

Verhuellia is a segregate lineage in Piperaceae: more evidence from flower, fruit and pollen morphology, anatomy and development

Marie-Stéphanie Samain^{1*}, Alexander Vrijdaghs², Michael Hesse³, Paul Goetghebeur¹, Francisco Jiménez Rodríguez⁴, Alexandra Stoll⁵, Christoph Neinhuis⁶ and Stefan Wanke⁶

¹Ghent University, Department of Biology, Research Group Spermatophytes, B-9000 Gent, Belgium, ²Katholieke Universiteit Leuven, Institute of Botany and Microbiology, Laboratory of Plant Systematics, Kasteelpark Arenberg 31, B-3001 Leuven, Belgium, ³University of Vienna, Institute of Botany, Department of Palynology and Structural Botany, A-1030 Vienna, Austria,

⁴Jardín Botánico Nacional Dr. Rafael M. Moscoso, Herbarium, Santo Domingo, Dominican Republic, ⁵Centro de Estudios Avanzados en Zonas Áridas – CEAZA, Casilla 599, Campus Andres Bello, Colina El Pino s/n., La Serena, Chile and

⁶Technische Universität Dresden, Institut für Botanik, D-01062 Dresden, Germany

*For correspondence. E-mail MarieStephanie.Samain@UGent.be

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• **Background and Aims** The perianthless Piperales, i.e. Saururaceae and Piperaceae, have simple reduced flowers strikingly different from the other families of the order (e.g. Aristolochiaceae). Recent molecular phylogenies proved *Verhuellia* to be the first branch in Piperaceae, making it a promising subject to study the detailed structure and development of the flowers. Based on recently collected material, the first detailed study since 1872 was conducted with respect to morphology, anatomy and development of the inflorescence, pollen ultrastructure and fruit anatomy.

• **Methods** Original scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy (LM) observations on *Verhuellia lunaria* were compared with those of Piperaceae, Saururaceae and fossils.

• **Key Results** The inflorescence is an indeterminate spike with sessile flowers, each in the axil of a bract, developing in acropetal, helical succession. Flowers consist of two (occasionally three) stamens with basifixed tetrasporangiate anthers and latrorse dehiscence by a longitudinal slit. The gynoeceum lacks a style but has 3–4 stigma branches and a single, basal orthotropous and unitegmatic ovule. The fruit is a drupe with large multicellular epidermal protuberances. The pollen is very small, inaperturate and areolate, with hemispherical microechinate exine elements.

• **Conclusions** Despite the superficial similarities with different genera of Piperaceae and Saururaceae, the segregate position of *Verhuellia* revealed by molecular phylogenetics is supported by morphological, developmental and anatomical data presented here. Unitegmatic ovules and inaperturate pollen, which are synapomorphies for the genus *Peperomia*, are also present in *Verhuellia*.

Key words: *Verhuellia lunaria*, Piperales, *Peperomia*, *Appomattoxia ancistrophora*, floral development, floral anatomy, fruit morphology, pollen morphology, unitegmatic ovule, inaperturate pollen.

INTRODUCTION

Verhuellia is a Piperaceae genus of three species, known from very few collections and localities on Cuba and Hispaniola (Haiti and Dominican Republic). It is a small, herbaceous, saxicolous perennial with monopodial architecture, whorled leaves and perianthless flowers organized in solitary lateral spikes (Fig. 1). These flowers, each subtended by a bract, consist of a gynoeceum with three to four stigmas and two tetrasporangiate stamens. With the exception of Schmitz (1872a, b), who only described some morphological characters based on herbarium specimens, detailed morphological, anatomical or developmental studies have never been conducted. This is probably due to the very limited number of collections and its absence from living collections and also to the presumed close relationship with the genus *Peperomia*.

The absence of some floral organs in the flowers of perianthless Piperales has recently re-enhanced the focus on the representatives of the sister families Saururaceae and Piperaceae,

presenting them as an interesting model for studying floral development and evolution (Jaramillo and Kramer, 2007; Arias and Williams, 2008; Madrid and Friedman, 2009). Furthermore, several phylogenetic studies in the order Piperales are now available as a robust framework to interpret character evolution (Wanke *et al.*, 2007a, b). With the exception of the two Piperaceae genera *Verhuellia* and *Manekia*, floral characters of nearly all genera have been extensively studied in earlier years (Tucker, 1975, 1976, 1979, 1980, 1981, 1982a, b, 1985; Liang and Tucker, 1989, 1990, 1995; Tucker *et al.*, 1993; Igersheim and Endress, 1998; Lei and Liang, 1998, 1999). In addition, Smith and Stockey (2007) studied the pollen of the four Saururaceae genera. Detailed observations on the fruit morphology in this family are lacking. In Piperaceae, pollen and fruit development have not been studied extensively despite the large number of species.

Verhuellia was generally considered to be part of or at least closely related to *Peperomia* (e.g. Tebbs, 1993), due to its



FIG. 1. General habit of *Verhuellia lunaria*, grown in greenhouse conditions.

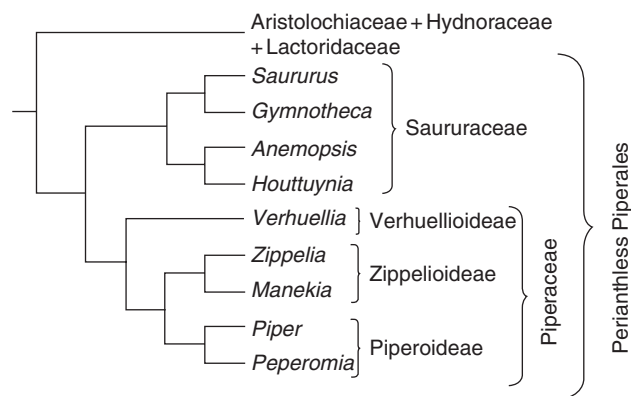


FIG. 2. Simplified cladogram of perianthless Piperales (after Wanke *et al.*, 2007b).

superficial similarities with this genus. However, Wanke *et al.* (2007b) recently showed that *Verhuellia* is sister to all other Piperaceae, a position formerly thought to be occupied by *Zippelia* and *Manekia* (Jaramillo *et al.*, 2004; Wanke *et al.*, 2007a). As a consequence, the traditional sub-division of the family Piperaceae into Piperioideae and Peperomioideae has been revised, resulting in three subfamilies congruent with the clades recovered by Wanke *et al.* (2007b): Verhuellioideae (with *Verhuellia*), Zippelioideae (with *Zippelia* and *Manekia*) and Piperioideae (with *Piper* and *Peperomia*) (Samain *et al.*, 2008). Figure 2 shows a summary cladogram of the perianthless Piperales.

