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**Journal of Plant Research**

ISSN 0918-9440

J Plant Res

DOI 10.1007/s10265-017-0963-z



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# Perianth organs in Nymphaeaceae: comparative study on epidermal and structural characters

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Received: 17 October 2016 / Accepted: 13 June 2017  
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**Abstract** The perianth organs of six species of Nymphaeaceae, representing *Euryale*, *Nymphaea* and *Victoria*, were studied on the basis of macroscopical, micromorphological, and anatomical characters. The aims were to determine whether perianth is differentiated among tepal whorls considering the presence of sepaloid and petaloid characters, and to evaluate the occurrence of both features in individual tepals. Selected perianth series were examined macroscopically, with light microscopy, and scanning electron microscopy. Osmophores were detected using neutral red and Sudan. In all tepals examined, stomata and hydrotopes were present on the abaxial and adaxial surfaces. These are anomocytic or stephanocytic; hydrotopes of irregular type are also present. The outer series of tepals display morpho-anatomical characters in most part related with photosynthetic and protective functions. Osmophore activity is very scarce and petaloid epidermal morphology is present only in *N. lotus*, thus allowing interpretation of this whorl as primarily sepaloid. The second series exhibits both petal-like and sepal-like characters; in *N. amazonum* and *N. gardneriana* sepaloid and petaloid group of cells are present on the abaxial surface of individual tepals. Therefore, this whorl is

transitional between the outer and the innermost ones. Both the morpho-anatomy and presence of osmophore activity indicate that the innermost series is entirely petaloid. Inner tepals of *E. ferox*, *N. alba*, and *V. cruziana* share the presence of epidermal cells with predominantly smooth cuticle, whereas those of *N. amazonum*, *N. gardneriana*, and *N. lotus* share a cuticular ornamentation consisting of numerous papillae on each cell. Morphological characters of the perianth epidermis are in some respects congruent with the molecular phylogeny of Nymphaeaceae. Our results support the co-expression of sepaloidy and petaloidy within individual tepals and the mosaic model of perianth evolution proposed for the angiosperms.

**Keywords** Anatomy · Cuticular ornamentation · *Euryale* · *Nymphaea* · Perianth · *Victoria*

## Introduction

The perianth of the flower is composed of sterile organs localized immediately beyond the bracts if present, and outside the fertile parts (Zanis et al. 2003). According to its position, morphology and function, the perianth may be differentiated in sepals and petals (bipartite perianth), or may exhibit organs very similar among them that receive the denomination of tepals (unipartite perianth). In the flower, tepals may be macroscopically uniform, or the outer tepals may be sepaloid and the inner ones petaloid (Endress 1994, 2001; Endress and Doyle 2009). Comparative criteria are used to distinguish between sepals and petals, and these are the following: position in the flower (external vs. internal), vasculature (three vascular bundles vs. one trace), development (early and spiral sequence vs. late and simultaneous), caducity (persistent vs. ephemeral), anatomy (flat

**Electronic supplementary material** The online version of this article (doi:10.1007/s10265-017-0963-z) contains supplementary material, which is available to authorized users.

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epidermal cells, leaf-like anatomy vs. papillate epidermal cells, homogeneous mesophyll), and function (protection vs. attraction) (e.g. Endress 2004, 2005; Hiepko 1965; Jaramillo and Kramer 2007; Ronse De Craene 2008; Soltis et al. 2005). It was recently demonstrated that none of these criteria including the developmental genetic can provide a unifying principle with which to homologize the perianth parts across angiosperms (Ronse De Craene and Brockington 2013). From a functional perspective, sepals are generally photosynthetic and protect the inner floral organs, and the petals are specialized to attract pollinators by means of the morphology, color, and emission of fragrance (Endress 1994, 2001; Soltis et al. 2005). Alternatively, tepals are considered to have overlapping functions (Endress 2002; Weberling 1989).

The core eudicots generally have a bipartite perianth, whereas the basal angiosperms, monocots and some basal eudicots display perianth organs differing from typical sepals and petals in some respects, so the term tepal is collectively used in these groups (Endress 2002, 2006; Endress and Doyle 2009; Ronse De Craene 2008; Ronse De Craene and Brockington 2013). The order Nymphaeales is one of the three earliest lineages that diverge prior origin of the vast majority of extant angiosperms (magnoliids, monocots, and eudicots) and it comprises the families Cabombaceae, Nymphaeaceae, and Hydatellaceae (APG 2016). Floral variation, particularly in basal angiosperms, is extensive and labile. Due to the phylogenetic position of Nymphaeaceae, the knowledge of the character states of each feature are a useful framework for ancestral traits reconstructions using the molecular phylogeny of the angiosperms, and for reviewing the morphological evolution of the flower (Doyle and Endress 2011; Ronse De Craene et al. 2003; Soltis et al. 2009). Nymphaeaceae consists of five genera, with about 60 species (Borsch et al. 2011). *Euryale* Salisb. (1 sp.), *Nymphaea* L. (50 spp.), and *Victoria* Lindl. (2 spp.) are clustered into the Nymphaeoidae subfamily. The most notable evolutionary trend in this clade is the floral pleiomery, which was acquired secondarily in correlation with the pollination by coleoptera (Les et al. 1999; Schneider 1979). The perianth is whorled and it has been interpreted as tetramerous (Endress 2001) or dimerous (Ronse De Craene et al. 2003) in Nymphaeoidae. The outer or sometimes the successive whorls arise in a unidirectional order, whereas the remaining inner organs are initiated simultaneously. Because both ontogenetic patterns are present within the flower, the term series is used to identify the set of pieces that appear in a whorl at maturity (e.g. Endress 2008; Warner et al. 2008).

Different criteria were applied to characterize the perianth organs in both Cabombaceae and Nymphaeaceae, so that *Barclaya* Wall., *Cabomba* Aubl., *Nuphar* Sm., *Nymphaea*, and *Victoria* are described as possessing a bipartite

perianth (Conard 1905; Endress and Doyle 2009; Ito 1987; Les et al. 1999; Moseley et al. 1993; Ronse De Craene et al. 2003; Schneider et al. 2003; Wiersema 1987; Zanis et al. 2003) or an unipartite perianth (Endress 2008; Endress and Doyle 2009; Irish 2009; Soltis et al. 2005). Particularly in Nymphaeaceae, the perianth has also been considered as undifferentiated with gradations from outer sepaloid tepals toward inner petaloid tepals (Endress 2001, 2008; Ronse De Craene 2008; Warner et al. 2008). In support of the last idea, Endress (2008) mentioned that in Nymphaeaceae the organs show a gradual change of coloration, but all have wide base and persist after anthesis. Ronse De Craene et al. (2003) suggest that flowers exhibit sepals developmentally homologous to bracts, and petals with similarities to stamens but with uncertain homology due to the presence of transitional stages between sepals and stamens. Hiepko (1965) mentions that development of petals is retarded relative to development of sepals and stamens. Within Nymphaeales, however, only *Cabomba* (Endress 2001) and *Nuphar* (Endress 2001; Hiepko 1965) clearly fit with this criterion. Furthermore, in Nymphaeaceae the distinction of perianth organs on the basis of the vascularization is not useful because of their relatively large flowers; the vascular bundles supplying the organs are variable among species and among different perianth series in a flower (Ito 1984; Moseley 1958, 1961; Moseley et al. 1993; Schneider 1976; reviewed in; Endress 2001; Warner et al. 2008). Within the distinction between sepals and petals or tepals, a statement of homology generally was not explicit, these terms are applied in a loose homology-neutral sense, or the perianth homology was defined on the basis of unreliable indicators of homology, such as morphology, ontogeny, and number of vascular traces (Ronse De Craene and Brockington 2013).

