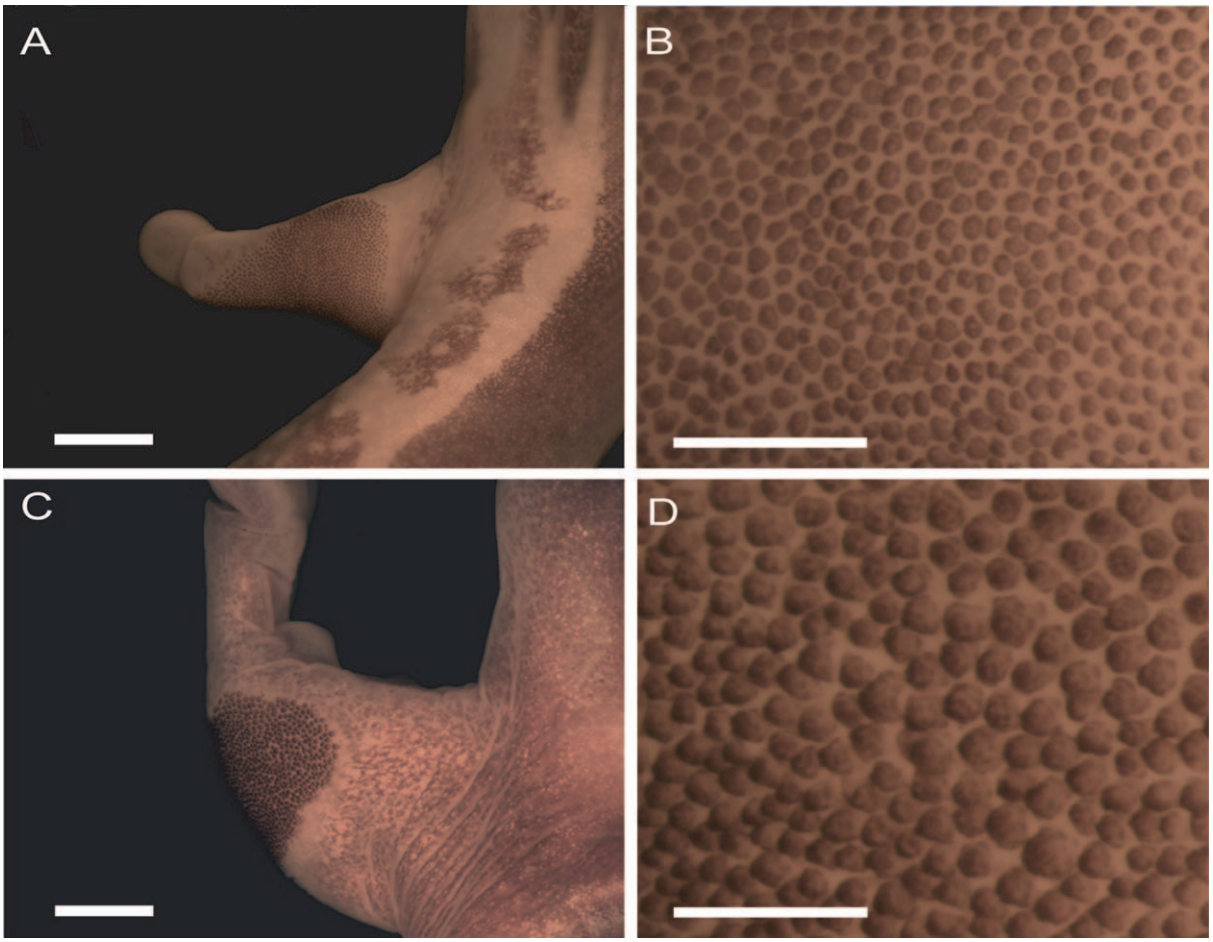

Fig. 1. Nuptial pads of exemplar species of *Phyllomedusa*. (**A**) *P. azurea* (MNRJ 40408). The pad covers the base of the digit and the basal phalanx, tapering off anteriorly. Scale bar = 1 mm (**B**) Higher magnification of nuptial pad showing EPs. Scale bar = 250 μm. (**C**) *P. perinesos* (MNRJ 74788). The pad extends from the distal half of the base of the digit until the beginning of the basal phalanx. Scale bar = 1 mm. (**D**) Higher magnification of nuptial pad showing EPs. Scale bar = 250 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Our histological study shows that each EP consists of five to seven layers of epidermal cells surrounding a dermal papillar core. The outermost cell layer of the epidermis (stratum corneum) consists of a monolayer of highly keratinized cells as revealed by PAAB. At the top of the EPs, the cell layer is thicker than at the sides and base; this same layer is dark brown colored. Beneath the stratum corneum, the stratum granulosum consists of a monolayer of swollen cells presenting an apical surface that resembles the ornamentation of the stratum corneum (Fig. 5A). This apical part, presenting cytoplasmic projections stains positively for PAAB, indicating the existence of disulfide bonds, putatively attributable to keratin. The stratum spinosum consists of three to four layers of cuboidal cells. Finally, the stratum basale consists of a monolayer of low columnar cells on the basal membrane and dermis (Fig. 5B). The stratum spongiosum of the latter holds two different kinds of glands. Melanophores are seen in this stratum above and beneath breeding glands.

Mucous glands are alveolar glands composed of a secretory compartment, a duct, and an intermediate region. The secretory portion consists of a monolayer of cells distributed along the periphery of the secretory compartment. The glands are filled with weakly basophilic, nongranular material. They yield a positive reaction for PAS and AB.

Breeding glands are alveolar glands, which show an ovoidal profile. They consist of a secretory compartment composed of a monolayer of densely packed columnar secretory cells, an intermediate region, and a duct. The secretory cells are lodged with acidophilic granules that yield a strong positive reaction to PAS (Fig. 5B). In some cells of the glands, there is also strong AB+ material (Fig. 5C).