Given the hypothesis that *Verhuellia* is sister to the four other Piperaceae genera (Wanke *et al.*, 2007b), intermediate characters between Piperaceae and Saururaceae could be expected. This is supported by the fact that *Zippelia begoniifolia*, placed in the next branching clade in Piperaceae (Jaramillo *et al.*, 2004; Wanke *et al.*, 2007a, b), also shows some similarities to Saururaceae (Igersheim and Endress, 1998). Tucker *et al.* (1993) and Liang and Tucker (1995) consider these characters as plesiomorphic. *Zippelia* has sometimes been considered a member of Saururaceae (e.g. Blume,

1830; Wu and Wang, 1957; Heywood, 1993) whereas it has never been questioned that *Verhuellia* belongs to Piperaceae.

The aims of this study are: (a) to present a detailed study on morphology, anatomy and development of the *Verhuellia* inflorescence, flower, pollen and fruit based on fresh material to overcome the lack of knowledge on this genus; and (b) to discuss the floral ontogenetic findings in the light of its new position in the family Piperaceae and in relation to available data of other perianthless Piperales, including the presumed fossil relative *Appomattoxia ancistrophora*.

MATERIALS AND METHODS

Plants of *Verhuellia lunaria* (Ham.) C.DC., the type species of the genus, originating from the Dominican Republic were cultivated in the Botanical Gardens of Ghent University and Dresden Technical University. Inflorescences, flowers and fruits of all stages were collected and preserved. Voucher specimens are deposited in the herbaria of Ghent University (GENT) and Dresden Technical University (DR).

Material for scanning electron microscopy (SEM) was prepared by fixation in Kew mix (53 % industrial methylated spirit, 37 % water, 5 % formaldehyde solution and 5 % glycerol), followed by dehydration and critical point drying with carbon dioxide in a BAL-TEC CPD 030 critical-point dryer. Subsequently, samples were fixed to aluminium stubs (Plano GmbH, Lünen, Germany) using a carbon adhesive tape (LEIT-TABS, Plano GmbH) and sputter-coated with gold (20 nm) under an argon atmosphere using an EMITECH K550 sputter-coater. Images were obtained with a LEO 420 scanning electron microscope at acceleration voltage of 15 kV.

Light microscopy (LM) samples were prepared in 70 % ethanol and subsequently gradually transferred to 100 % ethanol. The samples were then transferred to LR White Resin, hard grade (London Resin Company Ltd, Reading, UK) in a graded LR White Resin/ethanol series using solutions of 25/75, 50/50, 75/25, 100/0 resin/100 % ethanol for at least 5 h each. Subsequently, the samples were placed in a closed capsule filled with fresh resin and hardened at 60 °C for 48 h. Sections of 2 µm were made with a rotation microtome (Microm HM360, Walldorf, Germany) and subsequently stained with 0.1 % toluidine blue. The stained sections were fixed on microscopy slides using Eukitt® quick hardening mounting medium (Fluka Chemie GmbH, Buchs, Switzerland). Observations were done with a light microscope (Leitz Dialux 20, Van Hopplinyus, Brussels, Belgium) equipped with a camera (PixelINK PL-B622CF, Ottawa, Canada) with specially developed software (Microscopica v1.3, Orbicule, Leuven, Belgium).

For TEM pictures, pollen of *V. lunaria* was fixed in 50 % alcohol, embedded in Agar Low Viscosity Resin (Agar Scientific Ltd, Stansted, Essex, UK); ultrathin sections were post-stained using uranyl acetate–lead citrate, KMnO₄ and the Thiéry reaction for polysaccharides. Images were obtained with a Zeiss EM-900 transmission electron microscope. For SEM pictures, fixed *V. lunaria* pollen was rehydrated, critical-point dried and sputter-coated. Images were obtained with a JEOL JSM 6390 scanning electron microscope.

SEM and LM pictures were also taken from accessions of the genus *Peperomia* for comparison with *Verhuellia* morphology. This material was taken from the living collection of the Botanical Garden of Ghent University.

RESULTS

The inflorescence is initiated in the axil of one of the 3–5 whorled leaves on the stem (Figs 3A and 4A, B). Its primordium is surrounded by large, multicellular trichomes (Figs 4A, B and 5C). The inflorescence is an indeterminate spike with sessile flowers on a filiform axis (Fig. 4C). The inflorescence apex produces bracts in acropetal, helical succession (Figs 3A, 4A and 5A–C). Each flower primordium appears in the axil of a peltate bract, which originated considerably earlier (Figs 3A, 4A, 5A–D and 10F). The bract develops large trichomes at its margin, which are especially conspicuous during the young stages of the bract (Figs 3A, 4A, 5B, C and 6A). Subsequently, two lateral stamen primordia are formed, followed by the gynoecium, which appears as an annular structure (Figs 5D and 6A, B). Meanwhile, the bract increases in size, overarching the developing flower (Figs 3A, 4A and 5A–C).