Perianth differentiation in Nymphaeaceae was also analyzed in terms of the link between petaloidy and epidermal micromorphology. Warner et al. (2008, 2009) found that in *Nymphaea caerulea* Savigny the adaxial surface of outer tepals and the abaxial surface of second series of tepals show sepaloid and petaloid areas in a single organ. Other studied species with similar results were *Barclaya longifolia* Wall., *Nuphar advena* (Aiton) W.T. Aiton and *N. lutea* (L.) Sm. Therefore, these authors suggested that the coexistence of petal-like and sepal-like features within individual tepals is distinctive for Nymphaeaceae. However, information on the morphological and anatomical characters of tepals in the family is still very limited. Therefore, the aims of the present study are to: (1) examine tepal epidermis in selected species of *Nymphaea* subgenera *Hydrocallis* (*Nymphaea amazonum* Mart. & Zucc. subsp. *pedersenii* Wiersema, *Nymphaea gardneriana* Planch.), *Lotos* (*Nymphaea lotus* L.) and *Nymphaea* (*Nymphaea alba* L.), in *Euryale ferox* Salisb. and *Victoria cruziana* Orb., (2)

document macroscopic and anatomical observations of the perianth organs, (3) analyze comparatively the results and assess them in the context of the molecular phylogeny of the family.

## Materials and methods

Flowers of *Euryale ferox* and *Nymphaea lotus* were collected from individuals cultivated in the botanical garden of the Faculty of Agronomy, University of Buenos Aires, Argentina. Other species examined with vouchers deposited at the Northeast Institute of Botany Herbarium (CTES) are: *Nymphaea alba*. Scotland. County of Argyll. Pedersen 10627. *Nymphaea amazonum* subsp. *pedersenii*. Argentina. Province of Corrientes. Department of San Martín. Zini et al. 17. *Nymphaea caerulea*. Argentina. Province of Corrientes. Department of San Martín. Zini et al. 16. *Nymphaea gardneriana*. Argentina. Province of Corrientes. Department of Empedrado. Zini et al. 10. *Victoria cruziana*. Argentina. Province of Chaco. Resistencia. Zini et al. 18.

The tepals were sampled from three flowers at anthesis in all species except for *N. alba* in which only one flower of the herbarium specimen was available. A single tepal for each differentiable whorl was examined and they were: the outer series, the subsequent whorl or second series and the innermost whorl of tepals, which precedes outer staminodia (*Euryale*, *Victoria*) or petaloid stamens (*Nymphaea*). Staminodia and stamens of *E. ferox* and *V. cruziana* were analyzed as well as the inner stamens of *Nymphaea* species were considered (see Online Resources). Observations of the epidermis surface with light microscopy (LM) were made from flowers fixed with FAA (formalin, acetic acid, 70% ethanol, 5:5:90) or from hydrated herbarium samples of *N. alba*. The excised samples of tepals were clarified with sodium hypochlorite (50%) and then rinsed with distilled water, or by peeling the epidermis with forceps. For anatomical studies, floral buds and mature flowers in FAA were dehydrated in an ethanol series with a rinse using pre-impregnant Biopur® (Gonzalez and Cristóbal 1997). The material was embedded in Histoplast® (Biopack, Buenos Aires, Argentina) according to the technique of Johansen (1940). Sections (12–15 µm) were made with a rotary microtome (Microm, Walldorf, Germany). They were stained with Astra blue and safranin (Luque et al. 1996), and then were mounted with synthetic Canada Balsam (Biopur, Buenos Aires, Argentina). Fresh inner tepals of *N. gardneriana* at first and second day of anthesis were cut transversely with a razor blade and some of them stained with Sudan IV to detect lipids (Johansen 1940). Photographs were obtained with a Leica DM LB2 compound LM. For visualize areas where osmophores were present,

first day anthesis flowers of *N. amazonum*, *N. caerulea*, *N. gardneriana*, and *V. cruziana* were immersed in neutral red solution (1:10,000; Vogel 1990) for 1 h and then were rinsed with water. For scanning electron microscopy (SEM) observations, fixed and herbarium samples of tepals and anthers (in alcohol 70%) of approximately 10 mm were dehydrated by transfer through an acetone series, critical point dried, and sputter coated with gold–palladium. The micrographs were obtained with a JEOL 5800 LV at 20 kv. Stomata and hydropotes were classified following the terminology used by Carpenter (2005, 2006). The hydropotes are multicellular trichomes consisting of a unicellular or multicellular uniseriate hair like portion that is abscised at maturity, leaving a base of three or four specialized epidermal cells (Carpenter 2006). In this study, petaloidy are considered as morphological and structural attributes presumably related with attractive function like ‘petals’, such as color, texture, epidermal morphology, and scent production, and those traits like ‘sepals’, perceived as related with protection and with a leaf appearance, define sepaloidy (Endress 1994).

## Results

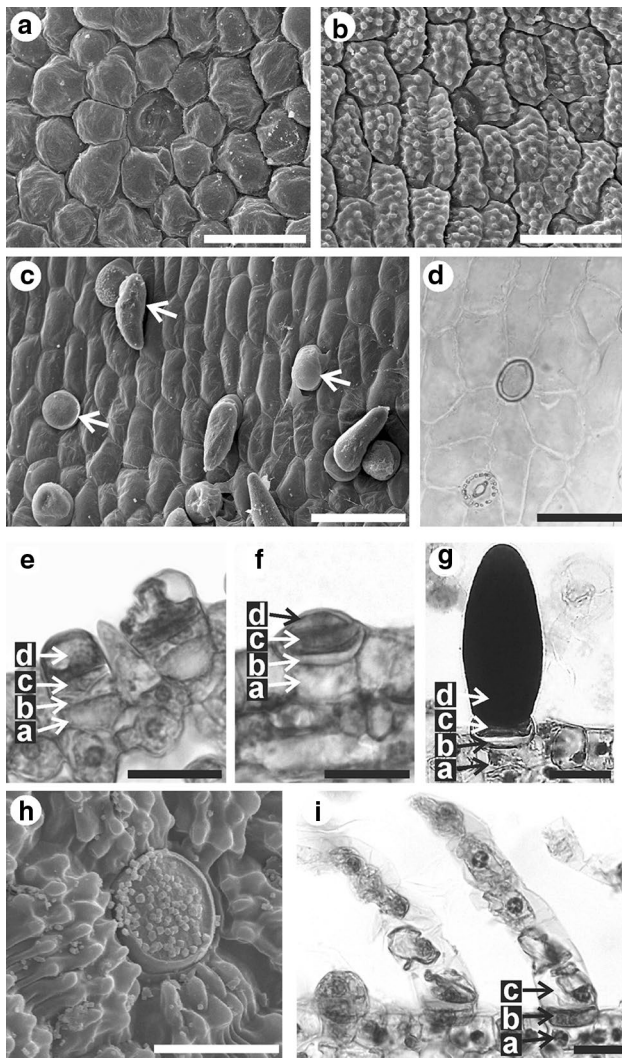
### Micromorphology and anatomy

#### *Stomata and hydropotes*

All the studied species have stomata and hydropotes on the abaxial and adaxial surfaces in all floral pieces. On the adaxial surface, stomata are scarcer than the hydropotes. Both are anomocytic or stephanocytic, but hydropotes can also exhibit irregular architectures (Figs. 1a–d, 2g). The hydropote is four-celled and is composed of: (a) a foot cell, located in the lower middle of the epidermis level; (b) a cell located slightly above the epidermis which at maturity appear tangentially compressed and boat-shaped; (c) a cell usually disk-shaped that fits in the concavity; (d) a terminal cell which is papillose, lens-shaped, or finger-like and tanniferous (Fig. 1e–g). The terminal cell is lacking in the majority of the observed samples; after its abscission, crystals are commonly found on the hydropote surface (Fig. 1h). Another type of hydropote is observed in *E. ferox* and *V. cruziana*. The basal portion of this trichome consists of a foot cell and a basal cell with dense cytoplasm, and the terminal part is pluricellular and seems non-glandular (Fig. 1i).