The duct of the breeding glands is formed by PAAB+ cells that cross the epidermal layers to form a keratinous lining (Fig. 6A). These cells seem to be keratinocytes. The skin of the surrounding area invaginates forming a gap or

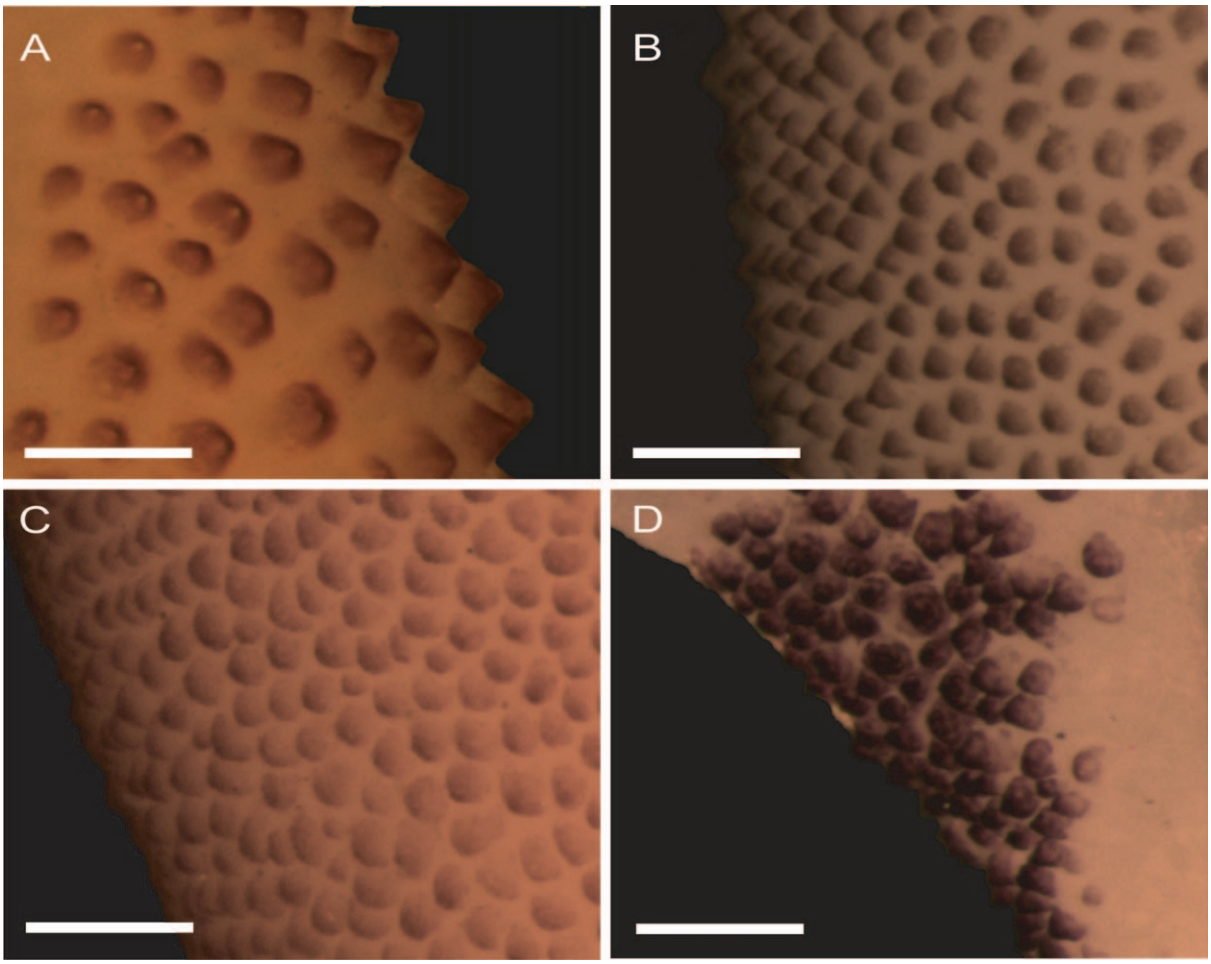

Fig. 2. Scanning electron micrographs of nuptial pads of some phyllomedusines. (**A**) *Phrynomedusa appendiculata* (MZUSP 35191); (**B**) *Cruziohyla calcarifer* (MNRJ 74789); (**C**) *Agalychnis moreletii* (CENAI 7821); (**D**) *Phasmahyla jandaia* (MNRJ 71953). Scale bar = 250 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

depression in the circumadjacent space of the pore. In *C. calcarifer* and the species of *Phyllomedusa*, the outermost duct cell emits a projection that delimits the pore of the duct. The adjacent keratinocytes from the skin surround but do not contact the outermost duct cell at its extreme (Fig. 6A,B). In the four species of *Agalychnis* for which samples were available for histological sections (*A. dacnicolor, A. hulli, A. lemur, A. morelettii*), the outermost duct cell protrudes outside the limits of the interstitial space between the EPs and defines a conspicuous keratinized epidermal rim (Fig. 6C,D).

Although pores are not visible on scanning electron micrographs of any of the available species of *Phyllomedusa*, *Agalychnis aspera*, or *A. dacnicolor*, the preparation of a stratum corneum of *Phyllomedusa tetraploidea* that was separated from the pad allowed to see on its undersurface the multiple keratinized ducts of the breeding glands that are present along the nuptial pad (Fig. 7A). A higher magnification of the area shows the same SEM structure for both the EPs and the ducts (Fig. 7C,D). In the same way, on the surface of the stratum corneum, as the EPs are more spaced (presumably as an artifact of the separation), it is possible to see the pores, otherwise invisible under normal conditions (Fig. 7B).