Next, the stigma primordia become apparent on the upgrowing ovary wall (Figs 6A, B and 8A–C), followed by the differentiation of each stamen into a short filament and a basifixed anther (Fig. 6A). The anthers are tetrasporangiate (Figs 6A–D and 7A–E). The lateral stamens develop simultaneously. Exceptionally, a third stamen is formed adaxially (Fig. 6B). In immature stamens, the connective ends in a bulge in between the two thecae (Fig. 6A, B). This bulge consists of one large gland (Fig. 7E). At maturity, the stamen is

T-shaped as the connective grows out laterally between the pollen sacs in each theca, forming a broad, oblate, saddle-shaped connection between the thecae (Figs 6C, D and 7A–C). The remnant of the apical bulge, which was prominent in immature stamens, is still visible in mature stamens (Fig. 7B, C). The anthers dehisce laterally with a longitudinal slit (Fig. 7B, C). After dehiscence, the anther wall recurves (Fig. 7B, C). Shortly after dehiscence, the stamens are shed.

On the annular young gynoecium (Figs 6B and 8A) generally four stigma primordia develop: one abaxial, one adaxial and two lateral stigmas (Fig. 8B–D). Occasionally, only three stigma branches are present (Figs 6D and 8E). This variation in stigma number can occur within a single inflorescence. As long as the ovary is open, no ovule development is visible (Fig. 8B, C). The ovule is unitegmic, orthotropous and basal (Fig. 10A–C). The micropyle is situated at the apex of the ovule (Fig. 10C, D). The stigma branches become papillate in later development (Figs 7A–C and 8F). During maturation of the fruit, the outer surface of the ovary is covered by initially unicellular protuberances that undergo cell divisions (Figs 6C, D, 7C, 8D–F, 9A, B, E, F and 10A–C, E, F). The fruit develops into a drupe with a relatively thin mesocarp and a stony endocarp and large multicellular protuberances on its wall (Figs 9C–F and 10E, F). The drupe is situated on a short gynophore, which slightly elongates during maturation of the fruit (Fig. 10A, B, F).

The pollen grains are shed as very small spherical monads only approx. 8–10 µm in diameter (Fig. 11A, B). Pollen is inaperturate and areolate with evenly distributed hemispherical microechinate exine elements (Fig. 11A–D). These exine elements are separated by narrow channels, forming a negative reticulum (Fig. 11C, D). Sometimes, as an artificial result of

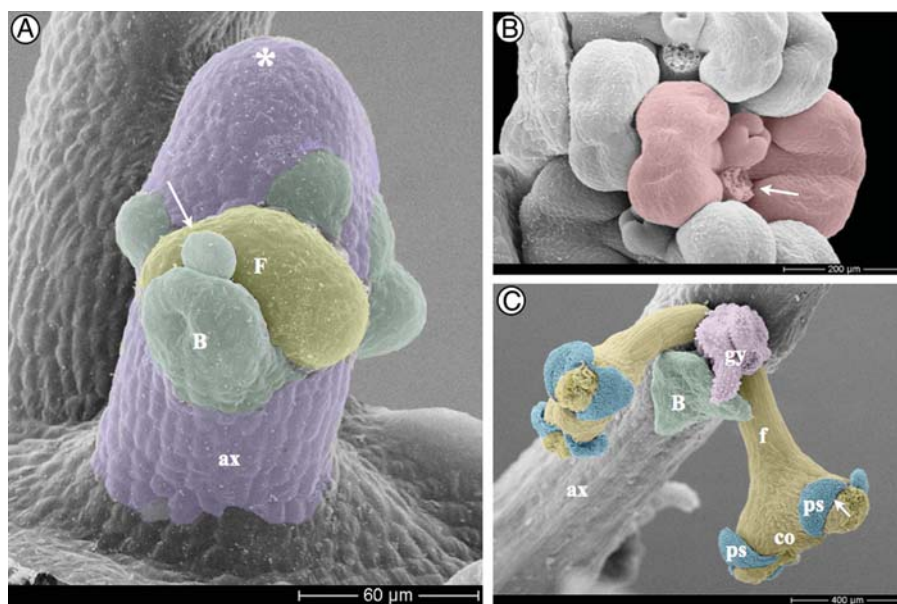


FIG. 3. SEM images of the most important floral structures in *Verhuellia lunaria* discussed in the text. (A) Young spike developing in the leaf axil. The axis is coloured blue, bracts and bract primordia are green and the young flower is yellow. The arrow points to a trichome on the adaxial side of the bract. (B) Detail of the spike with developing flowers. One flower, with its two developing stamens and its gynoecium in primordial stage, is coloured pink. The floral bract has been removed (arrowed). (C) Mature flower with green coloured bract, yellow stamens, with opened thecae in blue, and purple gynoecium. The arrow points to the tapetum that is visible because the anther wall has recurved. ax, axis of the spike; B, bract; co, connective; F, flower primordium; f, filament; gy, gynoecium; ps, pollen sac; *, apex of the spike.