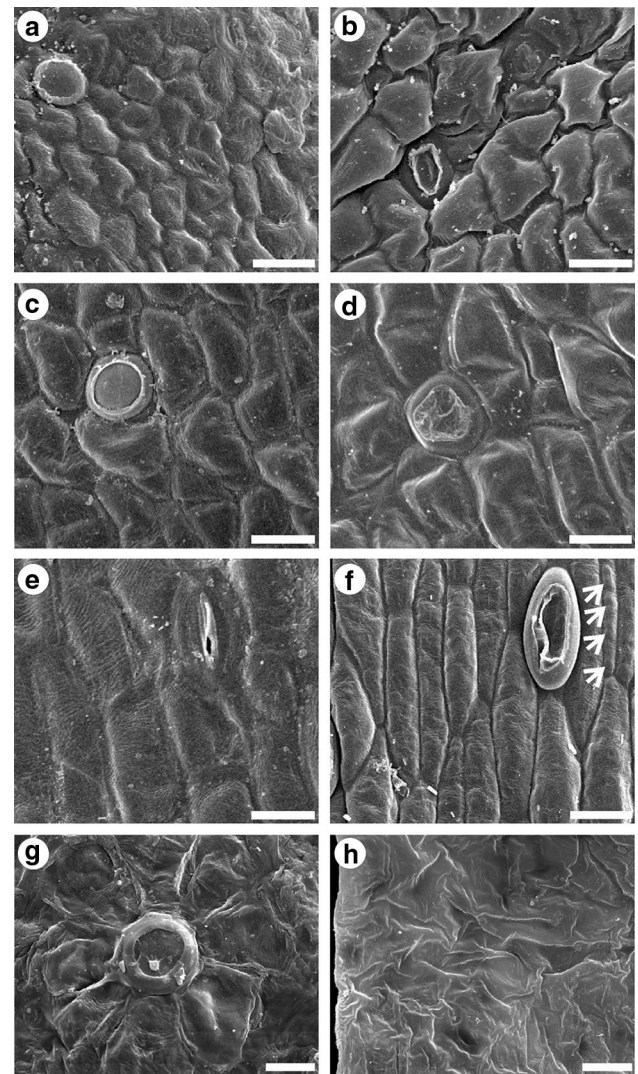
#### *Outer tepals*

In *Nymphaea* species, the epidermal cells are isodiametric or elongated (Fig. 2a–g). In *E. ferox* and *V. cruziana*, the



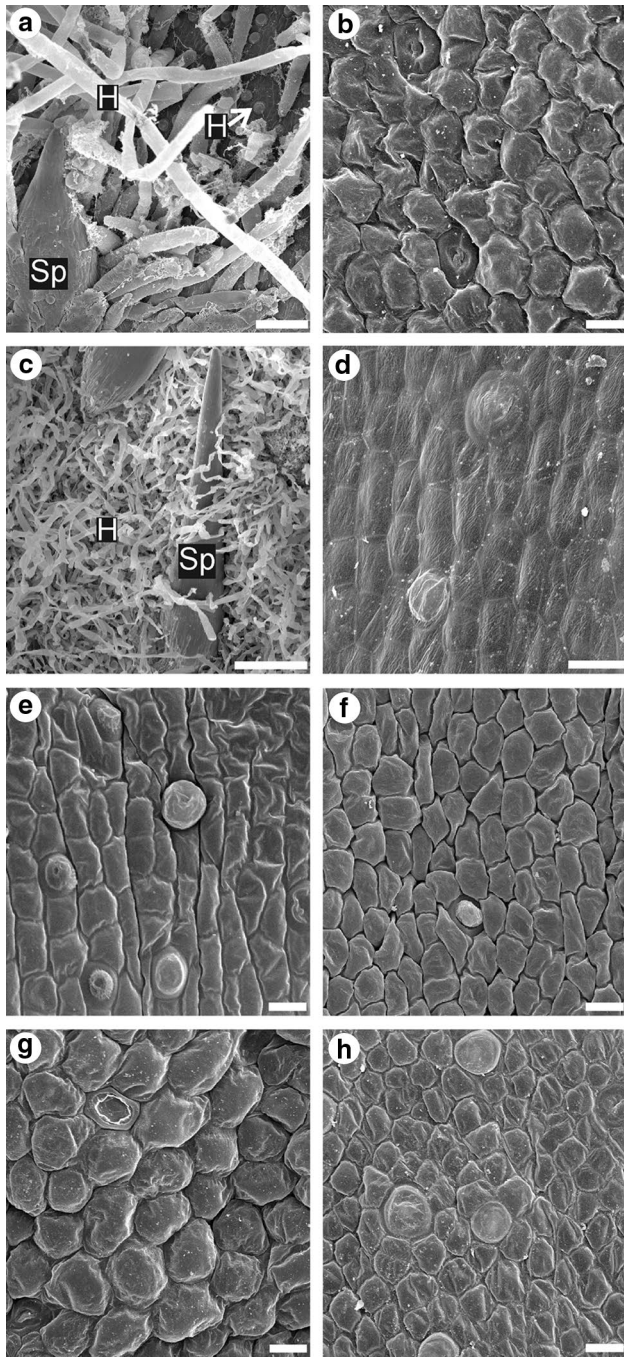
**Fig. 1** Hydrotypes and stomata of tepals in Nymphaeaceae. **a** Stephanocytic stoma on the inner tepal of *V. cruziana*. **b, c** Anomocytic stomata on the inner tepal and anomocytic hydrotypes (arrows) on the outer tepal of *N. amazonum*, respectively. **d** Stephanocytic hydropote devoid of terminal cell in *N. amazonum*. **e** Transverse section of hydropote in *E. ferox*. **f** Longitudinal section of hydropote in *N. gardneriana*. **g** Hydropote with a terminal cell in *V. cruziana*. **h** Hydropote with crystals in *N. amazonum*. **i** A young hydropote (left) and two hydrotypes with pluricellular terminal portion (right) on outer tepal of *V. cruziana*. Letters *a, b, c, d* indicate the cells comprising the hydropote. Scale bars 50  $\mu\text{m}$  (**a–d**), 20  $\mu\text{m}$  (**e–i**)

epidermis is characterized by the presence of polygonal, isodiametric, and slightly elongated cells (Fig. 3b, d). Epidermal cells are flat or slightly domed (Figs. 2a–g, 3b, d). Hydrotypes density differs considerably between abaxial and adaxial surfaces; they are more numerous on the abaxial surface (see Online Resources). The cuticle is smooth to finely striated in both surfaces of *Nymphaea* species, *E. ferox*, and *V. cruziana* (Figs. 2a–e, g, h, 3b, d), except for *N. lotus*, which in addition to striations, each epidermal cell



**Fig. 2** SEM images of outer tepals in *Nymphaea*. **a, b** Abaxial surface and adaxial surfaces in *N. amazonum*, respectively. **c, d** Abaxial and adaxial surfaces in *N. gardneriana*, respectively. **e, f** Abaxial surface and adaxial surface with cuticular domes per cell (arrows) in *N. lotus*, respectively. **g** Abaxial surface with stephanocytic hydropote and adaxial surface in *N. alba*, respectively. Scale bars 20  $\mu\text{m}$

exhibits several dome-shaped protuberances on the adaxial surface (Fig. 2f). Spiniform emergences and hydrotypes with terminal pluricellular portion are restricted to the abaxial surface of the outer perianth series of *E. ferox* and *V. cruziana* (Figs. 3a, c, 4c, see also Online Resources). Spiniform emergences are present in the whole tepal of *E. ferox*, whereas they are found only in the base of the tepal of *V. cruziana*. Outer tepals have unlayered epidermis. The mesophyll is homogeneous and mostly aerenchymatic, with air canals (Fig. 4a, c, d). Astrosclereids are associated with the air canals, and in *N. gardneriana* fusiform sclereids are located among the mesophyll cells at the abaxial side; both types of sclereids have crystals in their walls



**Fig. 3** SEM images of outer (a–d) and second series (e–h) of tepals in *Euryale* and *Victoria*. **a, b** Abaxial and adaxial surfaces in *E. ferox*, respectively. **c, d** Abaxial and adaxial surfaces in *V. cruziana*, respectively. **e, f** Abaxial and adaxial surfaces in *E. ferox*, respectively. **g, h** Abaxial and adaxial surfaces in *V. cruziana*, respectively. *H* hydropote, *Sp* spiniform emergence. Scale bars 50  $\mu$ m (a), 20  $\mu$ m (b, d–h), 500  $\mu$ m (c)

(Fig. 4a–d). Mesophyll cells are rounded or radially elongated in *N. gardneriana*, rounded in *E. ferox* and *V. cruziana* (Fig. 4a–d). In *N. gardneriana* the chlorenchyma is abundant at the abaxial side (Fig. 4a, b). Subepidermal

collenchyma is present at both sides of the mesophyll (Fig. 4a, c). Particularly in *N. gardneriana*, numerous articulated laticifers are observed among mesophyll cells and in association with the vascular bundles (Fig. 4b).