DISCUSSION

The nuptial pads in phyllomedusines are always a single structure. The covered surface presents some differences in the extension of the pad. However, this variation is not as extensive as in Pelodryadinae (Tyler, 1968; Tyler and Davies, 1978, 1979), the sister taxon of Phyllomedusinae, where some species present more than one pad (e.g., *Litoria infrafenata*), or in some cases the pad is absent as in *Litoria multiplica* (Tyler, 1964).

Tyler and Lungershausen (1986) examined 28 species now included in 17 species groups of *Litoria*, plus two species unassigned to any group.

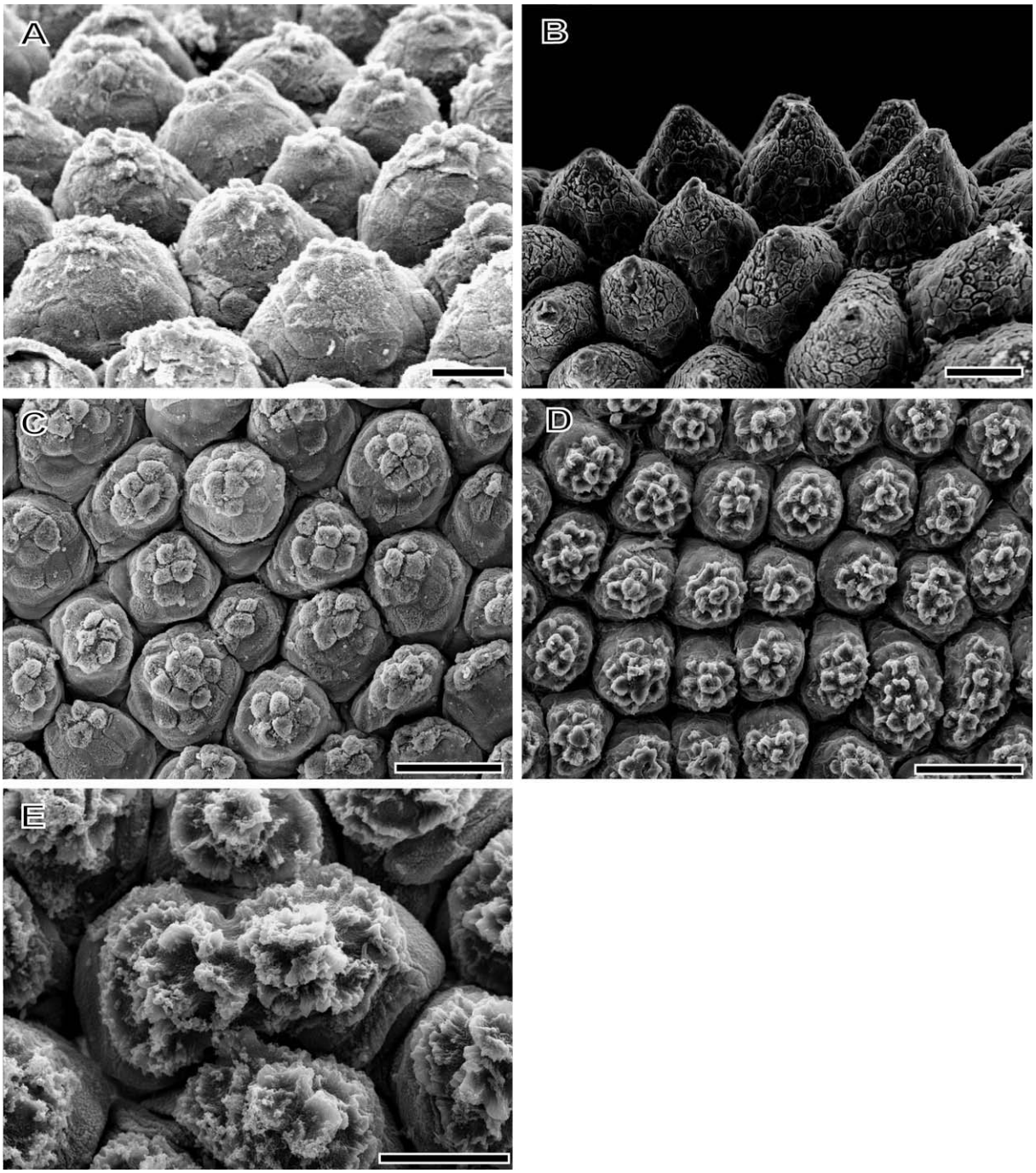

Fig. 3. Scanning electron micrographs of nuptial pads of Phyllomedusines. (**A**) Hemispherical EPs of *A. moreletii* (CENAI 7821). Scale bar = 20 μm. (**B**) Spine-shaped EPs of *Phrynomedusa appendiculata* (MZUSP 35191). Scale bar = 50 μm. (**C**) Nuptial pad of *Phyllomedusa bicolor* (MNRJ 52970) viewed from above showing the irregularly sized EPs. Scale bar = 50 μm. (**D**) Nuptial pad of *Phyllomedusa camba* (CFBH 21725) showing regularly sized and ordered EPs. Scale bar = 50 μm. (**E**) Detail of partially fused EPs of *Phyllomedusa azurea* (MNRJ 40408). Scale bar = 20 μm.

Using SEM, they described four different structures in this genus. All these correspond to different modifications associated with the EPs (that they call elevations). We found no obvious similarity between the morphologies of the EPs described in *Litoria* and our observations. The spine-shaped EPs of *C. calcarifer* and *Phrynomedusa appendiculata*, however, reminds to the morphology described by these authors for limnodynastids as a large thorn, having a diameter of 66 ± 8/μm (n = 33) and 96 ± 14/μm (n = 27), respectively, versus the 50-μm diameter that they reported for that structure.