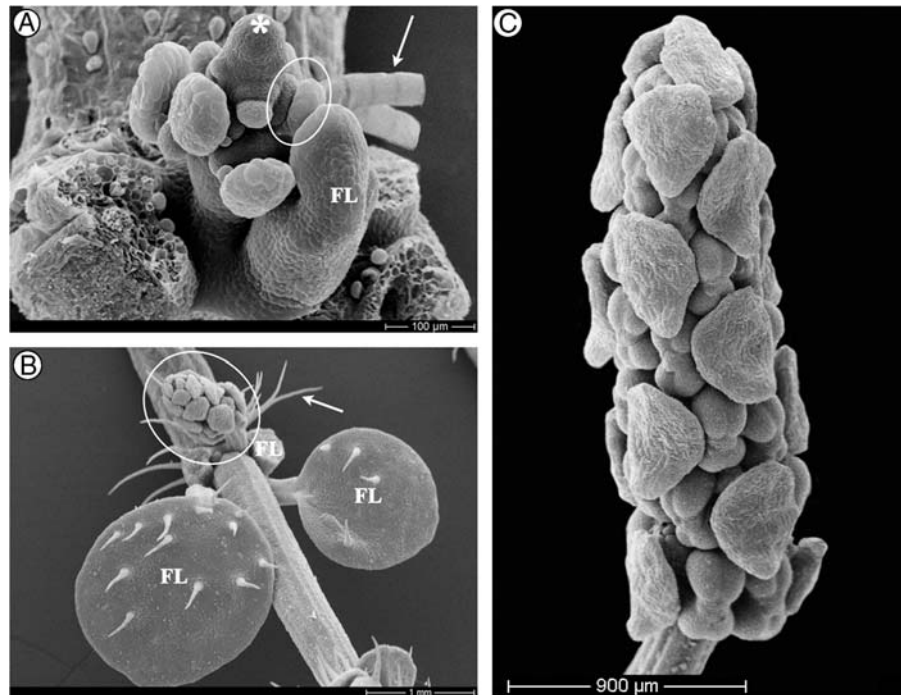


FIG. 4. SEM images of inflorescence development in *Verhuellia lunaria*. (A) Young spike with developing floral bracts with young flowers in their axils (one young flower with its subtending bract is circled). A young leaf develops next to the spike. Multicellular trichomes (arrowed) surround the spike. The older leaves on the main stem are removed. (B) View of a stem node with two older leaves still present and the immature spike (encircled) in the axil of the removed leaf. Multicellular trichomes (arrowed) surround the spike. (C) Lateral view of the maturing spike shortly before anther dehiscence. The peltate bracts partially cover the nearly mature anthers which, in turn, cover the young gynoecia. FL, foliage leaf; *, apex of the spike.

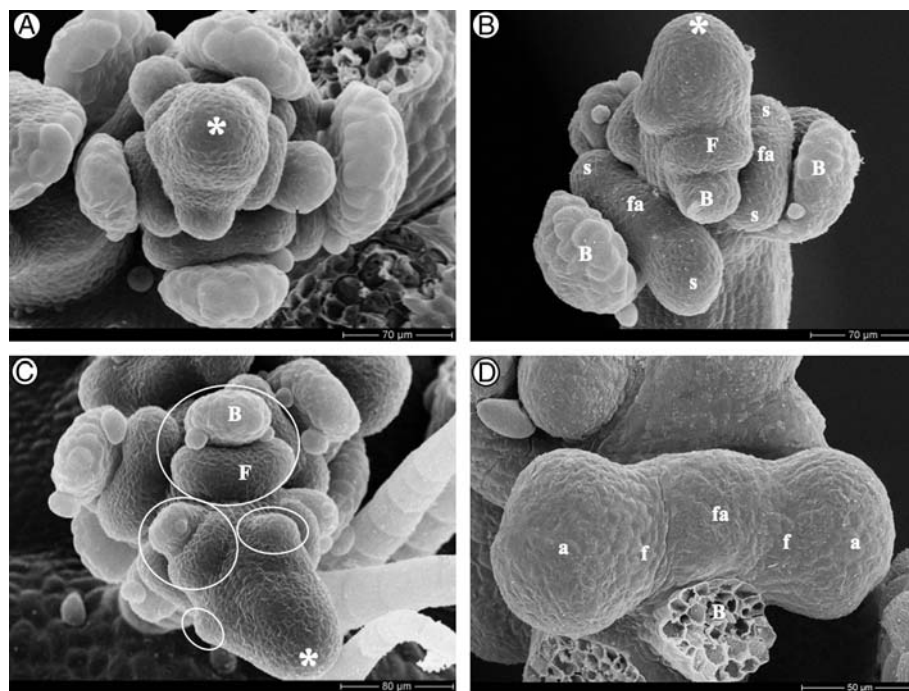


FIG. 5. SEM images of inflorescence and floral development in *Verhuellia lunaria*. (A) Apical view of the spike apex and bract and young flowers in successive stages of development. (B) Lateral view of the spike apex with developing bracts partially covering flowers at successive stages of development. In the most developed floral primordia visible, two stamen primordia and the gynoecium primordium are present. On the margins of the bracts, trichomes are present. (C) Lateral view of a spike at the same developmental stage as in B. The youngest flowers and bracts are circled. Multicellular trichomes are present at the base of the spike. (D) Apical view of a developing flower with two lateral stamens and the gynoecium primordium (floral bract removed). a, anther; B, bract; F, flower; f, filament; fa, floral apex; s, stamen; *, apex of the spike.