#### Second series of tepals

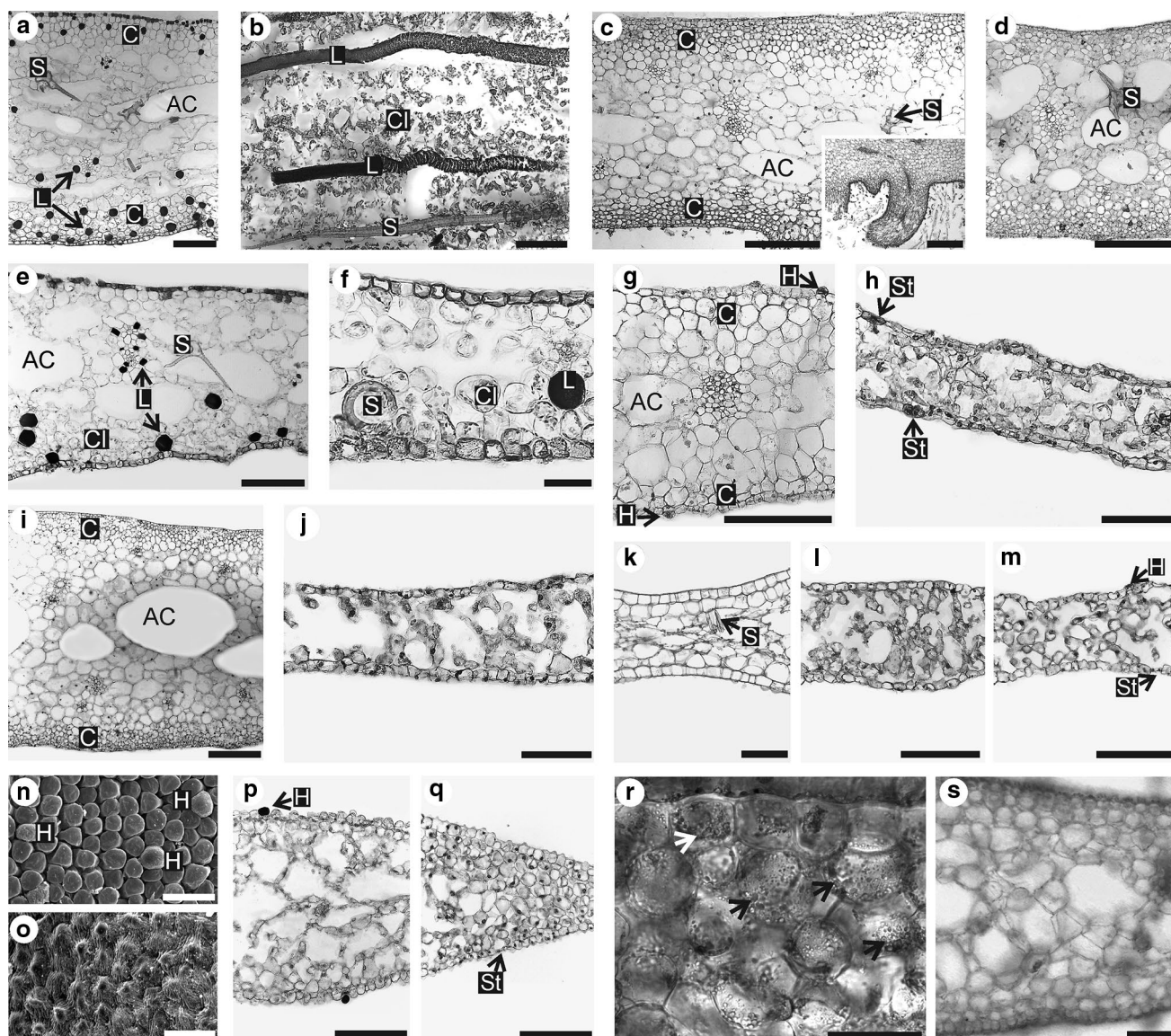
As in the outer tepals, epidermal cells are elongated or polygonal in superficial view (Figs. 3e–h, 5a–g). In *N. amazonum* and *N. gardneriana*, the abaxial surface present epidermal cells with smooth to finely striated cuticle (sepaloid morphology) and cells with numerous more or less prominent cuticular papillae per cell (petaloid morphology) (Fig. 5a, c). The latter morphology predominates in the adaxial surface (Fig. 5b, d); only the tepal base is devoid of cuticular ornamentation. In *N. lotus*, petaloid morphology is uniform in both surfaces, although the papillae are less pronounced (Fig. 5e, f), except at the tepal base where they are absent. Epidermal cells of *N. alba*, *E. ferox*, and *V. cruziana* are morphologically similar to those of the outer tepals (Figs. 5g, h, 6a–d). The second series shows similar anatomy compared with the anatomy of the outer tepals (Figs. 3e–h, 5g, h). In *E. ferox* and *V. cruziana* there is great reduction of the mesophyll at the tepal margin, and this region consists of elongated or lobed cells with large intercellular spaces (Fig. 4h, j).

#### Innermost tepals

Epidermal cells are elongated or isodiametric on both the abaxial and adaxial surfaces. In *N. amazonum*, *N. gardneriana*, and *N. lotus*, the epidermis is evenly ornamented with striations and well-defined cuticular papillae (Fig. 6a–f). In *N. alba*, *E. ferox* and *V. cruziana*, the epidermis does not vary with respect to the other two examined whorls, i.e., the cuticle is smooth or slightly striated (Fig. 7a–f). Epidermal cells are either flat or domed (Fig. 4k–l), principally domed in *N. lotus* (Fig. 6e, f). This perianth whorl and the petaloid stamens share the same epidermal surface (see Online Resources). Mesophyll is homogeneous (Fig. 4k–m); it is composed of one or more subepidermal cell-layers with minute intercellular spaces (collenchymatic or not) and, more internally, with aerenchymatic tissue (Fig. 4k, s). The margins have an entirely aerenchymatic mesophyll consisting of rounded or lobed cells with large intercellular spaces (Fig. 4l, m). Collenchyma and sclereids are occasionally observed (Fig. 4k).

#### Outer staminodia of *E. ferox* and *V. cruziana*

With exception of the staminodia basis, most part of the epidermis consists of papillose cells with one papilla per cell (Fig. 4n–q; see Online Resources); in *V. cruziana* also



**Fig. 4** LM and SEM images of outer tepals (a–d), second series of tepals (e–j), innermost tepals (k–m, r, s), and staminodia (n–q). a, b Details of transverse section and paradermal section at the level of abaxial chlorenchyma in *N. gardneriana*, respectively. c Transverse section, with a detail of the spine base (inset) in *V. cruziana*. d Detail of transverse section in *E. ferox*. e–j Transverse sections of *N. gardneriana* (e, f), *E. ferox* (g, h), and *V. cruziana* (i, j). Note that f, h and j correspond to the tepal margin. k–m Transverse sections of tepal near the margin in *N. gardneriana* (k), *E. ferox* (l), and *V. cruziana* (m).

