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The origin of the bifurcating style in Asteraceae (Compositae)

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● **Background and Aims** The plant family Asteraceae (Compositae) exhibits remarkable morphological variation in the styles of its members. Lack of studies on the styles of the sister families to Asteraceae, Goodeniaceae and Calyceraceae, obscures our understanding of the origin and evolution of this reproductive feature in these groups. The aim of this work was to perform a comparative study of style morphology and to discuss the relevance of important features in the evolution of Asteraceae and its sister families.
● **Methods** The histochemistry, venation and general morphology of the styles of members of Goodeniaceae, Calyceraceae and early branching lineages of Asteraceae were analysed and put in a phylogenetic framework to discuss the relevance of style features in the evolution of these families.
● **Key Results** The location of lipophilic substances allowed differentiation of receptive from non-receptive style papillae, and the style venation in Goodeniaceae and Calyceraceae proved to be distinctive. There were several stages of style evolution from Goodeniaceae to Asteraceae involving connation and elongation of veins, development of bilobation from an initially cup-shaped style, and a redistribution of the receptive and non-receptive papillae.
● **Conclusions** These developments resulted in bifurcation in the styles of Asteraceae, with each branch face having a different function, and it is suggested here as a mechanism that promoted outcrossing, which in turn led to the great diversification in the family.

Key words: Asteraceae, Calyceraceae, Goodeniaceae, homology, morphology, histochemistry, style evolution, venation.

INTRODUCTION

The recognition and identification of members of the daisy family (Asteraceae/Compositae; ~1600 genera and 24 000 species), has long been centred on its basic type of inflorescence (the capitulum), the connate and introrse anthers, a dispersal element or pappus (modified calyx) perched on the achene, and its bifid style. Of these characters, the morphology and evolution of the capitulum has received substantial attention (e.g. Harris, 1995; Katinas *et al.*, 2008*a*; Pozner *et al.*, 2012; Bello *et al.*, 2013) because of its crucial reproductive function. However, morphological and structural adaptations of the stigmatic surface and style are also key to reproduction: they represent the morphological basis of different breeding systems, are critical for optimum capture of pollen, and are frequently well-defined features of both taxonomic and phylogenetic value. In general, studies on the styles of Asteraceae have focused on pollen–style interaction (e.g. Torres, 2000) and on the morphology within the different taxonomic groups (e.g. Cassini, 1813; Wetter, 1983; Torres and Galetto, 2007; Erbar and Leins, 2015*a*). Style morphology also plays a key role in secondary pollen presentation syndromes. In the Asterales, these mechanisms comprise a male phase (proterandry) in which the pollen is not presented directly out of the anthers but instead is transferred to certain other structures (such as the styles) just before or at the onset of anthesis, followed by a female phase where the stigmatic surfaces of the styles become receptive to pollen (Erbar and Leins 1995, 2015*b*; Leins and Erbar, 2006). However, studies of style evolution within the family and among related families are lacking.

The sister groups of Asteraceae have been discussed for a long time and several different families were suggested as possible candidates. Among the families related to Asteraceae, most evidence (Albach *et al.*, 2001; Lundberg and Bremer, 2003; Funk *et al.*, 2009*a*) indicates that the Australian-centred Goodeniaceae (12 genera, ~420 species, including Brunoniaceae; Jabaily *et al.*, 2012) is the sister group of the Calyceraceae–Asteraceae, thus establishing the endemic South American Calyceraceae (four to six genera, ~60 species; Hellwig, 2007; Zanotti and Pozner, 2008) as the morphologically and phylogenetically closest relative of the Asteraceae. Some possible synapomorphies for the Goodeniaceae–Calyceraceae–Asteraceae clade are the presence of secondary pollen presentation (with different modes in each family and with parallelisms in other Asterales), a dry and papillate stigma and a persistent calyx. The Calyceraceae–Asteraceae clade is supported by many synapomorphies, such as the presence of capitula-type inflorescences, pollen morphology and unilocular ovaries with a single ovule (Lundberg, 2009).

From a morphological point of view, the divided style of Asteraceae with stigmatic surfaces on the inside of the two initially appressed style arms is different from that of
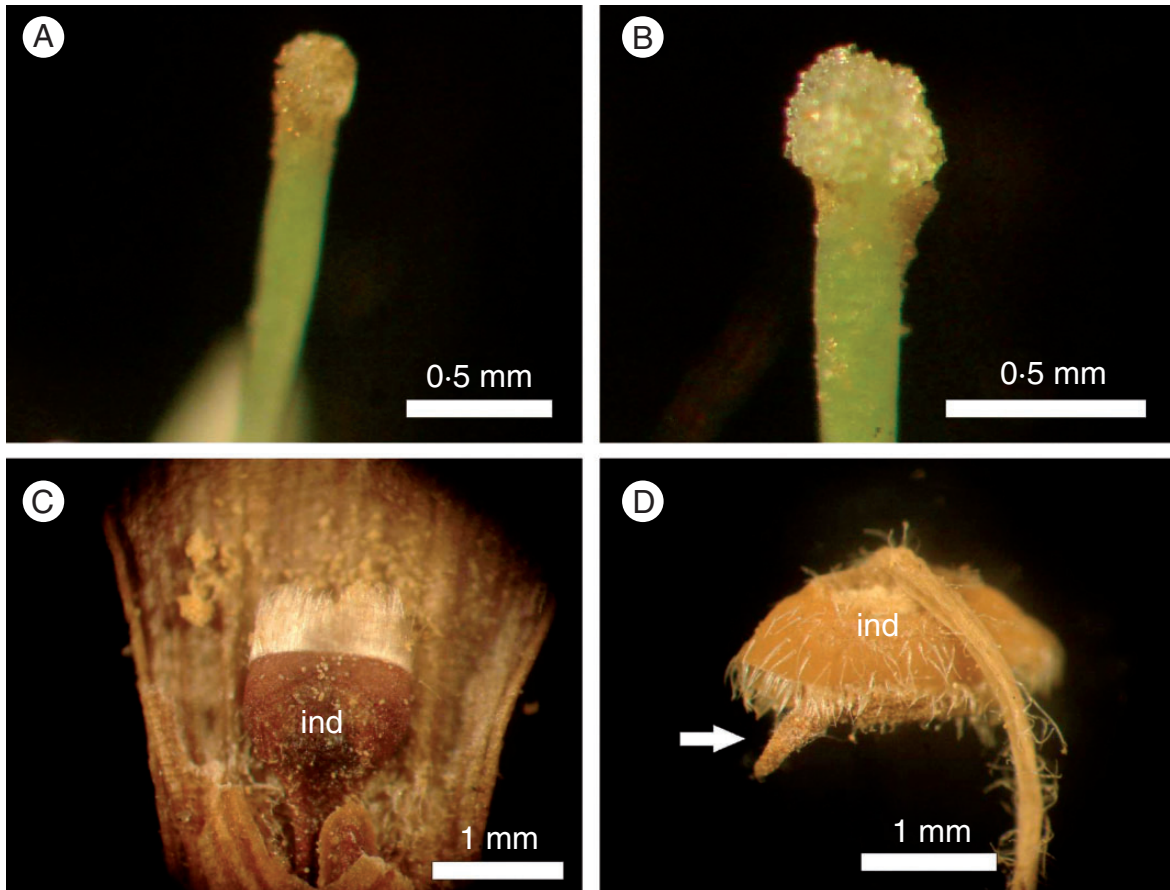
FɪG. 1. Stereomicroscope observation of styles of Calyceraceae and Goodeniaceae. (A, B) Styles of Calyceraceae. (A) *Acicarpha procumbens* at the beginning of anthesis (*Guerrero s.n.*, LP). (B) *Acicarpha procumbens* at the end of anthesis (*Guerrero s.n.*, LP). (C, D) Styles of Goodeniaceae. (C) *Velleia trinervis* at the beginning of the anthesis. Note the hairy indusium (ind) and the pollen grains deposited on the open corolla (*Pritzel 772*, US). (D) *Scaevola nitida* at the end of anthesis. The arrow points to the stigmatic surface emerging from the indusium (US 916958).