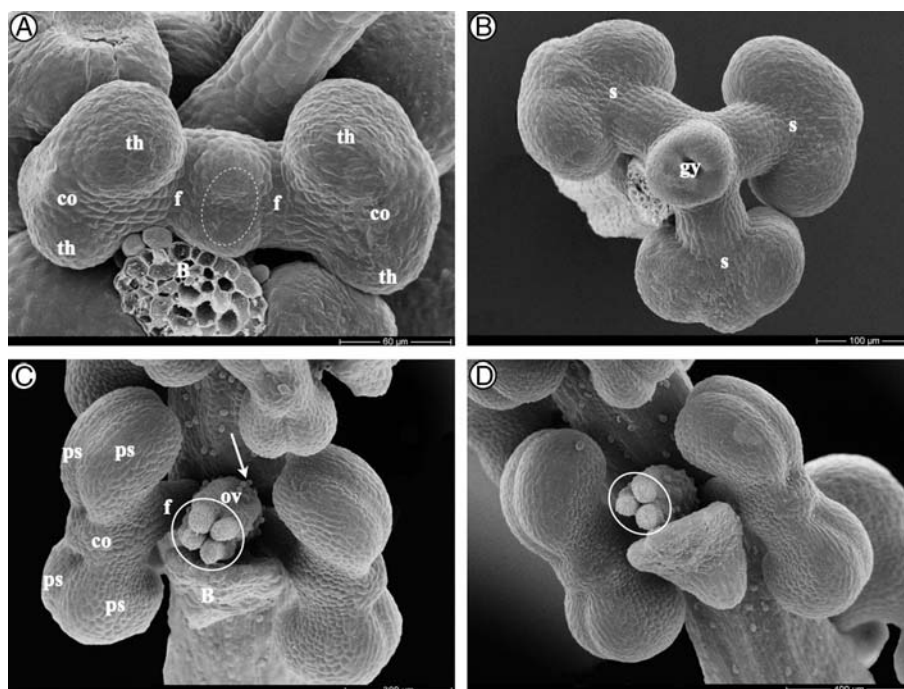


FIG. 6. SEM images of floral development in *Verhuellia lunaria*. (A) Flower with bract removed and developing tetrasporangiate anthers. The two thecae of each anther are visible and the connective is beginning to develop as a small bulge. On top of the developing ovary, adaxial and abaxial stigma primordia are apparent (encircled). Trichomes are visible on the remaining base of the bract. (B) Close-up of developing flower before closure of the ovary, with the subtending bract removed, three stamens and possibly three stigma primordia. The third stamen is positioned opposite the bract, which has been removed. (C) Almost mature flower with four stigmas (circled) and its subtending bract. In the stamens, the connective has broadened and its distal part is still recognizable as a small bulge in between the two thecae. The arrow points to a developing protuberance on the ovary wall. (D) Flower with three stigmas (circled) at the same developmental stage as in C. B, bract; co, connective; f, filament; gy, gynoecium; ov, ovary; ps, pollen sac; s, stamen; th, theca.

pollen expansion, these channels become conspicuously broader, and in such cases one or more apertural regions are mimicked (Fig. 11B, C). However, the absence of any intine thickening in ultrathin sections demonstrates the absence of apertures (Fig. 12A, B). The pollen wall consists of an ectexine (a massive and prominent tectum with very low columellae and a thin foot layer), an evenly thin endexine and an evenly thin intine (Fig. 12A, B). Extremely small very inconspicuous orbicules have been observed (not shown).

DISCUSSION

Wanke *et al.* (2007b), based on molecular data, showed that *Verhuellia* and *Peperomia* are not closely related, and this is supported by this study. Our morphological, anatomical and ontogenetic observations of flower, pollen and fruit in *V. lunaria* show that the genus is a clearly distinct lineage within Piperaceae and perianthless Piperales. An overview of important characters for Piperaceae and Saururaceae is given in Table 1.

The acropetal, helical succession of the initiation of bracts subtending the flowers in *Verhuellia* is comparable with the inflorescence development of all other Piperaceae studied. As in all other Piperaceae, there is a shift in position of the floral primordia with respect to their subtending bract as well as a considerable plastochron between the initiation of the bract and the floral primordium (Tucker, 1980, 1982a, b; Liang and Tucker, 1995). In contrast, Saururaceae have a

so-called common floral and bract primordium, which is a synapomorphy for the family (Tucker, 1975, 1981, 1985; Liang and Tucker, 1989). In many Piperaceae and Saururaceae investigated, trichomes around or on the developing and mature spikes as well as on the bracts and gynoecia occur in a wide range of shapes and sizes (unicellular, multicellular, pearl glands, etc.) (e.g. Tucker, 1975, 1976, 1982a, b; Fig. 11A, B). The function of these trichomes remains unknown. However, in his study of *Peperomia magnoliifolia*, Vogel (1998) suggested that the bract trichomes attract pollinators through secretion of small quantities of sugars.

All *Verhuellia* flowers observed are bisexual. However, the stamens are shed relatively soon after anthesis, which could explain why de Candolle (1866) reported 'female' flowers at the base of the inflorescence (see also Schmitz, 1872a). In addition, de Candolle (1866) described hermaphrodite flowers in the middle of the inflorescence and male flowers at the apex. However, this may be based on a misinterpretation as the flowers develop in acropetal succession: the stamens are already shed in the basal 'female flowers', while they still cover the small, developing gynoecia in the distal 'male flowers'. The arrangement of floral organs, number and order of initiation are highly variable among the perianthless Piperales investigated (see all abovementioned references, especially the floral diagrams in Tucker *et al.*, 1993; Liang and Tucker, 1995, and the ancestral state reconstruction in Wanke *et al.*, 2007b). Nevertheless, these characters are usually constant within Piperaceae and more or less variable