SEM images showing epidermis in detail of *E. ferox* (n) and *V. cruziana* (o). p, q Hand sections of *N. gardneriana* stained with Sudan IV. p Lipid globules within the epidermal cell (white arrow) and the mesophyll cells (black arrow). q Negative reaction with Sudan, except for the cuticle. AC air canal, C collenchyma, Cl chlorenchyma, H hydropote, L laticifer, S sclereid, St stoma. Scale bars 200 µm (a, b, e, g, l, m, p, q), 400 µm (c, d, i), 50 µm (f, k, n, o), 100 µm (h, j, s), 25 µm (r)

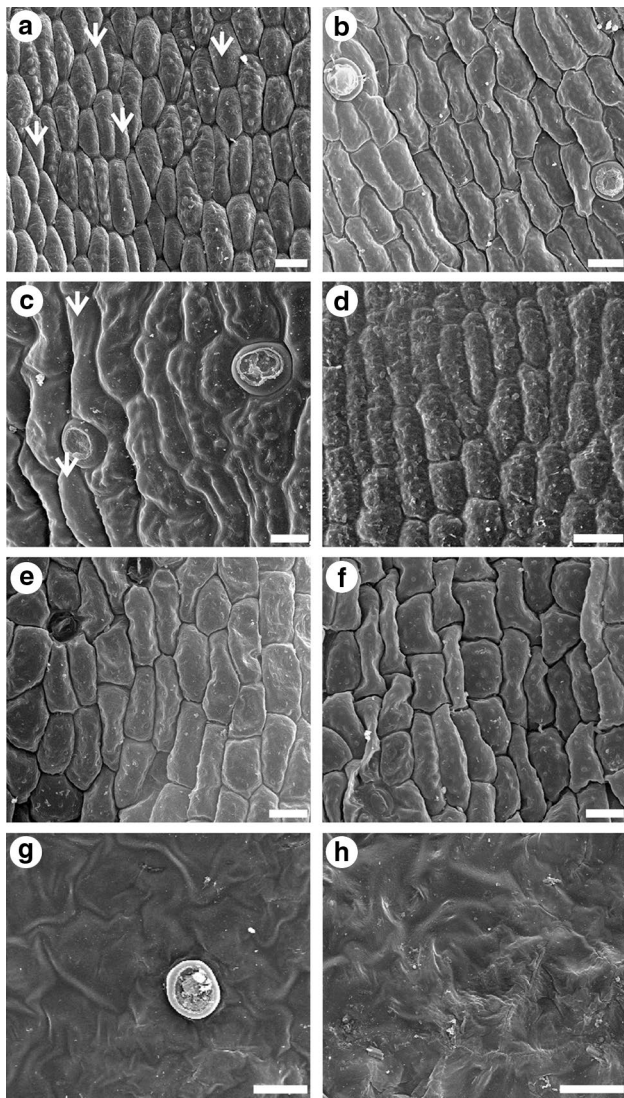
striations of the cuticle are observed with SEM (Fig. 4o). Hydropotes and stomata are present (Fig. 4n, p, q).

#### Color of tepals and osmophores detection

In *N. amazonum*, *N. caerulea* and *N. gardneriana*, outer tepals are of greenish color on the abaxial surface, with purple streaks in *N. amazonum* and *N. caerulea*, and

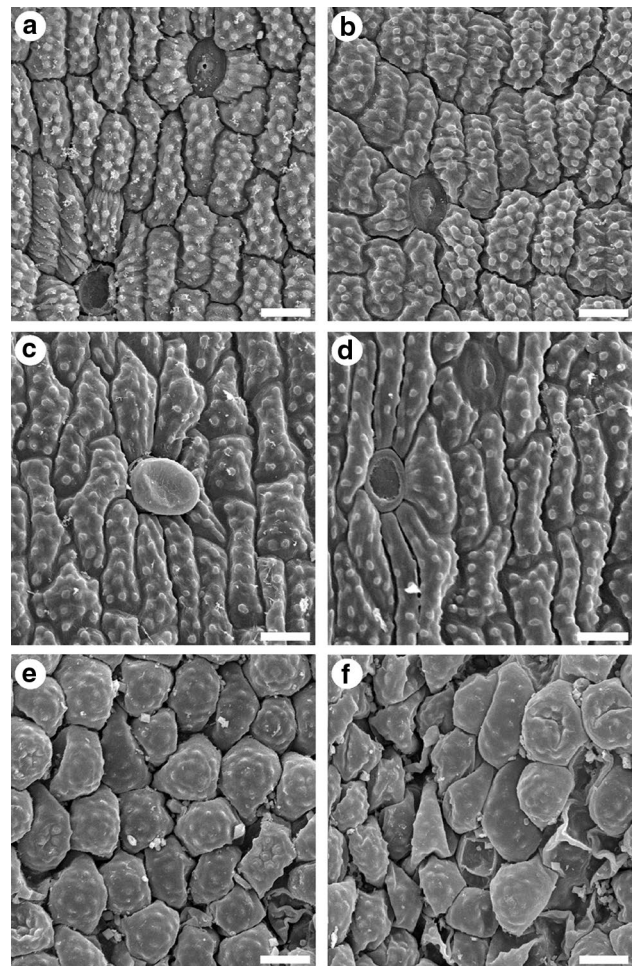
uniformly green or with reddish areas in *N. gardneriana* (Fig. 8a–c). The outer tepals of *V. cruziana* are reddish or combine green and reddish colors on the abaxial side, but they can also exhibit cream margins and soft texture when they were covered in the flower bud (Fig. 8d). The adaxial surface is white to greenish in all species (Fig. 8a, f, h, j). Tepals of the second perianth series are entirely greenish on the abaxial surface (Fig. 8a, b) or they are green only along





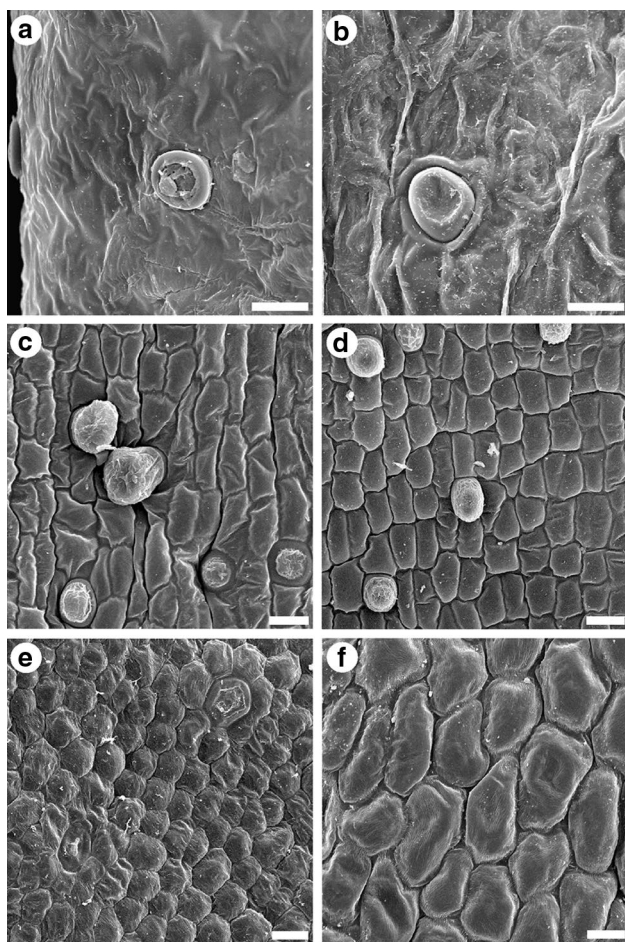
**Fig. 5** SEM images of second series of tepals in *Nymphaea*. **a, b** Abaxial surface with sepaloid (arrows) and petaloid areas and adaxial surface in *N. amazonum*, respectively. **c, d** Abaxial side with sepaloid (arrows) and petaloid areas and adaxial surface in *N. gardneriana*, respectively. **e, f** Abaxial side with petaloid morphology and adaxial surface in *N. lotus*, respectively. **g, h** Abaxial and adaxial surfaces in *N. alba*, respectively. Scale bars 20  $\mu$ m

the median surface (Fig. 8c). The adaxial surface is creamy white or greenish in *N. amazonum* and *N. gardneriana*; it is light blue in the margins and progressively cream and greenish toward the center and base in *N. caerulea* (Fig. 8a, f, h). Tepals of this series have more delicate texture than the outer ones. Innermost tepals are entirely cream in *N. amazonum* and *N. gardneriana*, whereas they are light blue with green-whitish base in *N. caerulea* (Fig. 8c, f, h). The first two species display transition from petaloid stamens to laminar stamens; *N. caerulea* does not show this transition (Fig. 8h). In *V. cruziana*, the inner whorls display cream