Calyceraceae, which is characterized by a terminal, club-like, papillose head (Funk *et al*., 2009*a*) (Fig. 1A, B). The literature, however, is contradictory regarding the style morphology in Calyceraceae. Most authors describe the apex of the style as capitate and undivided, but a shortly bilobed apex was mentioned by Funk *et al.* (2009*a*). Some authors (Pontiroli, 1963; Sorarú, 1974) pointed out that the style is glabrous at the apex whereas others described it as papillose (Erbar, 1993; Hellwig, 2007), or as 'indistinctly papillose ("glabrous")' (Hansen, 1992). The style morphology of Goodeniaceae, on the other hand, has been extensively studied because of its peculiar stigma subtended by a hairy pollen-collecting cup structure or 'indusium' (Fig. 1C, D), with a 'cup' mechanism of secondary pollen presentation (Leins and Erbar, 2006). However, other aspects of the Goodeniaceae and Calyceraceae style, such as style venation, have never been studied.

From an evolutionary point of view, Hansen (1992) postulated that the morphology-based phylogeny among the internal branches of Asteraceae could change if some features were repolarized, such as the shape of the style apex and the style's sweeping hairs (i.e. the outer papillae of the style branches). Even though current classifications within the family Asteraceae and its phylogenetic relationships with other families are mostly based on molecular data, the major taxonomic groups, such as tribes and subfamilies, are defined and supported by morphological characters. Because the style is one of the most prominent features for infrafamilial classification in Asteraceae, a distinction between apomorphic (more derived or more recently evolved) and plesiomorphic (more ancestral or earlier evolved) style character states is critical. This enhances the need to critically evaluate style features in Asteraceae and related families. Early branching lineages of Asteraceae were selected here because they constitute a representative sampling that can help our understanding of the evolution of the entire family. These lineages comprise the subfamilies Barnadesioideae (nine genera, ~90 species; Stuessy *et al*., 2009), Gochnatioideae (four genera, ~88 species), Mutisioideae (44 genera, ~630 species), Stifftioideae (nine genera, ~37 species) and Wunderlichioideae (four genera, 36 species) (Funk *et al*., 2009*a*).

The goal of this study was to perform a comparative analysis to discuss the relevance of style features in the evolution of Asteraceae and its sister families Goodeniaceae and Calyceraceae. We have divided this analysis into two parts: first, we will survey the morphology, histochemistry and venation in the groups under study. Whereas the style morphology of Goodeniaceae and basal lineages of Asteraceae (Fig. 2) are well known (e.g. Carolin *et al*., 1992; Cabrera, 1977; Katinas
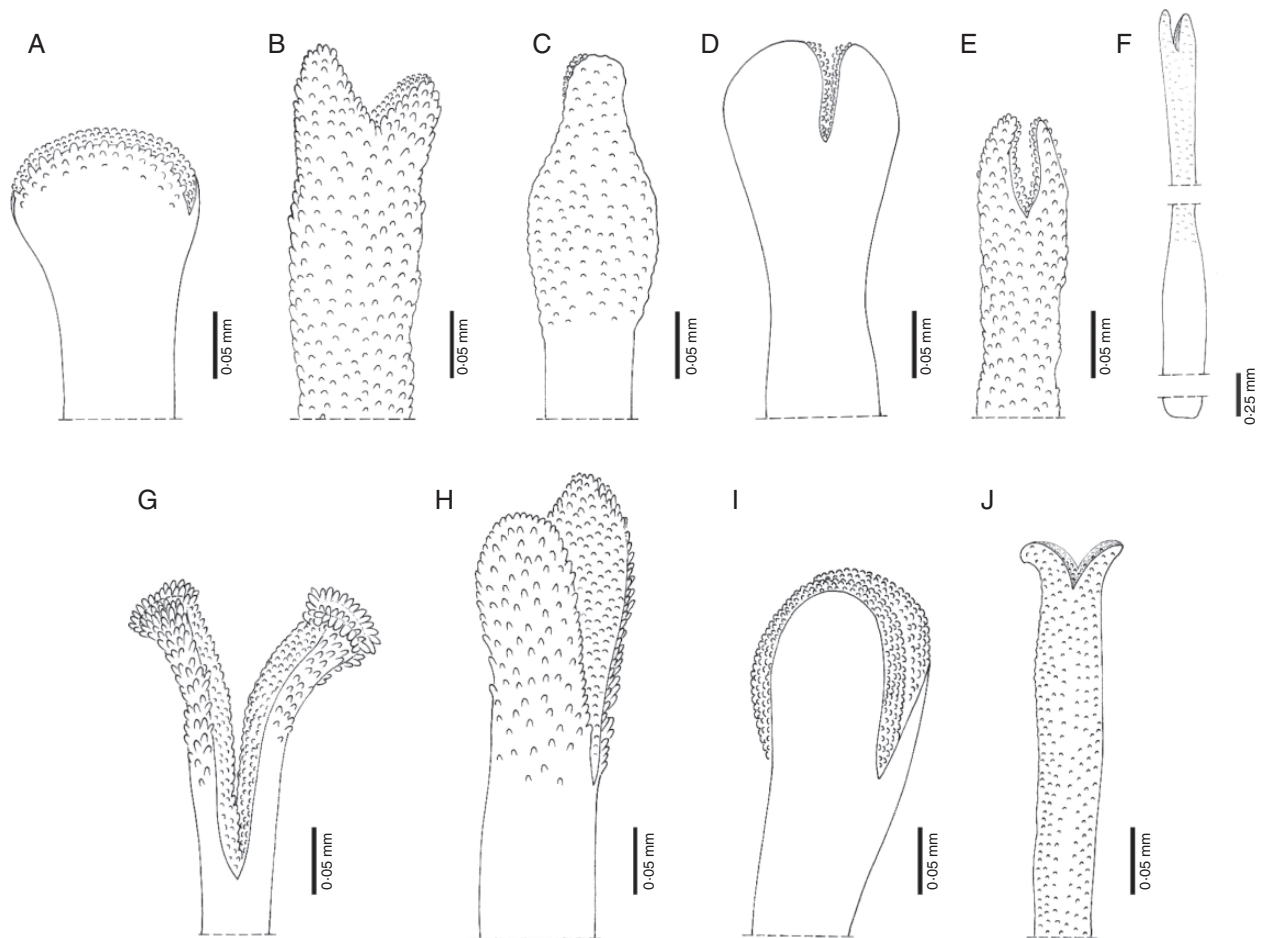
Fɪɢ. 2. Style morphology in early branching lineages of Asteraceae. (A) Style shortly bilobed with a lip of internal papillae and lobes externally scarcely papillose; lateral view [*Barnadesia odorata* (Barnadesioideae), *Fabris 4679*, LP]. (B) Style with lobes and shaft papillose; frontal view [*Doniophyton anomalum* (Barnadesioideae), *Chicchi 65*, LP]. (C) Style with shaft widened and papillose and stigma divided into two short, acute, externally scarcely papillose lobes; lateral view [*Fulcaldea laurifolia* (Barnadesioideae), *Ferreyra et al. 10932*, LP]. (D) Style with shaft and lobes glabrous, lobes with a lip of internal papillae; frontal view [*Huarpea andina* (Barnadesioideae), *Nicora et al. 8573* LP]. (E, F) *Schlechtendalia luzulifolia* (Barnadesioideae), frontal view. (E) Style apex and most of the shaft completely papillose, lobes with a lip of internal papillae. (F) Entire style showing the shaft base widened and glabrous [*Xifreda & S. Maldonado 585*, LP]. (G) Style branches externally covered by sweeping hairs which also constitute a tuft at the branch apex, and internal stigmatic papillae; frontal view [*Leucheria candidissima* (Mutisioideae), *Gentili 209*, LP]. (H) Style branches with external sweeping hairs and internal stigmatic papillae; lateral view [*Mutisia acerosa* (Mutisioideae), *Barros 1754*, LP]. (I) Style with shaft and lobes glabrous, lobes with a lip of internal stigmatic papillae; lateral view [*Salcedoa mirabaliarum* (Stifftioideae), *Veloz et al. 2383*, LP]. (J) Style apex and most of the shaft completely papillose; frontal view [*Stenopadus cucullatus* (Wunderlichioideae), *Liesner & Holst 21389*, MO].

et al., 2008*b*; Erbar and Leins, 2015*b*), an analysis of the morphological diversity of the styles of Calyceraceae is critical because it is poorly documented. Hernández *et al.* (2015) showed that the presence of lipophilic substances is a good way to differentiate between the receptive (to pollen) from the nonreceptive areas in the styles of Asteraceae. We will test whether this distinction also occurs in Goodeniaceae and Calyceraceae. In addition, style venation in Goodeniaceae, Calyceraceae and Asteraceae will be documented and analysed because such information is incomplete or lacking, focused on the ovary–style transition, or collateral to analyses of corolla venation (e.g. in Asteraceae – Small, 1917; Carlquist, 1957; Gustavsson, 1995; in Goodeniaceae – Carolin, 1959). Venation diversity may indicate high selective pressure acting on the plant organs (Roth-Nebelsick *et al.*, 2001) and also might be phylogenetically informative at several levels of classification (Nelson and Dengler, 1997). Changes in venation may also be correlated with other morphological changes in the styles. Second, the

characters listed above will be examined in a phylogenetic framework, allowing us to propose evolutionary hypotheses about the origin of the bifid style in Asteraceae (where the style branches have different external and internal surfaces).