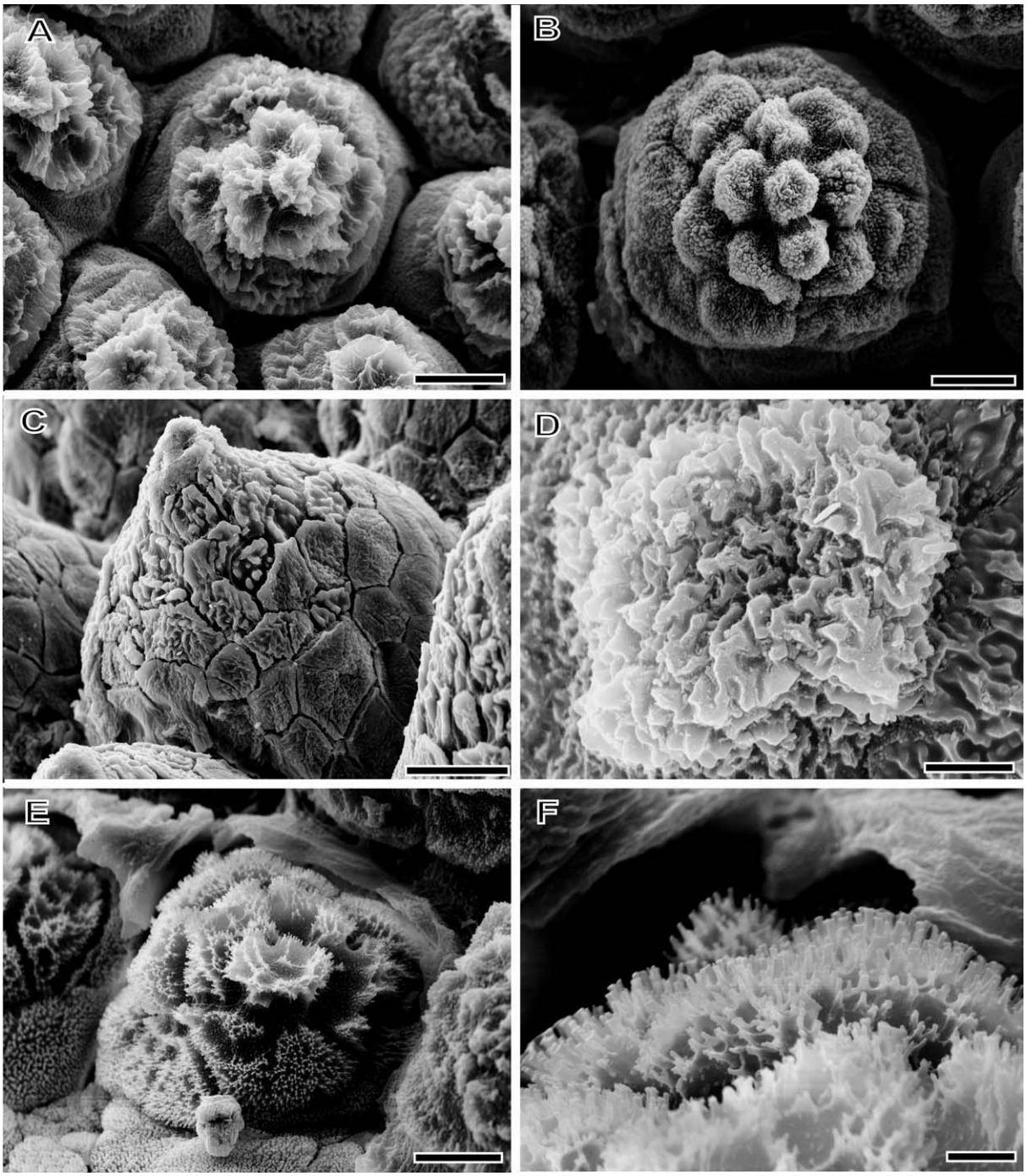

Fig. 4. Scanning electron micrographs of nuptial pads of Phyllomedusines (**A**–**C**) Detail of the disposition of the processes on the top of an EP. (A) *Phyllomedusa azurea* (MNRJ 40408) showing a central radiating cluster of irregular processes. Scale bar = 50 μm. (B) *Phyllomedusa bicolor* (MNRJ 52970) showing processes arranged in bundles. Scale bar = 10 μm. (C) Spine-shaped EP of *Phrynomedusa appendiculata* (MZUSP 35191). Notice the flattened cells of the stratum corneum covering the EP. Scale bar = 20 μm. (**D**) Ornamentation of *A. moreletii* (CENAI 7821) seen in high magnification showing structures resembling microridges. Scale bar = 2 μm. (**E**) Stratum granulosum of *A. lemur* (AMNH124161). Scale bar = 10 μm. (**F**) Higher magnification of the stratum granulosum showing radial projections. Scale bar = 2 μm.