**Fig. 6** SEM images of innermost tepals in *Nymphaea* subg. *Lotos* and *Hydrocallis*. **a, b** Abaxial and adaxial surfaces in *N. amazonum*, respectively. **c, d** Abaxial and adaxial surfaces in *N. gardneriana*, respectively. **e, f** Abaxial and adaxial surfaces in *N. lotus*, respectively. Scale bars 20  $\mu$ m

tepals on both surfaces and are of very delicate texture in contrast with the rigid texture of outer tepals (Fig. 8d, j). Neutral red staining reveals osmophore activity in the perianth organs of *N. amazonum*, *N. caerulea*, *N. gardneriana* and *V. cruziana* (Fig. 8e, g, i, k). However, there are differences in the degree of staining among whorls of the flower and the analyzed species. In all species, the two outer series of tepals show much less absorption of the stain than the inner ones. In addition, the innermost series of tepals exhibit homogeneous coloration throughout each individual organ. In *N. amazonum* and *N. gardneriana*, petaloid stamens stain, as do innermost tepals; *N. caerulea* is devoid of this type of stamens, and only the stamens tip are stained (Fig. 8i). In *V. cruziana*, the outer staminodia are also stained completely (Fig. 8k). The staining of the perianth is in general more intense in *N. amazonum*, *N. gardneriana* and *V. cruziana* than in *N. caerulea*. In the last species,



**Fig. 7** SEM images of innermost tepals in *Nymphaea alba*, *Euryale*, and *Victoria*. **a, b** Abaxial and adaxial sides in *N. alba*, respectively. **c, d** Abaxial and adaxial sides in *E. ferox*. **e, f** Abaxial and adaxial sides in *V. cruziana*, respectively. Scale bars 20  $\mu$ m

the basis of the tepal of any whorl does not stain (Fig. 8i). The presence of volatile oils in tepals is corroborated with Sudan IV in *N. gardneriana*. At the first day of anthesis, the inner tepals show a positive reaction to the staining in the epidermis and mesophyll (Fig. 4r), whereas after the second day of anthesis the oils droplets in the tissue disappeared almost completely (Fig. 4s).

## Discussion

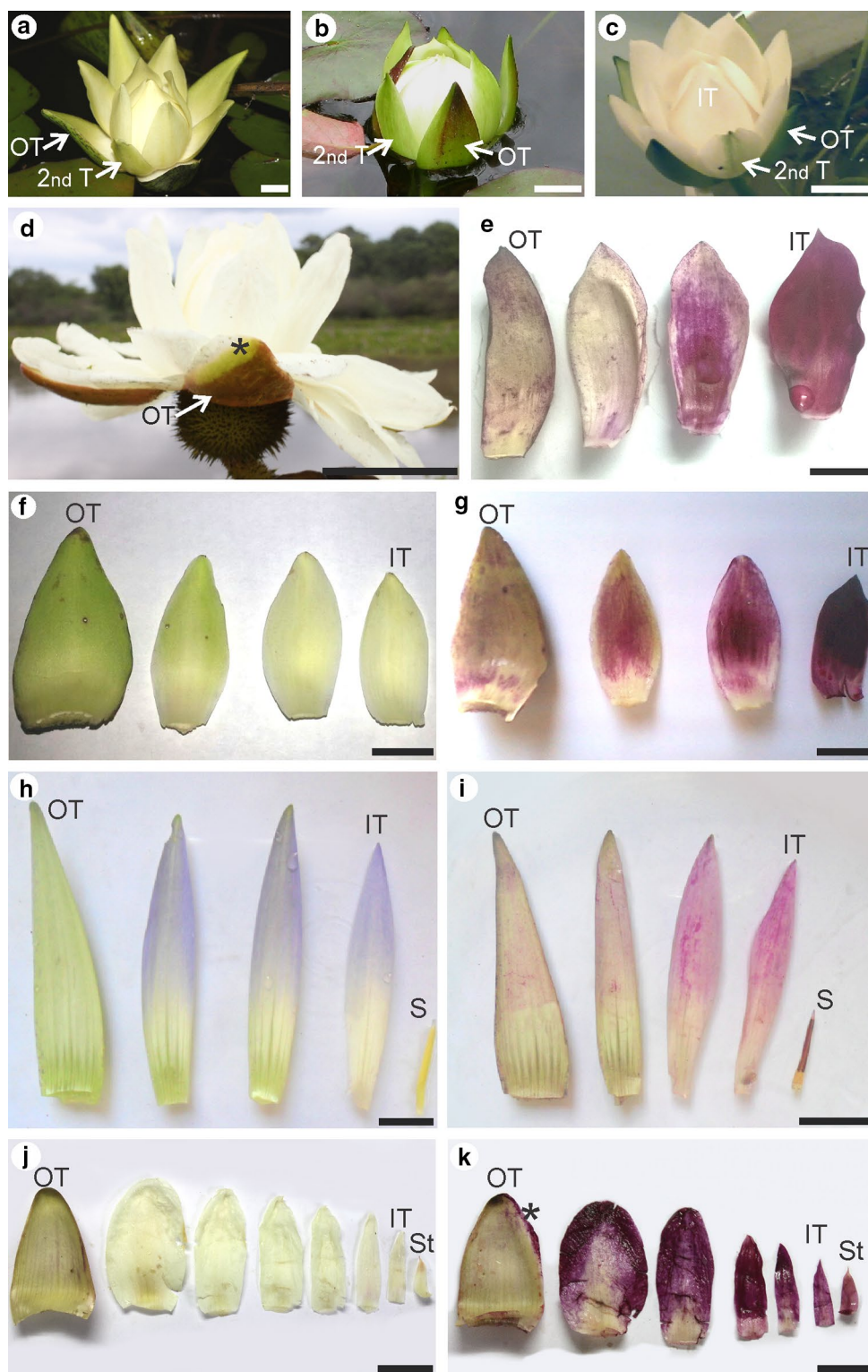
### Tepal differentiation and phylogenetic implication of the epidermal morphology

In the studied species of *Nymphaea* there is a gradual transition from outer to inner in the morphology of the tepals; the outer ones are more sepaloid and the inner ones more petaloid, as is described by other authors for this genus (e.g. Endress 2001, 2008; Warner et al. 2008, 2009; Yoo et al.

2010a). Furthermore, the micromorphological study of the epidermis in *Nymphaea* species of the subgenera *Hydrocallis* and *Lotos* agree with the interpretations of Warner et al. (2008, 2009) for *N. caerulea* (subgenus *Brachyceras*) and also for *Nuphar*, with regard to the occurrence of sepal-like and petal-like patches on individual tepals. In *N. lotus*, petaloid morphology of the epidermis is present on the adaxial surface of the outer tepals as well as in *N. caerulea* (Warner et al. 2008). However, in *N. amazonum* and *N. gardneriana* the entire outer tepals lacked cuticular papillae in the epidermis of all the samples examined, indicating variability in this perianth series for *Nymphaea*.