MATERIALS AND METHODS

This study was based on the literature (Pontiroli, 1963; Crisci, 1974; Cabrera, 1977; Carolin, 2007; Carolin *et al.*, 1992; Hellwig, 2007; Katinas *et al.*, 2008*b*, 2009; Stuessy *et al.*, 2009) and on the investigation of herbarium materials (Appendix S1) and some fresh specimens. For the general phylogeny of the groups under study and the taxonomy of Asteraceae we follow Funk *et al.* (2009*a*). Recently, the subfamily Famatinanthoideae was established (Panero *et al.*, 2014), containing one species, *Famatinanthus decussatus*, as the sister taxon of the entire Mutisioideae–Asteroideae. Support

for the relationships within the Mutisioideae and its closely related subfamilies is still not strong and the general morphology of this species, including styles, does not differ from that of other members of the subfamily where it was previously placed, the Mutisioideae (style shortly bifid, lobes rounded at the apex, with a lip of internal papillae, externally papillose, the papillae rounded). Because the morphology does not impact our study in any way and because we have not had a chance to examine the plant in detail we have not discussed it separately. For the taxonomy of Goodeniaceae we follow Carolin (2007). The number of genera of Calyceraceae ranges from four (Hellwig, 2007) to six (Zanotti and Pozner, 2008) according to the different classifications; we considered six genera: *Acicarpha* (three species), *Boopis* (∼30 species), *Calycera* (14 species), *Gamocarpha* (six or seven species), *Moschopsis* (seven species) and *Nastanthus* (six species).

Lipophilic components indicate areas that are receptive and non-receptive to pollen (Hernández *et al*., 2015). To test the lipophilic components, mature flowers were reconstituted in water in an oven at 30 °C for 24–72 h and then fixed in formalin-aceto-alcohol (FAA 70 %) (Johansen, 1940). The fixed material was dissected and the styles were bleached in sodium hypochlorite (50 %), washed twice with distilled water, then immersed in ethanol 70 % solution of Sudan IV for 15 min, differentiated with ethanol (70 %) for ∼1 min, and mounted in gelatin glycerin (Ruzin, 1999). A reddish colour on the cuticle and cell walls (and occasionally light red in the cytoplasm) indicates receptive areas, and non-receptive areas have a variable content of deep red drops filling the cells. For appreciating other details, such as venation or certain tissues, some styles were stained with 80 % safranin or left unstained. Florets of Calyceraceae are usually bisexual, but functionally male florets occur regularly in the centre of the capitula of *Acicarpha*; in these cases the bisexual marginal florets were sampled.

Style vascular bundles were analysed, beginning at the middle of the shaft and extending upwards; venation features below this area were not included in the analysis because the focus of this study was the upper part of the style.

Observations and drawings of morphological and anatomical features were carried out with a Leitz SM Lux and a Nikon Eclipse E200 light microscope equipped with a camera lucida. Light microscope photographs were taken with a Nikon Coolpix S10 and a Moticam 2300, which uses Motic Image Plus 2.0 software, and with a Wild M8 stereomicroscope with a digital camera (Sony W30).

RESULTS

As in Asteraceae, the location of lipophilic compounds proved to be effective in examining the styles of Goodeniaceae and Calyceraceae.

Goodeniaceae

Lipophilic compounds. The stigma was examined while it was enclosed in the indusium at the beginning of anthesis, and at the end of anthesis when it was emerging from the indusium. The enclosed stigma was exposed by mechanically breaking the indusium. The stigmatic surface is papillose and the papillae contain small, scarce droplets of lipophilic compounds (in red), revealing that they are non-receptive to pollen. The protruding papillose surface of the stigma (Fig. 3A) at the end of anthesis, on the other hand, shows colourless external papillae, without drops of lipophilic content, indicating that the external papillae are receptive to pollen. More internally located papillae still retain lipophilic drops (Fig. 3B)

Venation. The three or four main veins are parallel and run through most of the shaft of the style in the longitudinal median plane of the flower. Before ending, the veins may bifurcate and then each one of them develops a divergent branching of 4 to ∼15 complementary secondary veins, all of the same size, grouped in bundles with a broccoli-like appearance (Fig. 3C). The branched veins either end close to the base of the indusium or enter slightly into the indusium. The style shaft is widened in the area where the veins are branching. This widening is continuous with the turbinate-shaped indusium.

Calyceraceae

Lipophilic compounds. Our studies showed that the styles in Calyceraceae have three stages and that these stages correlate with the phases of anthesis: (1) an immature stage where all the stigmatic papillae have drops of lipophilic cytoplasmic that are deep red (non-receptive) (Fig. 3D), corresponding to the male phase of anthesis; (2) an intermediate stage where the basal and internal papillae have red content and the external papillae have no lipophilic content and are colourless (Fig. 3E); and (3) a mature stage where all the papillae have no content or very scarce content evidenced by a pale, light red colour in the cytoplasm (receptive papillae; Fig. 3F), corresponding to the female phase of anthesis. All of the cells of the style filament have red cytoplasmic content.

Venation. The two main veins (rarely three in some specimens of *Acicarpha*) run parallel through most of the shaft in the longitudinal median plane of the flower. Before ending, the veins develop a dramatic branching into 4 to ∼20 complementary secondary veins, all of the same size, grouped in two bundles, close to the style apex. Unlike Goodeniaceae, the bundles are not divergent; the veins are parallel and appressed at their margins. The branching causes a widening of the shaft in that area; above the widened area and below the head there is a constriction that is referred to as the 'neck'. The veins may: (1) reach the area below the head coincident with the base of the neck (all species analysed; Fig. 4A); (2) reach the base of the head via diffuse, scanty, provascular tissue (specimens of *Boopis gracilis*; Fig. 4B); (3) reach the base of the head through well-developed vascular tissue (specimens of *Acicarpha procumbens*, *Acicarpha spathulata*, *Acicarpha tribuloides*, *Boopis anthemoides* and *Boopis multicaulis*; Fig. 4C); or (4) pass the base of the head and enter the bilobed styles with each bundle of veins diverging slightly into each lobe (specimens of *A. tribuloides* and *B. multicaulis*; Fig. 4D).