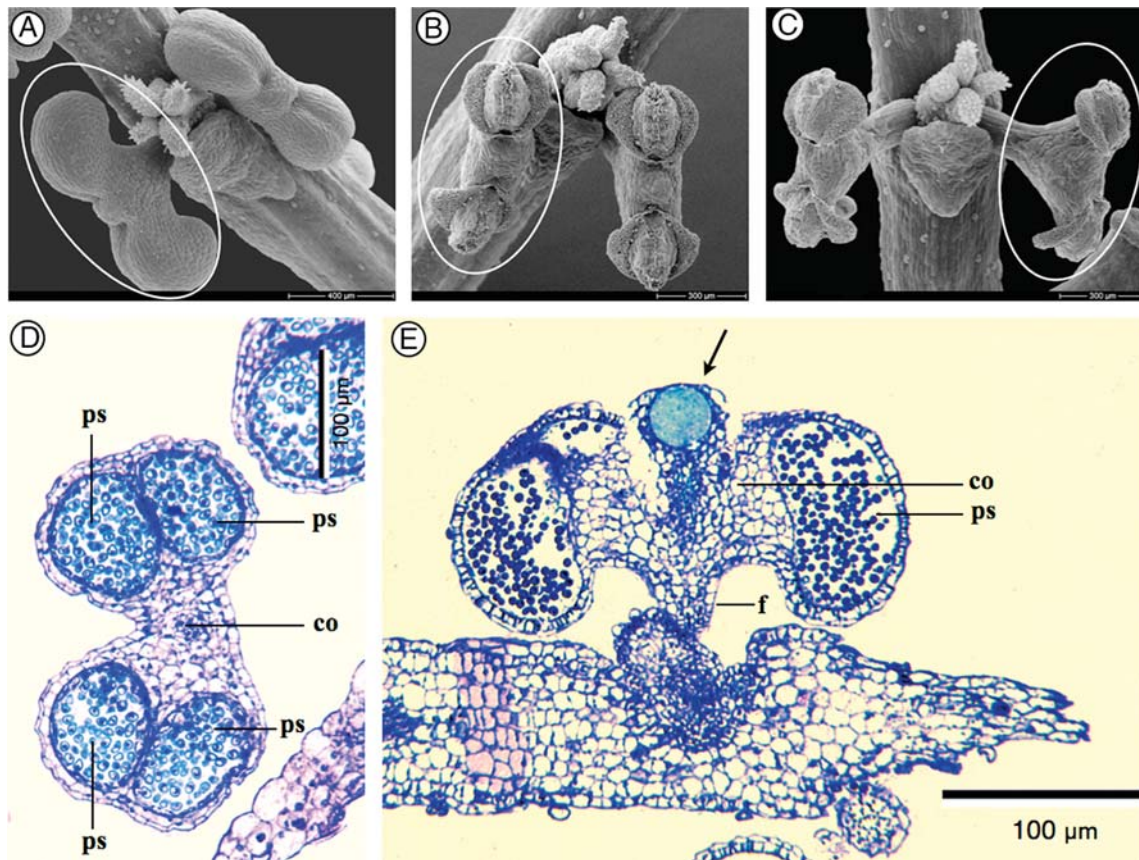


FIG. 7. SEM and LM images of floral development in *Verhuellia lunaria*. (A–C) SEM images of stamens at anthesis from early (A) to late (C). One entire stamen is encircled. (A) Flower with its subtending bract before anther dehiscence. The gynoecium has four receptive stigmas. (B) Flower with its subtending bract after anther dehiscence. The anther wall has recurved, exposing the tapetum. The connective is broadening and flattening. (C) Flower with bract after anther dehiscence and with four withering stigmas. The connective forms a broad, oblate, saddle-shaped connection between the thecae. (D) Transverse section through an anther showing the four pollen sacs. (E) Longitudinal section through a flower and inflorescence axis; in each stamen, one of the pollen sacs is visible. The connective ends in a bulge in between the two thecae and this bulge contains a single large oil cell. co, connective tissue; f, filament; ps, pollen sac.

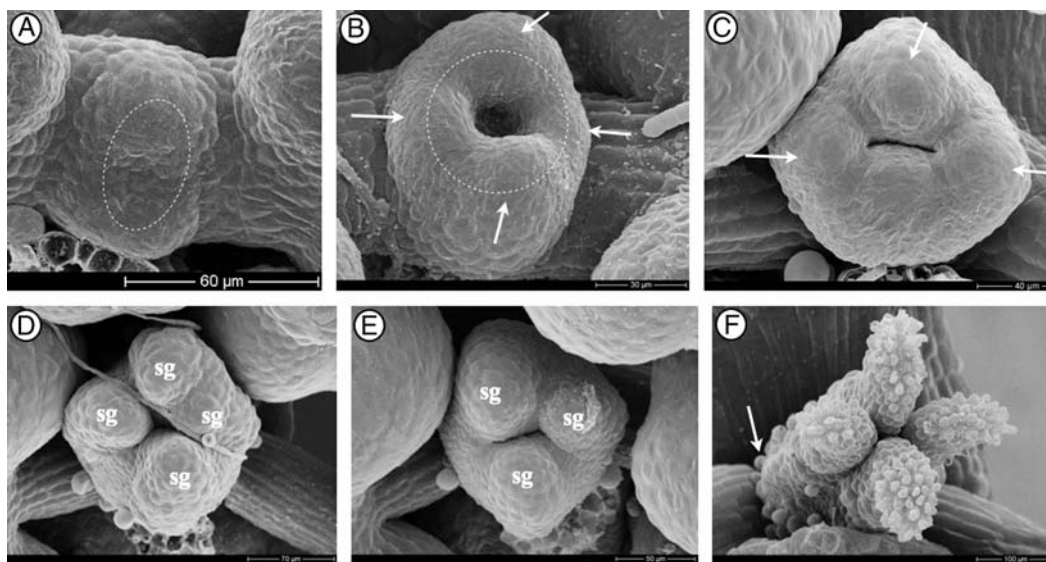


FIG. 8. SEM images of gynoecium development in *Verhuellia lunaria*. (A) The adaxial and abaxial stigma branches start to develop from the annular gynoecium base (circled). (B) Beginning closure of the ovary (encircled). The adaxial stigma branch develops first, followed by the two lateral ones. The abaxial stigma branch is the last to develop. The four stigma branches are arrowed. (C) Ovary closed. The three most developed stigma branches are arrowed. (D) All four stigma branches are distinct. (E) Gynoecium with three stigmas at the same stage as in (D). (F) Mature gynoecium with four recurved receptive stigmas with a papillose surface. One multicellular protuberance is arrowed. sg, stigma branch.