The results from this study at a micromorphological level have also revealed no clear distinction between sepaloid and petaloid regions within individual perianth members of *E. ferox* and *V. cruziana*, i.e., outer and inner tepals had either flat or domed cells with fine striations. In contrast, the outer staminodia have a papillate epidermis as in the stamens. These two epidermal cell forms are congruent with the petal epidermal characteristics recognized for other angiosperms by Kay et al. (1981) and Whitney et al. (2011), although they do not fit into the single conical papilla that is the most common cell shape. In *Euryale* and *Victoria*, the distinctive epidermis morphology of the outer staminodia suggests that these organs are derived from sterilized stamens and are functioning as petaloid tepals. The systematic importance of the epidermal morphology of petals has been recognized at familial or generic levels in angiosperms (Kay et al. 1981). We found some epidermal characters that are potentially useful for supporting clades within Nymphaeaceae. Research works on molecular phylogenetics confirm *Nuphar* as the first and *Barclaya* as the next lineage in Nymphaeaceae, with the latter as sister group of the subfamily Nymphaeoidae. Three clades are supported within *Nymphaea*: subgenera *Anecphyia-Brachyceras*, *Hydrocallis-Lotos*, and the subgenus *Nymphaea* (Borsch et al. 2008; Löhne et al. 2007). However, in one topology *Nymphaea* is monophyletic with respect to the clade *Euryale-Victoria*, but in an alternative result the *Euryale-Victoria* clade is nested within *Nymphaea* as sister group to all members of the genus except for the subgenus *Nymphaea* (Borsch et al. 2007, 2008; Les et al. 1999; Löhne et al. 2007). *Euryale ferox* and *V. cruziana* share unique characters, such as outer tepals with spines and hydro-potes with terminal pluricellular portion. These results show concordance with the close phylogenetic relationship between both genera revealed from the molecular phylogeny. The epidermal cell patterning of all tepals in *Nuphar*, possibly *Barclaya* (Warner et al. 2008), *Nymphaea alba* of the subgenus *Nymphaea*, *E. ferox* and *V. cruziana* is similar in terms of the presence of a smooth to slightly striated cuticle. Alternatively, epidermal

**Fig. 8** Perianth organs macroscopically studied. **a** Flower of *N. amazonum* beginning to open. **b, c** Flowers of *N. gardneriana* beginning to open, with differences between tepals of the second series. **d** Flower of *V. cruziana* at anthesis; left margin of the outer tepal (*asterisk*) was a covered area by the overlying tepal. **e** Tepals of *N. gardneriana* stained with neutral red. **f–k** Control (**f, h, j**) and staining (**g, i, k**) in *N. amazonum*, *N. caerulea*, and *V. cruziana*. In **k** note right margin of the outer tepal (*asterisk*) and staminode with positive result. *It* inner tepal, *Ot* outer tepal, *2nd T* second series tepal, *S* stamen, *St* staminode. Scale bars 20 mm (**a–c, e–i**), 80 mm (**d**), 40 mm (**j, k**)



morphology consisting of numerous papillae per cell was present in perianth organs of the studied species of *Nymphaea* subgenera *Brachyceras*, *Hydrocallis* and *Lotos*. The last morphology can be considered an apomorphic

state for Nymphaeoidae on the basis of outgroup comparison. Therefore, the epidermal features may constitute a morphological source potentially useful for phylogenetic studies.

**Characters that define sepal-like and petal-like series of tepals**

The function of sepals and petals is revealed at morphological and histological levels among different species of angiosperms. Sepals in most cases protect floral buds and therefore show a more leaf-like structure and are photosynthetic (Endress 1994). From this perspective, Table 1 presents macroscopic and microscopic features considered for defining sepaloidy and petaloidy on perianth organs of Nymphaeaceae. In *N. gardneriana*, *E. ferox*, and *V. cruziana* the outer tepals are sepaloid because they exhibit common elements to the leaf, such as chlorenchyma in the

exposed side of the mesophyll, numerous sclereids and abundant collenchyma, in addition to the presence of spiniform emergences and hydropotes with a pluricellular terminal portion in *E. ferox* and *V. cruziana* (Carpenter 2006; Catian and Scremin-Dias 2013, 2015; Conard 1905; Gonzalez 2002). However, sepalness in this series of organs is not exclusive as revealed for example by the presence of a petaloid epidermis on the adaxial surface in *N. lotus*. Furthermore, a contribution of outer tepals in fragrance emission is limited in *Nymphaea* species and *V. cruziana*. This observation confirms the results mentioned by Valla and Cirino (1972) for *V. cruziana* with the same method. Therefore, outer tepals exhibit a suite of characters that

**Table 1** Representative macroscopic and microscopic features defining sepaloidy and petaloidy in perianth organs of Nymphaeaceae

Taxa	Sepaloid features				Petaloid features				
	Smooth to finely striated cuticle	Chlorenchyma present (green color)	Firm texture	Another features	Smooth cuticle (1). Striated (2). Several papillae or domes per cell (3). One papilla per cell (4)	Color	Osmophore activity	Soft texture	Another features
<i>Nymphaea alba</i>	+	+ <sup>a</sup>	+ <sup>a</sup>	Striped with gray or reddish <sup>a</sup>	1	White <sup>a</sup>	?	+ <sup>a</sup>	–
<i>N. amazonum</i>	+	+	+	Purple streaks	2, 3	Cream	+	+	–
<i>N. gardneriana</i>	+	+	+	Brown striae (Laticifers)	2, 3	Cream-white	+	+	–
<i>N. caerulea</i>	+	+	+	Purple streaks	2, 3	Light-blue	+	+	–
<i>N. lotus</i>	+	+	+	Brown striae (Laticifers) <sup>d</sup>	3	Cream-white	?	+	–
<i>Euryale</i>	+	+	+	Red–purple margins. Spines and hydropotes with pluricellular portion	1, 2, 4	Purple-whitish	?	+	–
<i>Victoria</i>	+	+	+	Reddish-brown. Spines and hydropotes with pluricellular portion	2, 4	Cream -pink	+	+	–
<i>Barclaya</i> <sup>b</sup>	?	?	?	Hydropotes present	?	White to dark-red	?	?	Hydropotes absent
<i>Nuphar</i> <sup>b, c</sup>	+	+	?	Hydropotes present. Domed cells	1	Yellow–red	?	+	Hydropotes absent. Flat cells

? unknown, + present, – absent. References: <sup>a</sup>Conard (1905); <sup>b</sup>Warner et al. (2008); <sup>c</sup>Warner et al. (2009); <sup>d</sup>LM Zini, ined

allow to interpret them as primarily sepaloid and thus with primary function in protection. Particularly in *N. gardneriana*, articulated laticifers predominate in the two external most series of tepals and close to the epidermis, as might be expected if evidence supporting the view that latex has a protective function against herbivores and microorganisms (Fahn 2002). Little information is available on the latex-producing species in Nymphaeaceae. Previous anatomical studies reported laticifers in pedicel, outer tepals, stamens, and ovary of some *Nymphaea* species (Moseley 1961; Williamson and Moseley 1989) and in the pedicel of *Victoria* (Schneider 1976).