Bilobed style. The style apex is generally undivided in Calyceraceae. Only some specimens of *A. tribuloides*, *B. multicaulis* and *Nastanthus scapigerus* have a bilobate apex and,
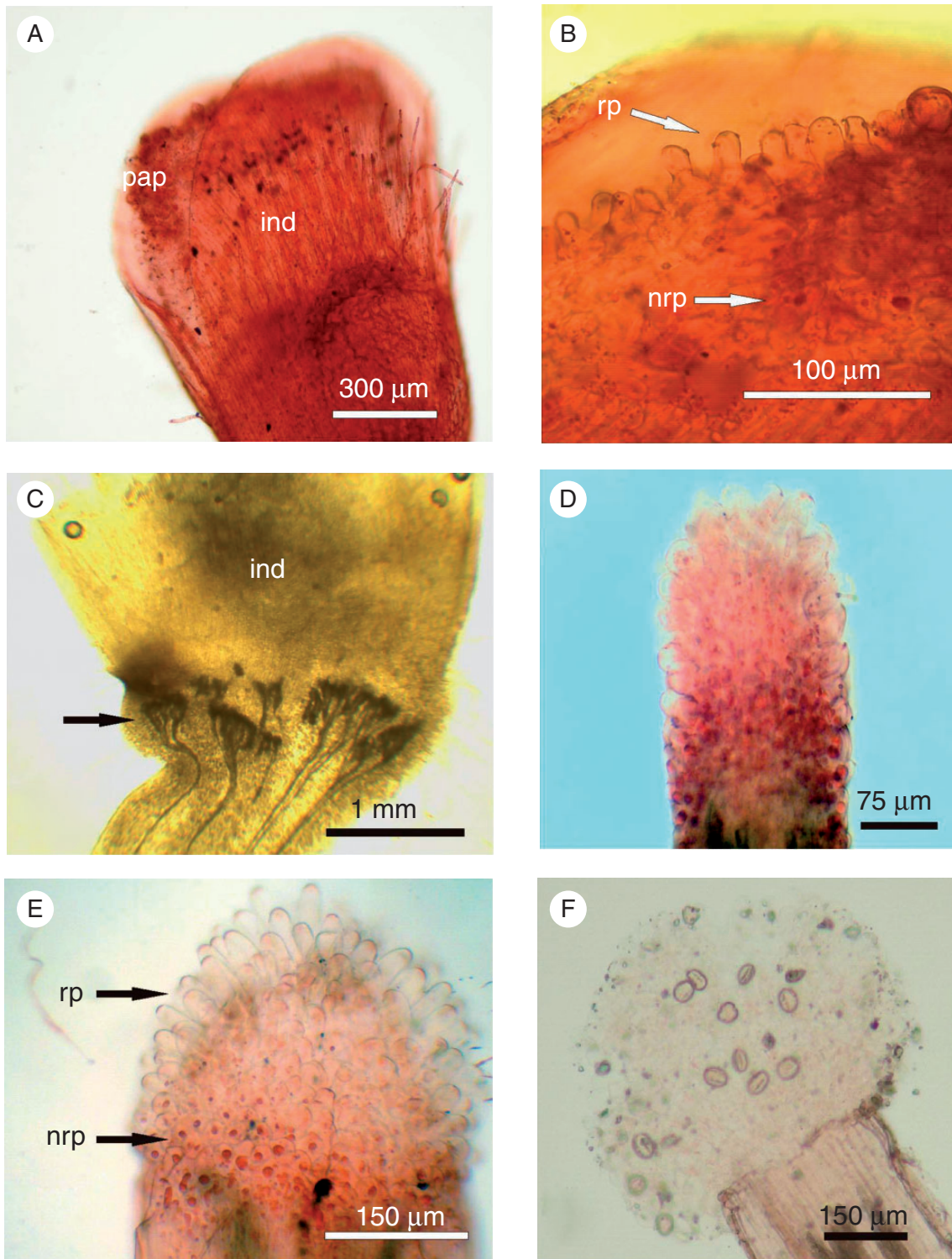
FIG. 3. Style features in Goodeniaceae and Calyceraceae. (A, B) Distinction between receptive and non-receptive papillae in *Brunonia australis* (Goodeniaceae), stained with Sudan IV (*Clemens s.n.*, US 3035598). (A) General view of the stigmatic surface emerging from the indusium. (B) Detail of the stigmatic papillae, showing the location of receptive papillae and non-receptive papillae to pollen. (C) Multiple, apically divergent branched veins (arrow) in *Selliera radicans* (Goodeniaceae) (*Lovis 90/15*, LP). (D–F) Distinction between receptive and non-receptive papillae in Calyceraceae, stained with Sudan IV. (D) Male phase in *Acicarpha procumbens* showing a reddish head due to cytoplasmic content of lipophilic compounds in all the papillae (*Guerrero s.n.*, LP). (E) Transitional phase in *Acicarpha tribuloides* showing a head with the basal papillae with reddish cytoplasmic content (non-receptive) and upper papillae without reddish content (receptive) (*Delucchi et al. 3255*, LP). (F) Female phase in *Nastanthus scapigerus* showing a colourless head due to the lack of lipophilic cytoplasmic content in fertile papillae (ex LPS 12937 in LP). ind, indusium; nrp, non-receptive papillae; pap, papillae; rp, receptive papillae.

even in these specimens, bilobed and non-bilobed styles are found in the same capitulum (Fig. 5). These style lobes vary from incipient (Fig. 5A) to well developed (Fig. 5B, C). No morphological or histochemical differences were found between the external papillae (covering the outer face of the lobes) and the internal papillae (covering the inner face of the lobes); the papillae simply do not undergo differentiation into receptive or non-receptive areas.

Morphology. Family description. The style consists of a shaft with a neck, and the stigma has an apical head (Fig. 4A). The shaft is cylindrical, smooth or papillose, variously widened below the neck due to the branching of the two veins in that area. The neck is the area between the shaft and the stigmatic head and is usually not widened: it can be an obvious narrowing or a gradual transition. The head shape can be capitate, rounded, cylindrical, elliptical (with the major axis horizontal) or conical and it can be undivided, or notched to bilobed. It is

covered by crowded papillae that are non-receptive to pollen in the male phase and receptive in the female phase. The papillae are short and with dense cytoplasmic content of lipophilic compounds in the male phase, and longer and without cytoplasmic lipophilic compounds in the female phase of floret maturation.

Generic descriptions. Acicarpha Juss. Head: rounded, conical; undivided or shortly bilobate (specimens of *A. tribuloides*); papillose. Neck: well demarcated (*A. procumbens*, *A. spathulata*, *A. tribuloides*) or transitional (*A. tribuloides*). Shaft: smooth or papillose (*A. tribuloides*). Veins: reaching the neck (*A. procumbens*), reaching the base of the head (*A. procumbens*, *A. spathulata*, *A. tribuloides*) or slightly surpassing the base of the head (*A. tribuloides*).

Boopis Juss. Head: rounded, conical or elliptical (with the major axis horizontal); undivided, notched, or bilobate (*B. multicaulis*). Neck: well-demarcated or transitional (*B. graminea*). Shaft: smooth or papillose (*B. multicaulis*). Veins: reaching the
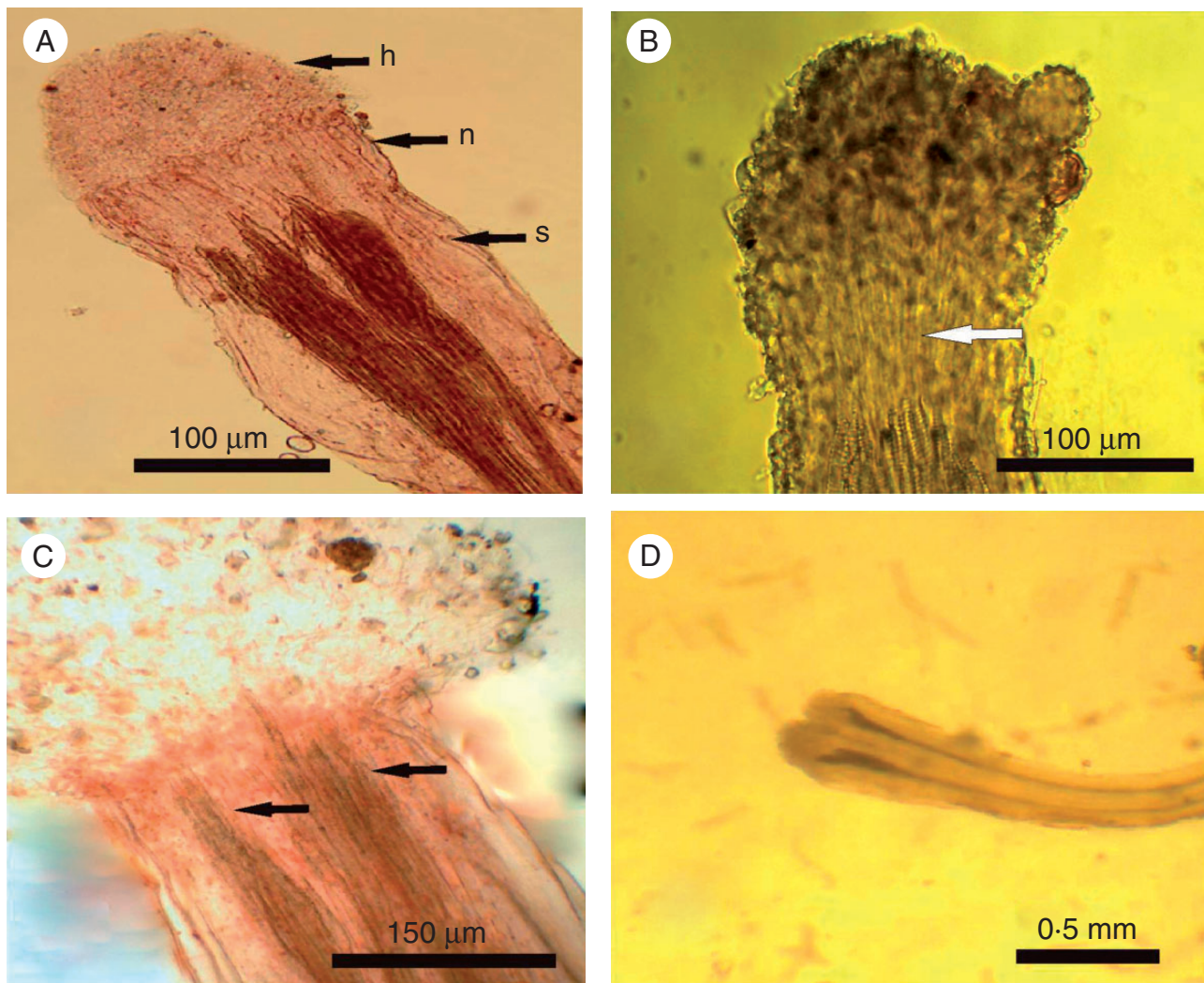