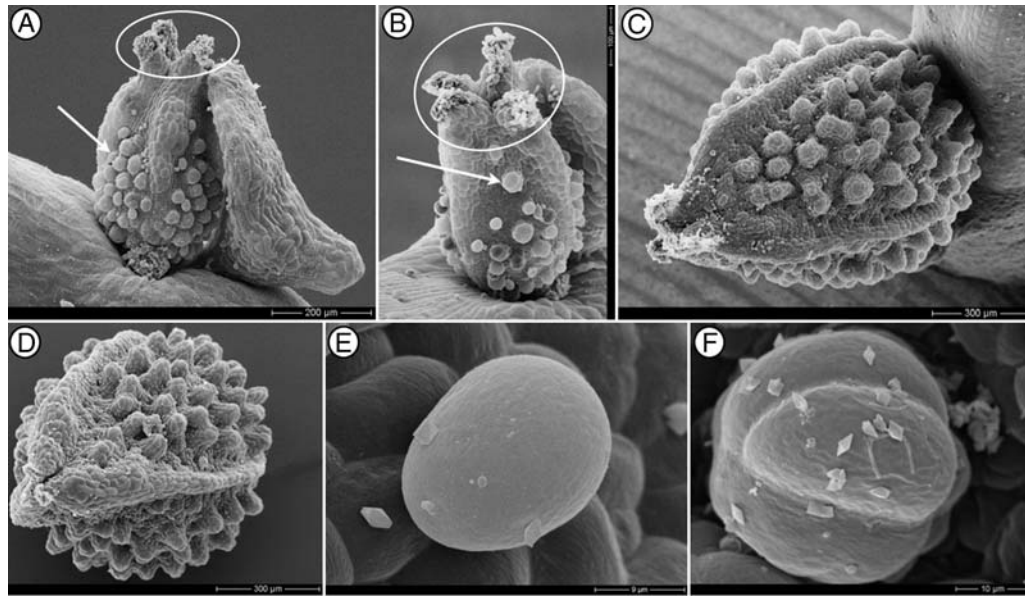


FIG. 9. SEM images of fruit development in *Verhuellia lunaria*. (A) Lateral view of immature fruit with bract; the four papillate stigmas are still visible (circled) and each stigma branch is decurrent on the fruit wall as a longitudinal rim. The initially single-celled wart-like protuberances on the fruit wall have started to divide (arrowed). (B) Lateral view of immature fruit with four papillate stigmas (circled) and bract, seen from the adaxial side. One protuberance is arrowed. (C) Mature fruit with three stigmas and three rims. The fruit is still attached to the inflorescence axis and the multicellular protuberances on the fruit wall are fully developed. (D) Mature fruit, fallen from the inflorescence axis. (E) Detail of a single-celled fruit wall protuberance. (F) Detail of a mature protuberance after cell division.

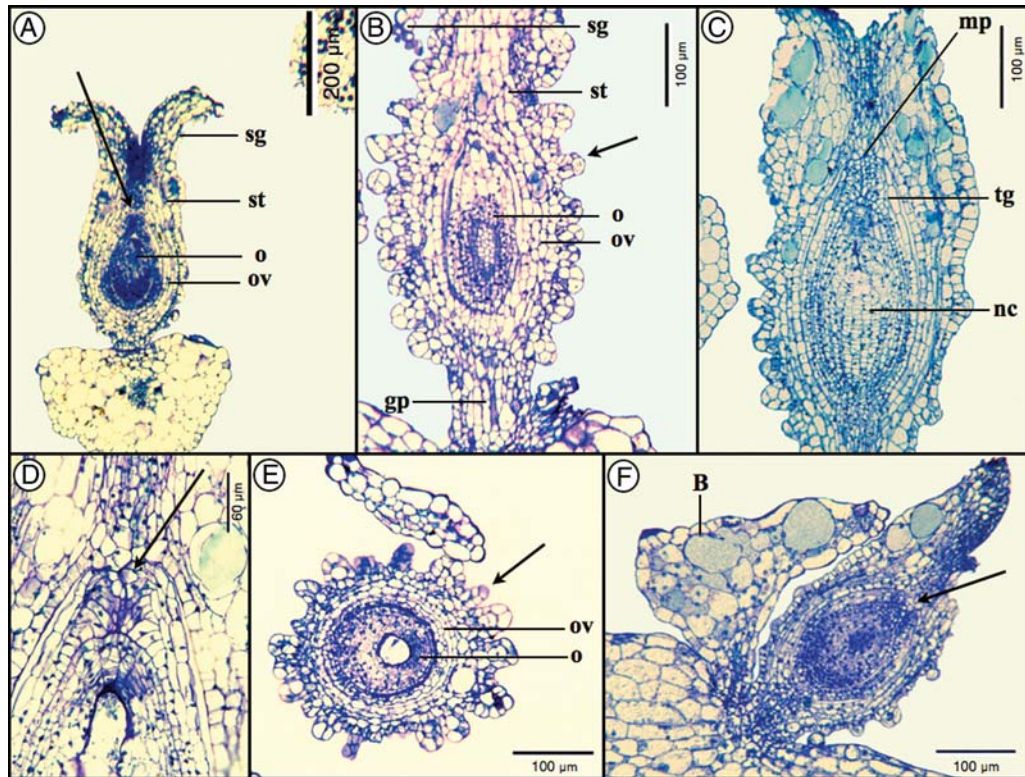


FIG. 10. LM images of sections through developing gynoecium and fruit of *Verhuellia lunaria*. The stamens are shed. (A) Longitudinal section through the mature gynoecium; two stigmatic branches are visible. The micropylar area is arrowed. The gynoecium stands on a short gynophore on the axis of the inflorescence (in transverse section). (B) Longitudinal section through the ripening fruit with the single ovule. The gynophore has elongated and the fruit wall protuberances are fully developed (arrowed). (C) Detail of a ripening fruit showing the single central ovule with micropyle formed by the single integument. (D) Detail of the micropylar area (micropyle arrowed), showing the single integument and the apical part of the nucellus. (E) Transversal section through mature fruit, with the single ovule. One of the multicellular protuberances on the fruit wall is arrowed. (F) Maturing fruit in the axil of the bract. Large glandular cells are present in the bract and the stigmatic area. The arrow points to the micropyle. B, bract; gp, gynophore; mp, micropyle; o, ovule; ov, ovary; nc, nucellus; sg, stigma; st, stigma branch; tg, integument.