Hydropotes are a synapomorphy of Nymphaeales (Carpenter 2006). The occurrence of hydropotes in tepals has been useful to discriminate sepaloid and petaloid regions in *Nuphar* and presumably in *Barclaya* (Warner et al. 2009; Table 1). However, this has no implication for the studied genera of Nymphaeaceae because hydropotes are regularly present in all perianth organs. Even so, possibly in association with the environmental exposure and its function, these trichomes were more common on the abaxial side of outer tepals than on the inner ones. In the adult foliar organs of *Nuphar* and *Nymphaea*, hydropotes are restricted to the abaxial surface (Carpenter 2006; Catian and Scremin-Dias 2015; Gonzalez 2002), whereas in *Euryale* and *Victoria* they are found on both leaf surfaces (Carpenter 2006). It was proposed that the two shorter cells of the hydropotes mediate the exchange of water and minerals (Wilkinson 1979). In leaves of *Cabomba*, the apical cell of the hydropote releases mucilaginous and tannic substances; therefore, defense against microorganisms is another possible function (Galati 1981). We have observed tanniferous terminal cells in some hydropotes of *Euryale*, *Nymphaea*, and *Victoria*, in agreement with previous reports (Conard 1905; Igersheim and Endress 1998; Lavid et al. 2001). In *Nuphar* and *Nymphaea*, these compounds are responsible for the antibacterial and antifungal activities (Nishizawa et al. 1990; Vergeer and Van der Velde 1997). In *Nymphaea* species of the subgenera *Hydrocallis* and *Lotos*, not only macroscopic but also anatomical results allow us to define the second series as intermediate because it combines sepaloid (abaxial chlorenchyma and smooth to finely striated cuticle) and petaloid attributes (cream color, absence of chlorenchyma, and strongly ornamented cuticle), whereas the innermost series of tepals is petaloid since the presence of characters exclusively associated with optical and olfactory functions (Table 1). However, in *E. ferox* and *V. cruziana* the second series of tepals is clearly petaloid due to a morphological and anatomical resemblance with the inner whorls, including the scent production in *V. cruziana*. In comparison with the outer series, the two innermost whorls of tepals (*Nymphaea*) or all inner tepals (*Victoria*) stained more with neutral red, suggesting

a main contribution of these last organs in the production of volatile compounds. In addition, the presence of entire innermost tepals with well-developed cuticular papillae or striations combined with the lenticular shape further support that this is an epidermal specialization for attraction, because this characteristic is generally linked with the efficient emission of fragrance and glossiness (Endress 1994; Kay et al. 1981; Martin et al. 2002; Weberling 1989).

### Link between petaloidy and developmental genetics and the environment

Investigations from developmental genetics reveal that contrary to eudicot model organisms that have a bipartite perianth and following the ABCE discrete model of floral organ identity, several basal angiosperms with undifferentiated perianth, such as *Amborella* Baill., *Cabomba*, *Illicium* L., *Nuphar* and *Nymphaea* present a gradient in the level of gene expression pattern that produce gradual transitions in organ morphology through the development of some features of adjacent organs (Buzgo et al. 2004; Kim et al. 2005; Soltis et al. 2007; Yoo et al. 2010a, b). *Nuphar* and *Nymphaea* exhibit broader A- and B-class gene expression compared with the eudicots, and with the B-gene homologs expression that extends across all floral organs early in development (Yoo et al. 2010a, b). In *Nymphaea odorata* Aiton, only the innermost tepals share similar gene expression patterns with the petaloid stamens (referred as staminodes) and are morphologically similar, supporting a common origin for the two former organ kinds (Yoo et al. 2010a). On the basis of the slight morpho-anatomical perianth differentiation in *Nymphaea*, Yoo et al. (2010a) proposed this to be probably linked with differences detected in expression levels of AG homologues among perianth organs. Integrating structural changes with changes in the gene expression is important for understanding perianth evolution and inferring ancestral character states (Ronse De Craene 2007; Ronse De Craene and Brockington 2013). Therefore, the present results on perianth differentiation may provide a useful framework within *Nymphaea* species, *Euryale*, and *Victoria* for future studies on gene expression patterns controlling organ identity and for testing the correlation or decoupling between the morphology and genetics.

Cabombaceae and Nymphaeaceae diverge remarkably in their floral organization despite both families being closely related. In Cabombaceae there are only two trimerous whorls of tepals and they are very similar in color and histology (Endress 2008); hydropotes were found only in the outer whorl, but do not possess morphologically distinct areas within individual tepals (Warner et al. 2008). Most part of this morphology was successfully correlated with the developmental genetic studies (Yoo et al. 2010a). In Nymphaeaceae, perianth organs occur in more than two

series and, at least macroscopically, sepaloid and petaloid features are observed in individual perianth organs of all genera studied to date. According to Warner et al. (2009), the 'fading borders' model is insufficient to explain perianth morphology in *Nuphar* and *Nymphaea* because sepaloidy and petaloidy are not organ-specific. These authors found that features of developing tepals are partly controlled by the environment in two ways: the light influences the production of sepaloid regions (e.g. non-papillate epidermis and/or green color) because they were differentiated even in experimental inner tepals, and the surface contact of one tepal with another tepal in bud stage produces petal-like areas. The presence of petaloidy in outer tepals was corroborated in this study for *V. cruziana* through macroscopic observations. Even if there are abrupt macroscopic changes (i.e. color, consistency, neutral red reaction) between the outer and the subsequent whorls in *V. cruziana*, this species showed some outer tepals with white margins and delicate texture (i.e. petaloid), similarly to previous reports in *Euryale*, *Nuphar*, and a *Nymphaea* hybrid (Warner et al. 2008). In *N. amazonum* and *N. gardneriana* petaloid margins in mature outer tepals are absent. The adaxial surface of some tepals is more whitish than others (more greenish). However, the cuticular ornamentation does not vary suggesting that the color and epidermal morphology developed independently at different developmental stages.

Ronse De Craene (2007) proposed the ancestral perianth for core eudicots is that of a bracteolar origin, with less restricted gene expression, spiral phyllotaxis, and little morphological distinction between sets of organs. Derived states in this clade are to extent petal features into the sepals often before or after petal loss takes place, and in a few clades are to produce petaloid staminodes, where petals have been lost and thus a visual attraction had to be reinvented (Ronse De Craene 2007, 2008). Perianth differentiation has also broken down several times due to forces acting to increase meristem size. The above mentioned range of possibilities demonstrates that the presence and location of petaloid structures are in fact constantly altered across angiosperms as result of developmental constraints, environmental stimuli, and ecological adaptations to pollinators (Ronse De Craene and Brockington 2013). In Nymphaeaceae, interacting events are similarly involved in the morphological differentiation of the perianth organs. We observed that the expression of sepaloidy and petaloidy at a morpho-anatomical level is plastic in the family and even within *Nymphaea*. In addition, the petaloid features differ between staminodial organs and the remaining tepals in *Euryale* and *Victoria*. The possibility that petaloidy can run along a gradient accompanied with mixed gene influences was emphasized (Ronse De Craene 2007). In *Nymphaea*, the inner tepals are morphologically similar to stamens; the common ontogenetic and genetic patterns have

been proposed to be responsible for the superficial similarity between both organs (Ronse De Craene et al. 2003; Yoo et al. 2010a, b), but also the environmental factors play a role in determining tepal features (Warner et al. 2009). The presence of flowers with low synorganization and progressive change from outer bract-like tepals to inner more stamen-like organs correlates to a much expanded floral apex, which result in higher and variable number of floral organs (Endress 2006, 2008; Schneider et al. 2003). In addition, it was stated that selective forces accompanying a shift in the pollination biology allowed the establishment of this character within some species of the Nymphaeaceae (Schneider 1979). Since diffuse boundaries between different perianth organs categories are not unique of Nymphaeaceae as they are also present in other basal angiosperms such as *Amborella* (Amborellaceae) and *Schisandra* Michx. (Schisandraceae), Warner et al. (2009) have proposed a model of perianth evolution, in which sepalness and petalness evolved early in the angiosperm history, but these features were not fixed to particular organs; at a later stage in the evolution, sepaloid and petaloid characteristics became developmentally fixed to whole organs and in specific whorls. Therefore, our results support the prevalence of co-expression of sepaloidy and petaloidy in individual tepals within basal angiosperms and the mosaic model of perianth evolution for angiosperms.

**Acknowledgements** Financial support for this research was provided by the Agencia Nacional de Promoción Científica, Tecnológica y de Innovación, Argentina (ANPCyT-UNNE, PICTO 2012-0202), the Universidad Nacional del Nordeste (PI A012-2013), and the Universidad de Buenos Aires (UBACyT20020120100056BA).

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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