FIG. 4. Venation in Calyceraceae. (A) Widening of the shaft and vein branching not reaching the head in *Boopis australis* (ex LPS 12810 in LP). h, head; n, neck; s, shaft. (B) Veins reaching the head base with provascular tissue in *Boopis gracilis*; arrow shows intermediate tissue between parenchyma and xylem (*Bridarolli 2205*, LP). (C) Veins (arrows) reaching the head base with vascular tissue in *Boopis anthemoides*. Some veins entering into the head to a short distance (*Fabris 907*, LP). (D) Veins surpassing the base head and diverging into the bilobed head in *Acicarpha tribuloides* (*Delucchi et al. 3255*, LP).
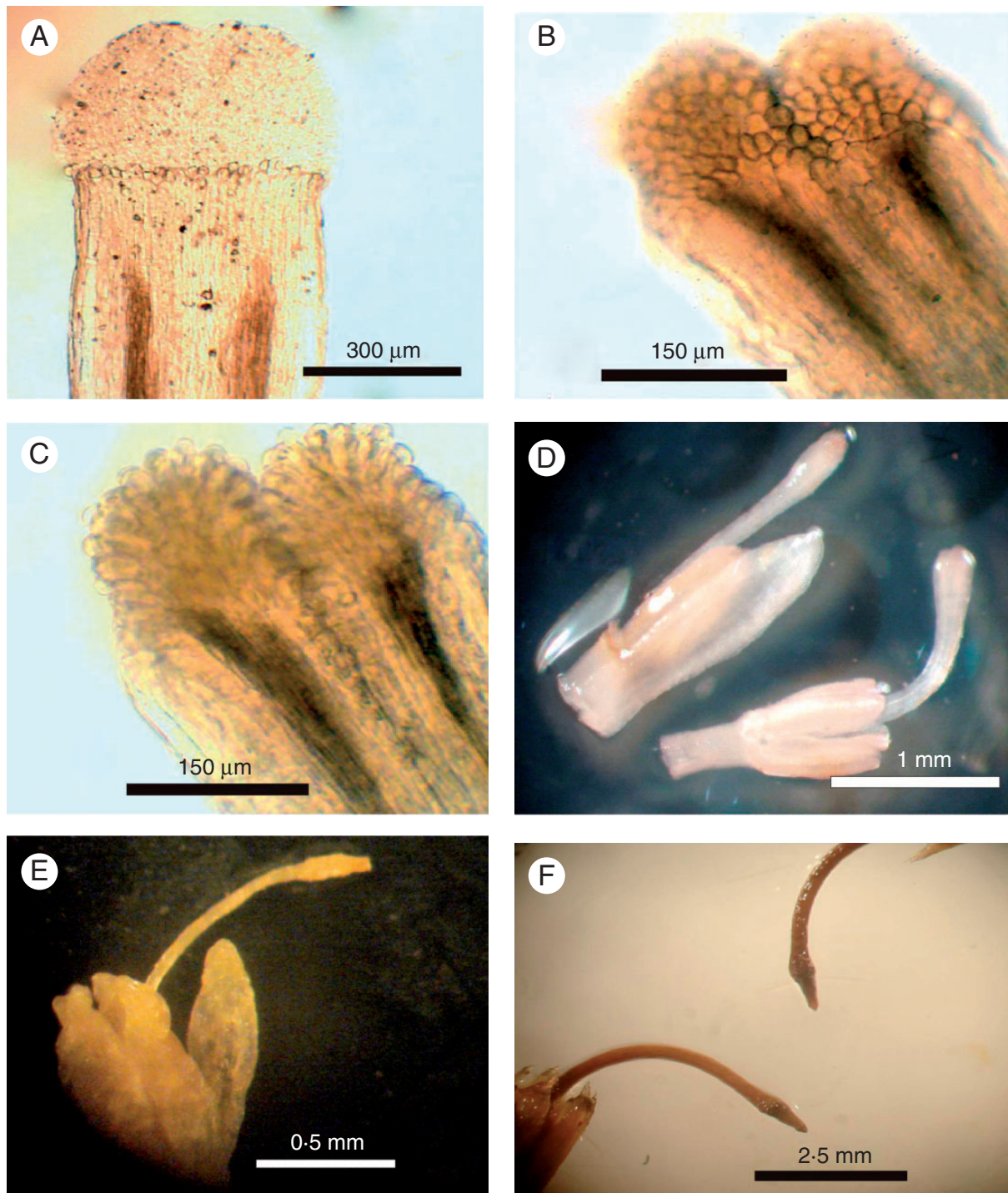
FIG. 5. Style bilobation in Calyceraceae. (A) Incipient bilobation in *Nastanthus scapigerus* (ex LPS 12937 in LP). (B) Conspicuous bilobation in *Acicarpha tribuloides*, male phase (*Delucchi et al. 3255*, LP). (C) Conspicuous bilobation in *Acicarpha tribuloides*, female phase; note the major development of the papillae compared with the male phase (*Delucchi et al. 3255*, LP). (D–F) Stereomicroscope comparison between the bilobed styles of Calyceraceae and Barnadesioideae (Asteraceae). (D) Two florets of *Acicarpha tribuloides* (Calyceraceae), one (left) with the style with entire head and the other (right) with notched head (*Delucchi et al. 3255*, LP). (E) Floret of *Acicarpha tribuloides* (Calyceraceae) showing style bilobation at the apex (*Berro 4344*, LP). (F) *Fulcaldea laurifolia* (Barnadesioideae, Asteraceae). Note the resemblance to the photograph in panel (E) (*Ferreyra et al. 10932*, LP).

neck with provascular tissue (*B. gracilis*); reaching the neck with vascular tissue (most species); surpassing the neck and reaching the head (*B. anthemoides*, *B. multicaulis*); or slightly surpassing the base of the head (*B. multicaulis*).

Calycera Cav. All six species analysed have the same style morphology. Head: rounded or elliptical (with the major axis horizontal); undivided; papillose. Neck: well-demarcated. Shaft: smooth. Veins: reaching the neck.

Gamocarpha DC. All four species analysed have the same style morphology. Head: rounded or conical; undivided; papillose. Neck: well-demarcated. Shaft: smooth. Veins: reaching the neck.

Moschopsis Phil. All three species analysed have the same style morphology. Head: rounded; undivided; papillose. Neck: well-demarcated. Shaft: smooth. Veins: reaching the neck.

Nastanthus Miers. All three species analysed have the same style morphology. Head: rounded or elliptical (with the major axis horizontal); undivided, notched, or bilobate (*N. scapigerus*); papillose. Neck: well-demarcated or transitional. Shaft: smooth. Veins: reaching the neck.