Zweifel (1983) estimated the EP density in *Litoria disrupta* and *L. trachydermis* (then included in the genus *Nyctimystes*) to be "about" 700/mm^2, whereas Tyler and Lungershausen (1986) reported EP density estimates between 15/mm^2 and 280/mm^2. While our estimates for a few species overlap in different degrees with those reported by Tyler and Lungerhousen (*A. moreletii*, *C. calcarifer*,

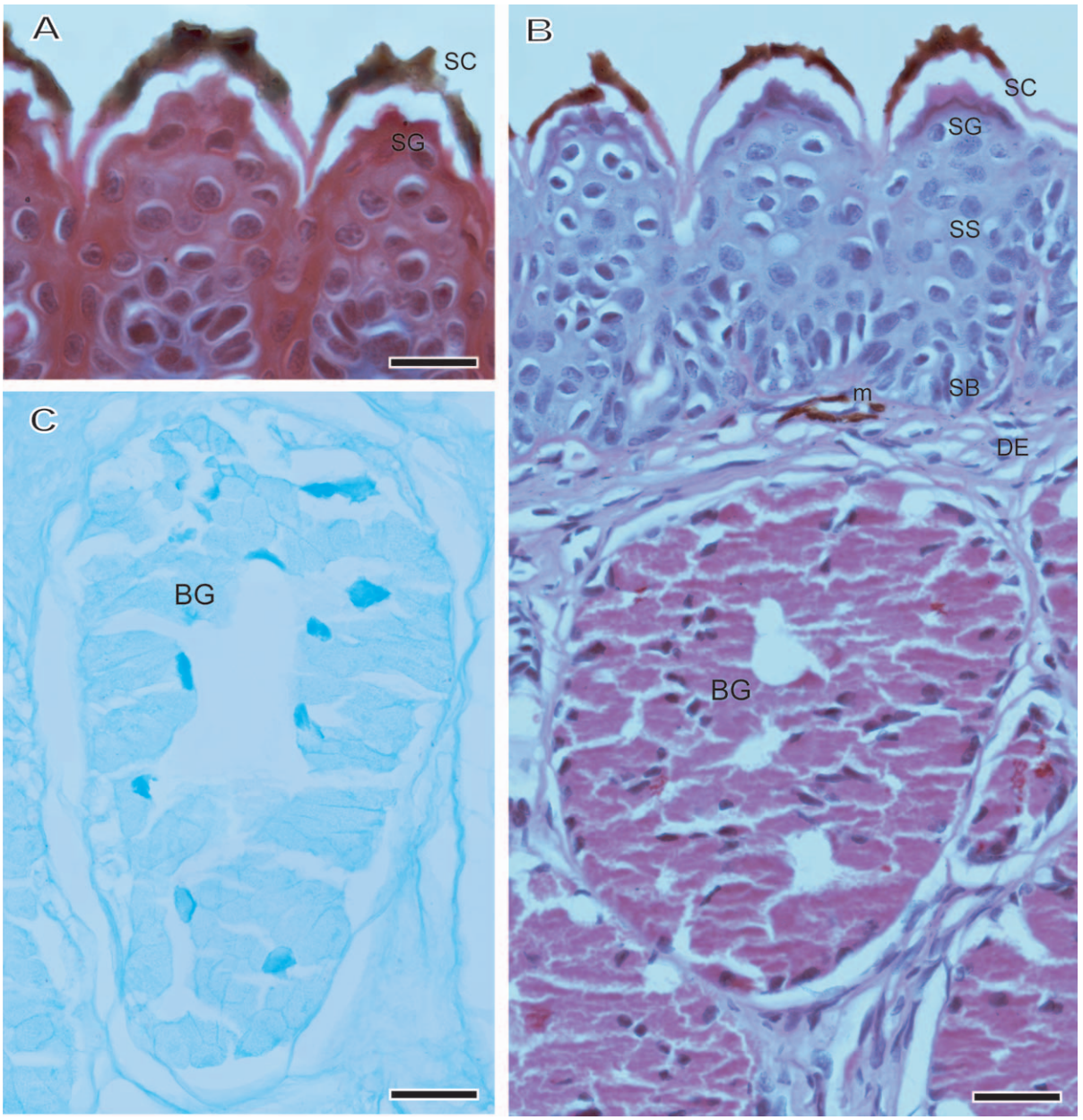

Fig. 5. Transverse section of nuptial pads of Phyllomedusines. (**A**) EPs of *Phyllomedusa camba* (CFBH 21725) stained with Masson's trichromic. Note the apical surface of the stratum granulosum resembling the ornamentation of the stratum corneum. (**B**) EPs of *P. camba* (CFBH 21725) showing epidermal stratum and a PAS-positive breeding gland. Counterstained with hematoxylin. Melanophores are seen in the stratum spongiosum above and beneath breeding glands. (**C**) Breeding gland of *Phyllomedusa sauvagii* (MACN 38136) showing the positive reaction to AB. Scale bar = 15 μm. BG = breeding gland; DE = dermis; m = melanophores; SB = stratum basale; SC = stratum corneum; SG = stratum granulosum; SS = stratum spinosum. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Phasmahyla guttata, *P. jandaia*, *Phrynomedusa appendiculata*; see Table 1), all other species have higher estimates, although several showing overlapping values with these ones, indicating continuous variation. However, there is a clear interval in EP density between the species in the *P. hypochondrialis* group and all other phyllomedusines, as our density estimates indicate that the former has the highest density counts within phyllomedusines (764 ± 58/mm^2 to 923 ± 160/mm^2; Table 1). This increase in EP density is here considered a putative synapomorphy of this group.