Asteraceae

Venation. Barnadesioideae. Two parallel veins run through the shaft in the longitudinal median plane of the flower and (1) end at the bifurcation point of the two apical lobes (Fig. 6A) or (2) each vein enters into each lobe but ends without reaching the lobe apex (Fig. 6B). The veins are broader at their apex because of the branching of the secondary veins while remaining parallel and laterally appressed to one another.

Gochnatioideae, Mutisioideae, Stifftioideae, Wunderlichioideae. In general, venation patterns agree in these four subfamilies. Two parallel veins run through the shaft in the longitudinal median plane of the flower and (1) each vein enters into each lobe or arm and ends at the lobe apex or (2) ends close to the apex. However, rarely there are four veins reaching the lobe apex (in Stifftioideae and Wunderlichioideae; see Discussion). The veins are branched and appressed (Fig. 6C) as in Barnadesioideae, or unbranched in the species with longer style arms (e.g. *Chaptalia*, *Dinoseris*) (Fig. 6D, E).

DISCUSSION

New findings in style morphology

For the first time style venation has been investigated in Goodeniaceae (Fig. 3), showing multiple branching in a divergent pattern close to the indusium. The stigma is papillose. When the stigma is enclosed in the protective sheath or indusium, the papillae are non-receptive to pollen, as evidenced by the reddish, scarce, small droplets (after treatment with Sudan IV). When the stigma is carried upwards and displayed, all the external papillae are colourless (with Sudan) and pollen-receptive. The changes in the papillae are thus correlated with the male and female phases of the cup mechanism of secondary pollen presentation in the family.

In Calyceraceae, the stigmatic head was papillose in the species analysed instead of glabrous, as suggested by some authors (e.g. Pontiroli, 1963; Sorarú, 1974), and proved to be remarkably morphologically homogeneous at specific and generic levels for most genera. Unlike Asteraceae, the different distribution of lipophilic substances reflected the double role of the same papillae during anthesis: firstly sterile and secondly fertile. Regarding the controversy about the apex of the style as undivided or shortly bilobed, we found that the head is generally undivided, although there are few exceptions. Bilobed styles do not display differences between the papillae covering the internal and external faces of the lobes. Style venation was analysed here for the first time, showing that a sudden branching of the two veins that run parallel through most of the shaft occurs in the area close to the neck before the veins end, producing an obvious widening of the shaft at that point. When bilobation is coupled with the style-widening of Calyceraceae, the styles have a close resemblance to the styles of *Fulcaldea* of Barnadesioideae (Asteraceae) (Figs 5D–F). Occasionally, there is provascular tissue at the base of the head. The provascular tissue appears as parenchyma that starts its differentiation into vessels by showing intermediate, transitional stages between parenchyma and xylem cells. According to Fukuda (1997), the earliest recognition of provascular cells is based on differential stain affinity; the cells become more densely stained, presumably through increased vacuolation, whereas their neighbours become less so. Provascular cells are also elongate and narrow, with a long axis parallel to the axis of the provascularized strand, by contrast to the more isodiametric ground cells, and characteristically divide longitudinally in the growth direction. These characteristics can be seen in *B*. *gracilis* (Fig. 4B).

In Asteraceae, the two veins of the style shaft are apically branched in most genera, with a major branching in Barnadesioideae. The veins do not enter or enter only a short way into the style lobes in Barnadesioideae, whereas veins do enter the style lobes or arms of the other basal subfamilies. Carlquist (1957) described four veins for the styles of *Chimantaea rupicola* (Wunderlichioideae) and *Gongylolepis pedunculata* (Stifftioideae): two external (dorsiventral) veins running from the style base and entering into the lobes, and two internal (lateral) ones running from the base of the style to just below the point of bifurcation. The same situation occurs in *Gongylolepis bracteata* (Stifftioideae), but the bundles branch and anastomose into two veins in the upper part of the style close to the lobes. In other species, such as *Achnopogon virgatus*, *Duidaea tatei*, *Glossarion rhodanthum*, *Gongylolepis fruticosa*, *Neblinaea promontoriorum*, *Quelchia cardonae* (Stifftioideae) and *Stenopadus cucullatus* (Wunderlichioideae), the extra pair of veins is only found at lower levels of the style and then they unite with the other two veins (Carlquist, 1957). The four veins found running from the base to the apex of the style in some species of Stifftioideae and Wunderlichioideae might be interpreted as vestiges of a primitive venation condition such as that found in Goodeniaceae and some Calyceraceae.

All these findings have significant implications for the interpretation of style evolution in these families, and are discussed below.

From temporal to spatio-temporal papillae presentation

When Bremer (1987) performed the first morphological phylogenetic analysis of the family Asteraceae, with the Calyceraceae as the outgroup rather than other families, such as Lobeliaceae or Campanulaceae, he concluded that only a few characters are apomorphic for the Asteraceae. One of these characters was the shortly bilobed, apically thickened style, such as that of *Barnadesia* and *Gochnatia*. This hypothesis can now be tested using the morphological findings presented here and the most recent phylogenetic evidence.

Phylogenetic trees based on molecular data (e.g. Funk *et al.*, 2009*a*) provide a fundamental tool for interpreting the evolution of reproductive features and are used here as a framework
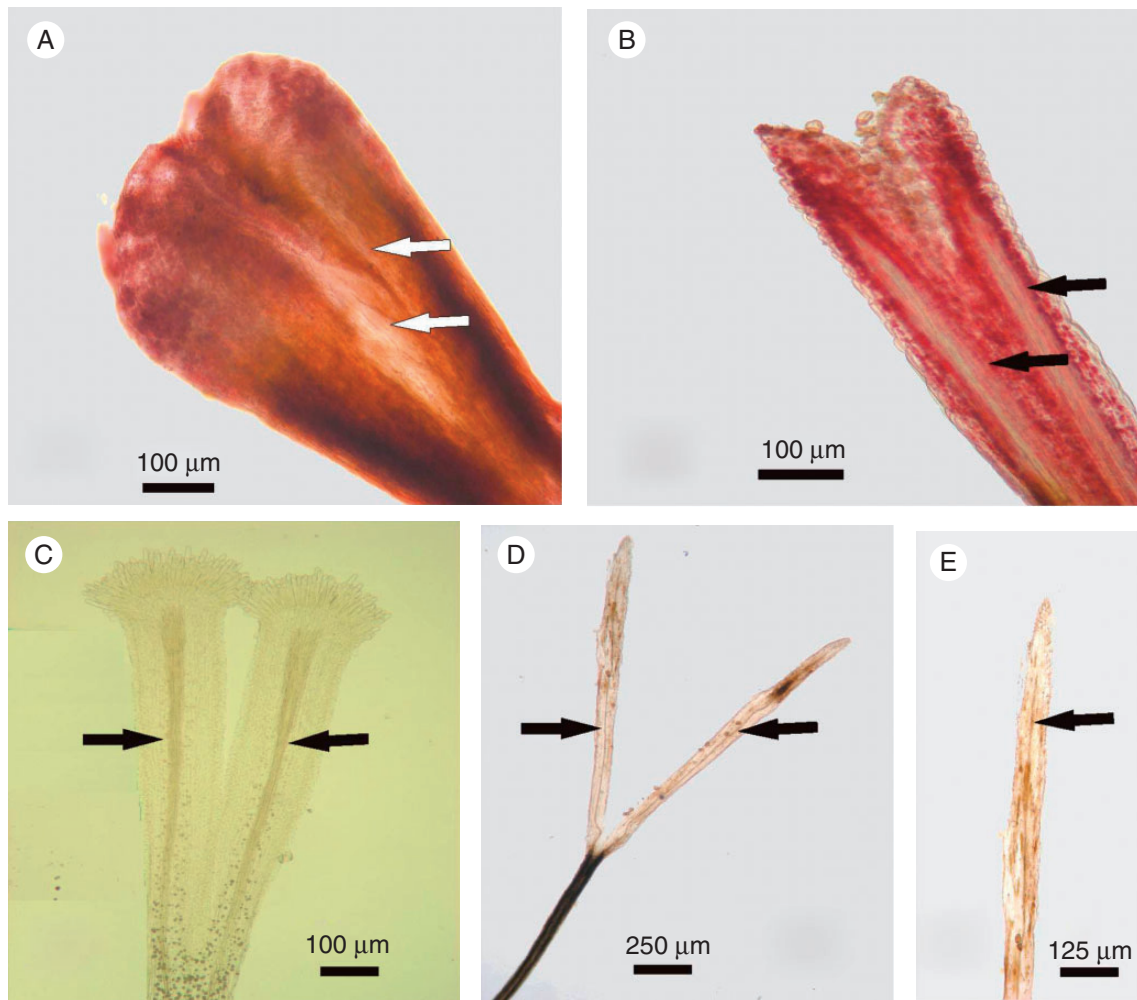
FIG. 6. Style venation in early branching lineages of Asteraceae. (A) Veins ending at the style lobe bifurcation point in *Huarpea andina* (Barnadesiodeae). Note the branching of the veins (*Nicora et al. 8573*, LP). (B) Veins entering each style lobe without reaching the style apex in *Doniophyton anomalum* (Barnadesioideae) (*Chicchi 65*, LP). (C) Veins ending close to the style branch apex and widened at the apex in *Jungia pauciflora* (Mutisioideae) (*Cabrera et al. 26051*, LP). (D) Veins not widened at the apex and ending at the branch apex in *Chaptalia integerrima* (Mutisioideae) (*Katinas & Iharlegui 136*, LP). (E) Detail of the branch apex in *Chaptalia integerrima* (Mutisioideae). Arrows indicate veins.

for our findings (Fig. 7). Lundberg (2009) and Funk *et al.* (2009*b*) suggested that since Calyceraceae and Goodeniaceae have a papillose, dry stigma that lacks a copious surface secretion, it is reasonable to assume that this is a synapomorphic character for Asteraceae (and therefore plesiomorphic within the family). The aquatic Menyanthaceae, on the other hand, the sister taxon to our three study families, have wet stigmas that vary greatly; they may be usually cup-shaped, cyathiform or crateriform, entire, two-lobed, petaloid, sessile, lacerate, lamelliform, fimbriate, finger-like or with long hairs (cf. Dulberger and Ornduff, 2000; Tippery *et al.*, 2009), and commonly papillate where a distinct surface secretion is present (Heslop-Harrison and Shivanna, 1977). Our observations of style venation in the Menyanthaceae show variation in the sampled species, with two to multiple veins, which may be branched or unbranched at their tips. In many members of this family, it is common for the maturation of the floral bud to be underwater and for species to be dioecious, undergo asexual reproduction, be self-compatible (Ornduff, 1966; Haddadchi *et al.*, 2014) and

have no secondary pollen presentation mechanism (Leins and Erbar, 2006). In contrast to Goodeniaceae, Calyceraceae and Asteraceae, we observed a lack of lipophilic compounds in the stigmatic papillae in the styles of Menyanthaceae, suggesting that there is probably a relationship between secondary pollen presentation mechanisms and papilla receptivity. In conclusion, it seems the mechanisms of secondary pollen presentation and a dry stigmatic surface are style features that support Asteraceae, Calyceraceae and Goodeniaceae as a monophyletic group (Lundberg, 2009).

Goodeniaceae is the only one of these three families with a 'cup' secondary pollen presentation mechanism (Leins and Erbar, 2006). The style has an apical hollow pollen cup or indusium enclosing a more or less lobulate stigma. The pollen from the stamens slides into the cup, where it is collected; it is retained there and is presented to the pollen vectors (male phase) (Carolin *et al.*, 1992). Later the stigma matures, becomes pollen-receptive, and grows out of the indusium in the form of one or more papillose lobes and the female phase of anthesis commences.
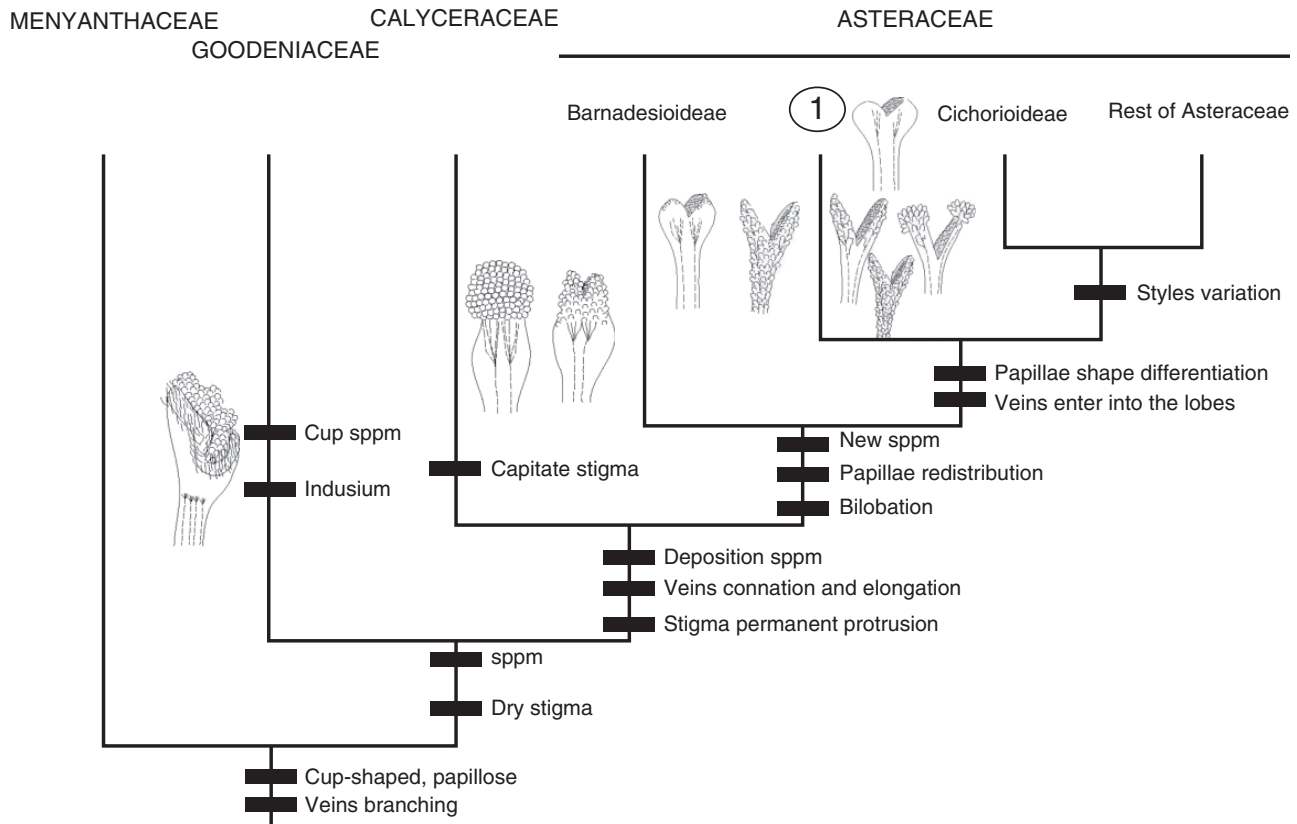
Fig. 7. Simplified phylogenetic tree of Asteraceae and related families of Funk *et al.* (2009*b*), showing the evolution of style features and the synapomorphies supporting the groups. 1, (Mutisioideae, (Stifftioideae, (Wunderlichioideae, (Gochnatioideae, Hecastocleidoideae)))). sppm, secondary pollen presentation mechanism.

Calyceraceae displays a trend towards vein cohesion and lateral adnation and elongation in the vascular tissue of the styles. The location of the termination of the veins in the style progresses acropetally from the neck to the head, as suggested by the observed transformation of provascular tissue in vascular tissue. The formation of a compact, more or less spherical stigmatic head is found in Calyceraceae. Although some styles have varying degrees of bifurcation, the external and internal papillae of the lobes are the same. This family shows a temporal differentiation of male–female phases, as in Goodeniaceae, but with a simple deposition mechanism of pollen presentation (Leins and Erbar, 1997). In the male phase, the papillae covering the capitate head are non-receptive and tightly grouped, which allows the deposition of pollen, held together by pollenkitt, and are presented to visiting pollinators. The male phase is followed by a female phase, in which the style lengthens and the same papillae of the male phase become fertile and receptive to pollen. At this stage it is common to see the mass of pollen attached to the widened area of the shaft below the fertile head of the style (Fig. 8). This mechanism is possible only if the head papillae are permanently exposed and not temporarily enclosed in a cup, as in Goodeniaceae. Therefore, it is possible that this stigmatic exposure was present in the ancestor of the Calyceraceae–Asteraceae clade.