The most distinctive EP morphology was found in the basal genera *Phrynomedusa* and *Cruziohyla*. Both have spine-shaped EPs, although SEM revealed that these structures have different ornamentations. The other spine-shaped EPs occur in one species of *Phasmahyla*, *P. jandaia*; in this spe-

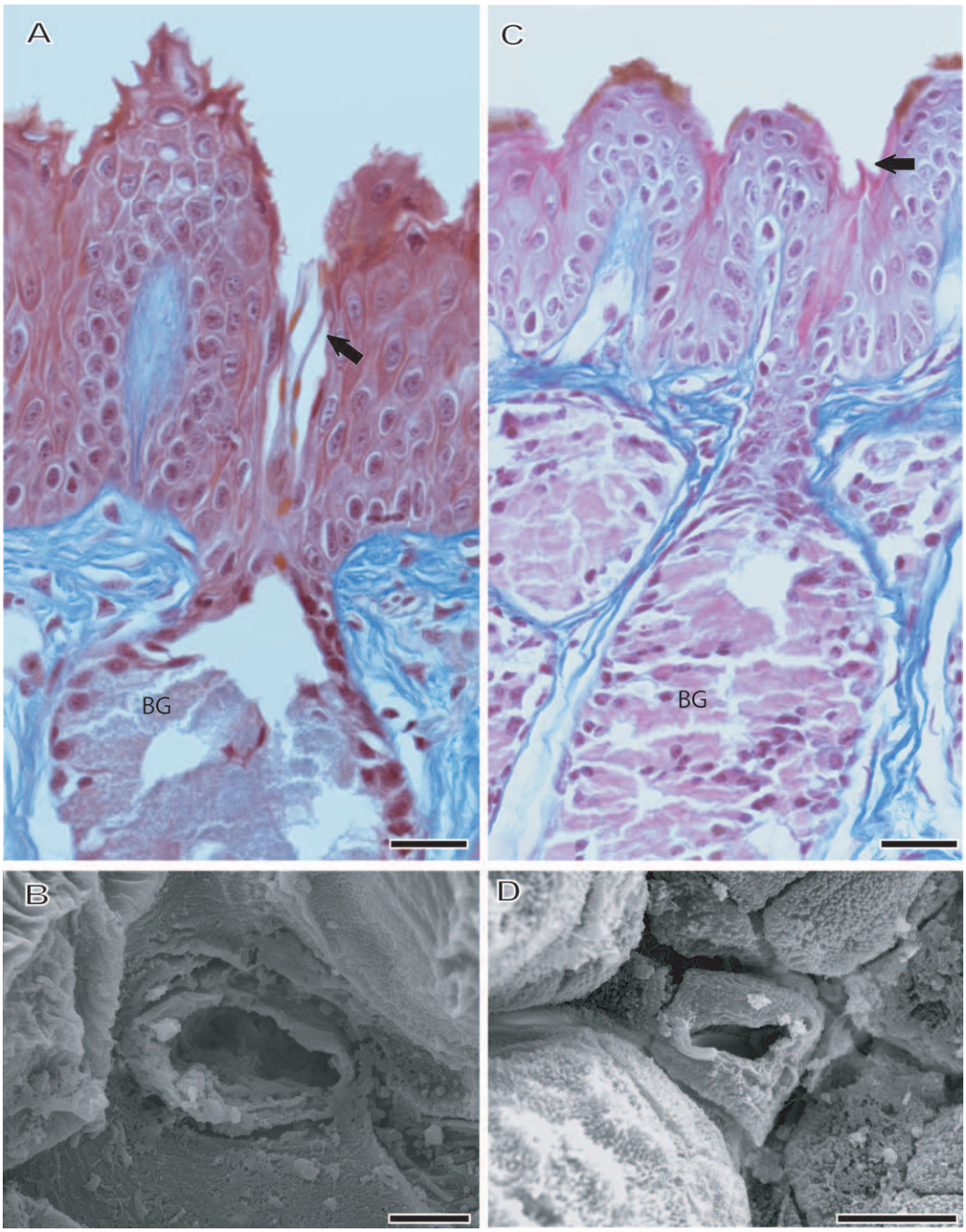

Fig. 6. Pores of breeding glands. (**A**) Transverse section of nuptial pad of *C. calcarifer* (MNRJ 74789) stained with Masson's trichromic. The outermost duct cell emits a projection that delimits the pore of the duct (arrow). The skin of the surrounding area invaginates forming a gap in the circumadjacent space of the pore. Scale bar = 15 μm. (**B**) Detail of a characteristic pore of *C. calcarifer* seen under SEM. Scale bar = 5 μm. (**C**) Transverse section of nuptial pad of *A. moreletii* (CENAI 7821) stained with Masson's trichromic. The outermost duct cell protrudes outside the limits of the interstitial space between the EPs defining a keratinized epidermal rim (arrow). Scale bar = 15 μm. (**D**) Detail of a characteristic pore of *A. moreletii* seen under SEM. Note the conspicuous epidermal rim. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cies, the EPs are smaller (diameter 56 ± 9 μm, n = 22) than in *Phrynomedusa appendiculata* (96 ± 14 μm, n = 27), and it is most parsimoniously interpreted as a reversion from the hemispherical EP, that occurs on all other studied species. In the context of the current phylogenetic hypothesis of Phyllomedusinae, the spine-shaped EPs in *Cruziohyla* and *Phrynomedusa* could be interpreted as a