Members of Asteraceae have the upper part of the style divided into two lobes, and what was a typically capitate head in Calyceraceae is, in the Asteraceae, clearly bifid. There is a definite and permanent redistribution of papillae, with the receptive ones restricted to the inner side of the lobes, whereas the outer part of the lobes is covered by non-receptive, pollen-collecting papillae. Thus, the styles show a clear division in the function of the papillae correlated with their different spatial distribution. In some members of early branching lineages, especially in Barnadesioideae, there are still thickened, shortly bilobed, style apices, possibly remnants of what was a capitate head. A common feature in very shortly bilobed styles is a bulging of the stigmatic papillae or 'lip' (e.g. *Barnadesia*, *Gochnatia*, *Schlechtendalia*) presumably to augment the pollen-receptive area due to the reduced internal surface of the short lobes (Fig. 2I). The two veins of the style remain highly branched in Barnadesioideae (e.g. *Dasyphyllum*, *Huarpea*) and the veins may barely enter into the lobes (e.g. *Dasyphyllum*) or not (e.g. *Huarpea*). Asteraceae genera in our study, other than Barnadesioideae, show a vascular progression, with the veins entering into the lobes and having slight branching at the apex, with the exception of those with the longest arms (e.g. *Chaptalia*, *Dinoseris*; Fig. 6D, E), where the veins are unbranched. In Barnadesioideae with externally papillose styles, the external and internal papillae are very short and generally morphologically similar. In the style papillae of the remaining basal taxa there is a morphological distinction between the internal stigmatic papillae, short and rounded, and the external papillae (sweeping hairs), long and rounded or obtuse at the apex. Indeed, the location of sweeping hairs can be specialized,

F ig . 8. Photograph of the inflorescence of a member of Calyceraceae showing an ant crawling among the florets. Note the different style phases, with the pollen covering the style apex (male phase) and the capitate apex free of pollen (female phase). Photograph by M. Bonifacino.

as in the tribe Nassauvieae, where they appear as a tuft at the apex of the style branches (Figs 2G and 6C).

According to Leins and Erbar (2006), a 'deposition mechanism' of secondary pollen presentation occurs in Barnadesioideae, as in Calyceraceae, along with a new but probably plesiomorphic 'brushing mechanism' (hairs at the back and apex of the style brush the pollen of the introrse anthers) and a 'pump mechanism' (the pollen released into the anther tube is pushed out by the growing style; Erbar and Leins, 2015*b*). The transition to the brushing mechanism can be seen in the different genera of the subfamily, ranging from externally very scarcely papillose (e.g. *Barnadesia*, *Huarpea*) to conspicuously papillose (e.g. *Doniophyton*, *Schlechtendalia*). In general, there is a link between the shape of the style apex and the number of papillae: the styles that are glabrous or scarcely papillose have the shortest styles and inflated lobes that are compatible with a deposition mode, whereas the longer lobes have more papillae and are more compatible with a brushing mode (Fig. 2A–F). Completely glabrous styles were found in *Huarpea* of Barnadesioideae, and it is therefore possible that their presence in other basal subfamilies, such as Gochnatioideae and Stifftioideae, may be plesiomorphic, but it must be emphasized that the venation is different, as has already been mentioned.

The temporal male–female phases that predominate in many Asterales is of course present in Asteraceae, but with the important innovation (apomorphy) that each phase is separated not only temporally but also spatially. The bilobation and bifaciality of the style resulted in a redistribution of the papillae. In the brushing mechanism, for example, when the initial dehiscence of the internal surface of the fused pollen sacs occurs, the sweeping hairs on the outer surface of the closed style branches brush the pollen through the anther tube, exposing it to the pollinators. Then, in the female phase, the style branches open, exposing the papillae of the stigmatic adaxial surface to the pollinators. Whereas in Goodeniaceae and Calyceraceae the same papillae play both roles – exposing and receiving pollen – the spatial separation of the papillae in Asteraceae can be viewed as a more successful mechanism because it is specially designed to promote outcrossing, with the receptive inner surface remaining closed and inaccessible to self-pollen until after the style branches separate and become receptive to cross-pollination. This physical separation of the two style surfaces (inner and outer) with separate functions (receptive papillae and sweeping hairs) is key to the prevention of self-pollination. This leads us to wonder whether self-pollination is common in Goodeniaceae and Calyceraceae.

Bifid styles and pollination

Insect and bird pollination as well as self-pollination are well-known in Goodeniaceae (e.g. Elmore, 2008; Liao, 2008; Jabaily *et al.*, 2014), but not in Calyceraceae. The presence of nectar glands and pollenkitt along with the corolla shape in Calyceraceae seems to suggest that insects may be involved in pollination. In fact, Erbar (1993) pointed out that insects with a thin proboscis, such as butterflies, might be possible pollinators for *Acicarpha*. However, although a sporophytic incompatibility

system was indicated for the calyceracean alpine *Nastanthus patagonicus* (under the name *Nastanthus spathulatus*), wind pollination was eliminated as an option and no flying insect visitors were observed in this species (Ladd and Arroyo, 2009), only ants crawling among the florets were seen (Kalin Arroyo *et al.*, 1982). Ants are very unlikely to cause cross-pollination (Proctor *et al.*, 1996), and are very probably attracted by the typical nectaries of Calyceraceae (Fig. 8), which alternate with the base of the stamens' filaments. We examined ∼450 herbarium sheets for this family and none of the labels contained any information on pollinators for the family. Also, we did not observe pollinators when collecting specimens of *Acicarpha procumbens* and *A. tribuloides* in the field. We think that self-pollination must be difficult to prevent when the same papillae must change their status from non-receptive to receptive to pollen during secondary pollen presentation in a relatively short period of time. In fact, a high percentage of self-pollination (78·9 %) was established for *Moschopsis rosulata* (Kalin Arroyo and Squeo, 1990). In addition, hand cross-pollination experiments in this species yield a low percentage (3·17 %) of fertile seeds. We do not discard entomophilous pollination in Calyceraceae, but more studies are needed to ascertain the pollination type and specific pollinators in the family.

Thus, a plausible hypothesis is that the innovative divided style of Asteraceae coupled with other floral variations (e.g. diverse corolla colours, nectaries, gender strategies) could have augmented the reproductive success of the family. The bifid style with differentially receptive surfaces could have acted as a selective factor favouring shifts to a wide range of pollinators, including hummingbirds, which are common in the early branching lineages of Asteraceae (cf. Ramírez, 1992; Ezcurra, 2002; Katinas *et al.*, 2009).

CONCLUSIONS

A detailed analysis of the morphology of the styles of Goodeniaceae and Calyceraceae viewed within a phylogenetic framework allowed us to interpret and understand the origin of the bifid–bifacial style that is so prevalent in Asteraceae. Histochemistry of the papillae and venation of styles, analysed here for first time, allowed us to propose a hypothesis about style evolution in these families. The clear distinction between a temporal and a spatial papilla distribution in Asteraceae through style morphology and function and accompanied by changes in venation and histochemistry is one of the most important outcomes of this contribution. Initial style widening in the upper shaft of Calyceraceae is probably correlated with the occurrence of extensive branching of the veins in this area, a characteristic identified in Goodeniaceae. Further vein connation, branching reduction and elongation were necessary steps leading to the narrowing and bilobation of the style apex, key to stigma bifaciality. The presence of bilobed styles in a few Calyceraceae resembling those of the genus *Fulcaldea* of Barnadesioideae (Asteraceae), put in a phylogenetic context, are critical to our ideas about what could be initial attempts towards bilobation. This would support Bremer's (1987) hypothesis that the bilobed style is apomorphic for Asteraceae.

The division of the style and the resulting array of style shaft and arm features are reflected in many subtribes, tribes or groups of tribes of Asteraceae and have led to an impressive taxonomic diversification of this cosmopolitan family and to the development of variations in secondary pollen presentation mechanisms (cf. Yeo, 1993; Erbar and Leins, 2015*b*). The capitulum has been commonly proposed as the functional unit responsible for the great diversification and adaptation of Asteraceae but the acquisition of a bifid–bifacial style should be considered as another fundamental keystone for the family's evolutionary success.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Appendix S1: list of specimens analysed with voucher information.

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