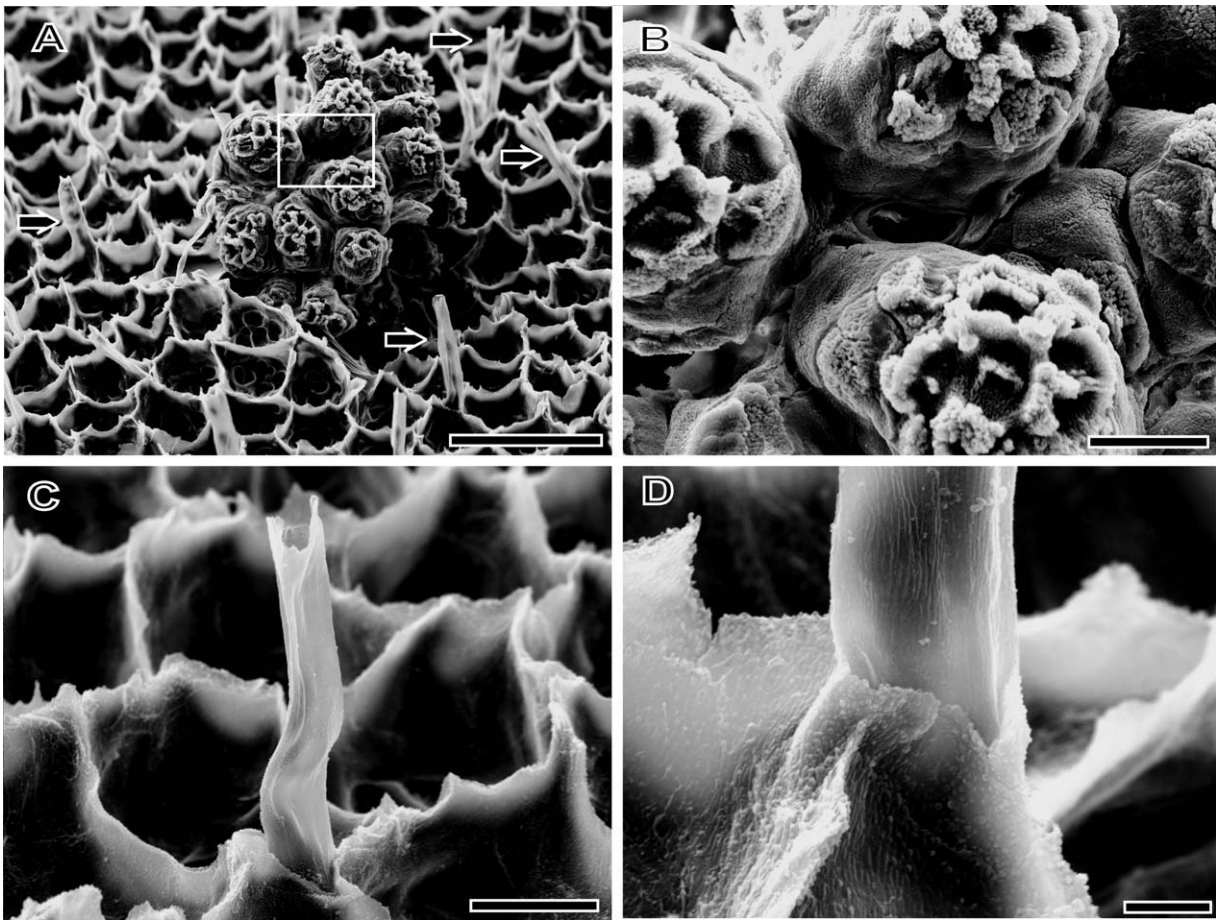

Fig. 7. Stratum corneum of *Phyllomedusa tetraploidea* (MACN 40751) observed with SEM. (**A**) Multiple ducts of breeding glands seen on the undersurface of the stratum corneum (arrows). Scale bar = 100 μm. (**B**) Enlargement of the area delimited in (A) showing the pore of *P. tetraploidea*. Note that the EPs are widely separated, because the stratum corneum has been bended; otherwise the pores would not be visible using SEM. Scale bar = 20 μm. (**C**) Detail of a single duct. Scale bar = 20 μm. (**D**) Higher magnification of the apical area of the duct. Note the same structure in the duct and the stratum corneum. Scale bar = 5 μm.

plesiomorphy within the clade or as two independent origins. This is dependent on the EP structure that optimizes at the base of pelodryadines; unfortunately knowledge of both phylogenetic relationships within this subfamily and taxonomic distribution of EP morphology is still poor.

The SEM observations indicate that EPs of nuptial pads in *Phyllomedusa camba* follow an ordered arrangement and are uniform in size, which does not occur in the *P. burmeisteri* group, *P. bicolor* or *P. sauvagii*. No species of the *P. tarsius* Group were available for this study, but we would expect to find the same arrangement on their nuptial pads.

A remarkable aspect of our SEM results is the external visibility and morphology of pores. Pores have been shown in *Pelophylax perezi* (Brizzi et al., 2003) and in *Buergeria japonica* (Kurabuchi, 1994) without any comment besides its shape as seen with SEM.

We found pores using SEM in only a few of the studied species, all occurring in the spaces between the bases of the EPs. In *Agalychnis moreletii* and *A. lemur*, the pores have the same structure, with a conspicuous epidermal rim; the same structure was confirmed through histological sections in *A. dacnicolor* and *A. hulli*. In *Cruziohyla calcarifer*, the pores lack that protusion. A similar morphology occurs in the sample of *Phyllomedusa tetraploidea* in which we were able to study only the stratum corneum, and therefore see the pores when the EPs were artificially separated. Scanning electron micrographs also confirmed the same keratinized structure of the ducts and stratum corneum of the EPs (Fig. 7D). Other than that particular sample of *P. tetraploidea*, we have carefully searched for pores in the other species analyzed with SEM but were unable to find them. For species studied by SEM and histological sections, we could prove the presence of pores by LM, even if these were not detected by SEM. The pores are difficult to see in SEM because of the position between the EPs, in particular when the EPs are closely packed. We consider this the most likely explanation for the negative result with SEM. In the

particular case of *P. appendiculata*, where unlike the species of *Phyllomedusa* we could see by SEM the base of EPs, we attributed the missing pores to the small size of the piece studied; for this same reason, we could not carry out histological sections for this species. Our survey predicts the presence of externally visible pores with the morphology seen in *C. calcarifer* in the other species of *Cruziohyla*, *C. craspedopus*, and the presence of ducts with conspicuos epidermal rim in the other species of *Agalychnis*. In the latter case, we further hypothesize that this peculiar structure of the pore is a putative synapomorphy of *Agalychnis*.

The visibility of pores in SEM is directly associated with how separated the EPs are. However, it is not necessarily something related with EP density. In species of *Phyllomedusa* (*P. bicolor*) or other species of *Agalychnis* where the density is similar to that of *A. moreletii*, the pores were not visible on scanning electron micrographs. The visibility of pores on SEM is considered here a consequence of the structural differences among EPs. Although our knowledge on chemistry of the pad breeding glands secretions is limited to that provided by standard histochemistry, our knowledge on its biological role is directly null. It could have an antislippery function for holding a tight grip on the female in addition to the surface of the pad during amplexus, as it has been repeatedly suggested (Lataste, 1876; Boulenger, 1897; Noble, 1931; Liu, 1936). It could also have a role in chemical communication with the female (Thomas et al., 1993) or other males, or serve in both roles. Regardless of which of these turns to be supported empirically, differences in overall exposition of the pores might have consequences in the way its secretions are spread on the pad and/or eventually on other surfaces this make contact with (like the female axilar/pectoral area).

Microridges have been assumed to be involved in holding mucous secretions to the cell surface and increasing the functional surface area (Olson and Fromm, 1973; Sperry and Wassersug, 1976). The presence of structures that resembles this reticulated morphology in the processes of the EPs could be involved in a similar function, offering pathways for an easier spread of breeding glands secretion along the nuptial pad area, regardless of its biological role.

The positive reaction to PAS was an expected result based on previous descriptions of breeding glands in other species (Fujikura et al., 1988; Thomas et al., 1993; Epstein and Blackburn, 1997). However, the positive AB reaction is an unexpected finding in all phyllomedusines for which we could make serial sections. Thomas et al. (1993) described sexually dimorphic glands in 14 anuran species and reported AB positive material only in the glands of the single specimen of *Leptodactylus bolivianus*, besides some positive reactions in a minimum percentage of *Xenopus laevis* breeding glands (3–5% of nuptial glands studied). Epstein and Blackburn (1997) could neither detect acidic glycoconjugates in *Lithobates pipiens* breeding glands. We have studied pads from two other hylids (Hylinae: *Dendropsophus labialis* and *Scinax perereca*) and stained them in parallel with phyllomedusines. These presented PAS+ but AB− breeding glands suggesting the lack of acidic mucosubstances but the presence of neutral glycoconjugates (data not shown). These results, added to the scarce data available in the literature, could suggest that the presence of acidic mucosubstances in the breeding glands could be a putative synapomorphy of at least Phyllomedusinae, or a slightly less inclusive clade if not confirmed in *Phrynomedusa*.

The coloration of the pads varies from light to dark brown. There are few hard keratinized epidermal structures in anurans. Among these are the labial teeth and jaw-sheaths of tadpoles, claws, spade-shaped inner metatarsal tubercles, and the nuptial pads having a distinctively thick and heavily keratinized epidermis. In most of these cases, the structures are conspicuously colored, from light brown to black. The basis for this coloration is unknown (Altig, 2007; Maddin et al., 2009), and no pigment is known to be involved. Treatment of histological sections with hydrogen peroxide for 30 h showed that melanophores were bleached, whereas the stratum corneum remained fully colored (data not shown). The dark coloration so far seems to be a property of the keratin, or of other proteins associated with the keratins, as was suggested for other anuran structures such as larval jaw sheaths (Alibardi et al., 2010a). Maddin et al. (2007) pointed out that the biochemical identity of the particular keratins of the dark claws of *X. laevis* is yet unknown. Although Alibardi et al. (2010a,b) provided useful insights into the nature of jaw sheath and *Xenopus*' claws keratins, they demonstrated that the situation is complex and that further research must be done to fully characterize the identity of keratins and keratin associated proteins. The PAAB reaction in this work indicated an increase in sulfhydryl and disulfide groups in nuptial pads in the outermost part of the cells of the stratum granulosum and the stratum corneum. This is consistent with previous results in jaw sheaths of tadpoles in which a progressive increase in sulfhydryl groups is detected form the beginning of formation of keratinocytes to the more external regions of the beak (Beaumont and Deunff, 1959). These results indicate an increase in the content of keratins or other sulfur rich associated protein.

The undefined limits of EPs found in one of the specimens of *Phyllomedusa azurea* and in one specimen of *P. tomopterna* could be related with an early stage of development of the pad. We ignore if

this is related to a cycle of seasonal development of this structure as has been shown in other species where the thickness of the epidermis and even the height of the glands changes within the breeding season (Iwasawa and Kobayashi, 1985; Kao et al., 1994).

It has been suggested that nuptial pads are an adaptation to aquatic amplexus, being more developed in species that have amplexus on streams than in those that reproduce in calm water, and less developed or absent in species with terrestrial amplexus (Parker, 1940). Kurabuchi (1993) found rigid and thorny structures on *Pelophylax porosus* inferring that this rough architecture provides friction for the male to firmly grasp the female in the axillae. As for the rhacophorid and hylid frogs, Kurabuchi (1994) reported that elevations and accessory protuberances of the two species of *Buergeria* he analyzed were larger than those observed in *Hyla japonica*, and the degree of development in *Rhacophorus schlegelii* was poor. He pointed out that these differences could be correlated with the site of amplexus of each species, however, he did not provide any specific evidence. All phyllomedusines have terrestrial, axillary amplexus, although in *Cruziohyla* and some species of *Agalychnis* the amplectant pair spends some time in the water during the bladder-filling behavior (For a review see Faivovich et al., 2010). The correlations so far reported between nuptial pad structure and the place where amplexus occurs are only general statements; in fact, the pad is better developed in all phyllomedusines than in several groups of hylids that do have aquatic amplexus (e.g., most Dendropsiphini and Cophomantini). As such it still needs a rigorous test, as a first step toward a better understanding of nuptial pad function and diversity in anurans.

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