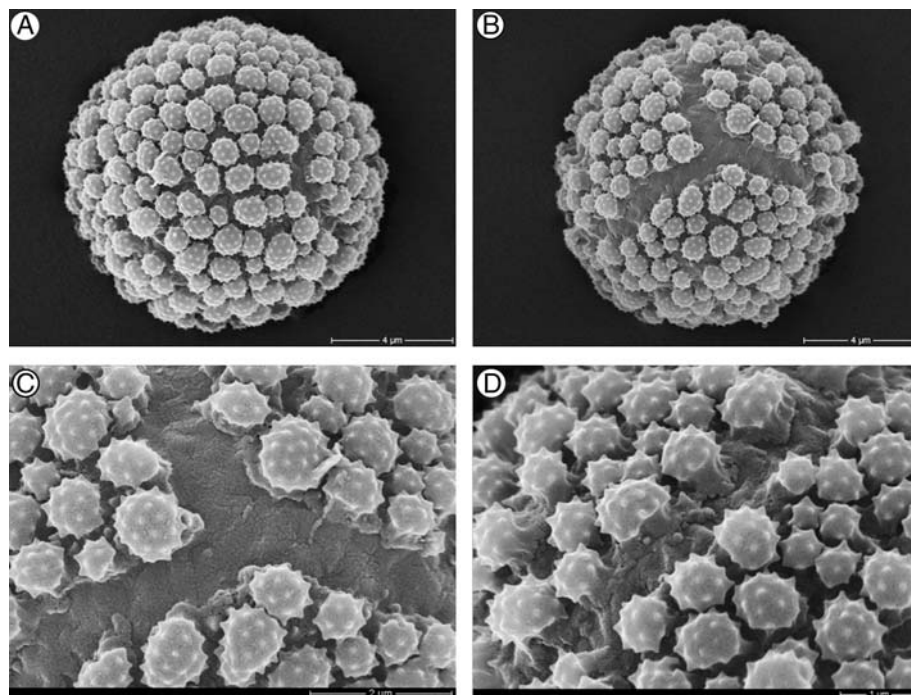


FIG. 11. SEM images of pollen morphology of *Verhuellia lunaria*. (A) Pollen grain with evenly distributed isolated microechinate exine elements. Note that the channels are uniformly very narrow. (B) Pollen grain with artificially broad channels between the microechinate elements, mimicking a furrow-like aperture. (C) Detail of the pollen surface, with microechinate exine elements and a channel mimicking an aperture. (D) Detail of the pollen surface with a very narrow channel.

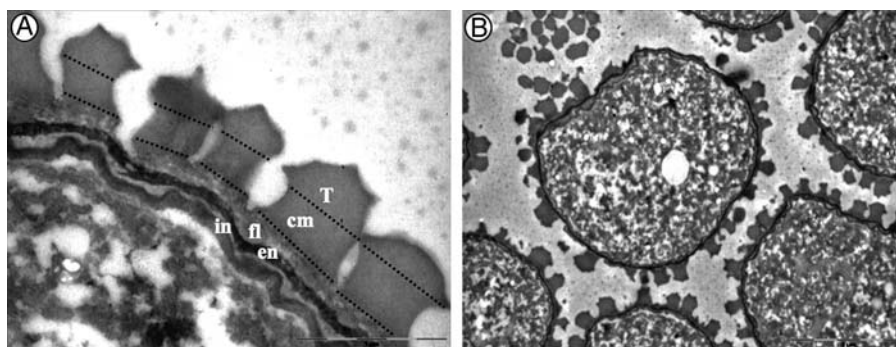


FIG. 12. TEM images of pollen ultrastructure of *Verhuellia lunaria*. (A) Pollen wall stratification: with ectexine (prominent tectum, short columellae, thin foot layer), meandering highly electron-dense endexine and medium-dense, evenly thin intine. (B) Pollen in cross-section. The intine (this is the layer below the highly electron-dense endexine) is meandering, however never distinctively thickened, thus indicating an inaperturate condition. cm, columella; en, endexine; fl, foot layer; in, intine; T, tectum.

in Saururaceae. However, being a member of Piperaceae, *Verhuellia* proves to be an exception to this rule as (a) the number of stamens and stigma branches can vary between 2 and 3, and 3 and 4, respectively (Fig. 6A–D); (b) the initiation sequence of the stigma branches is variable (Fig. 8B vs. C); and (c) in the case of three stigma branches, their position is not fixed (Figs. 6D vs. 8E). In contrast, in *Gymnotheca* (Saururaceae), the number of stigma branches and stamens can also vary but it usually is the abaxial stigmatic branch that is missing. In addition, the abaxial median stamen is also usually absent in these flowers (Liang and Tucker, 1989).

Despite this aberrant floral development in *Verhuellia*, the zygomorphic symmetry of its flowers is similar to that of all other perianthless Piperales (Figs 7B, C and 11A, B). The genus also shares the pairwise initiation of stamens and stigma branches and the simultaneous development of each pair of organs with most other Piperaceae and Saururaceae (Fig. 5D, although the two stamens in *Peperomia* as well as the second stamen pair in some *Piper* sometimes show disparity in size throughout development, Fig. 13B; Tucker 1980, 1982). The two stamens arise at the same position as the first two stamens in *Piper* and the only two stamens in *Peperomia* and a few other *Piper* species (Figs 7A–C and

TABLE 1. Overview of the most important characters of the five genera of Piperaceae and its sister family Saururaceae (from Samain, 2008)

	<i>Verhuellia</i>	<i>Manekia</i>	<i>Zippelia</i>	<i>Piper</i>	<i>Peperomia</i>	Saururaceae
Number of species	3	4–5	1	± 2000	± 1600	4 genera/6 species
Distribution	Cuba and Hispaniola	Central and South America	Southeast Asia	Pan-tropical with highest diversity in America and Asia	Pan-tropical with highest diversity in America	East Asia and North America
Life form	Perennial	Perennial	Perennial	Perennial	Perennial, rarely annual	Perennial
Growth form	Terrestrial; herbaceous	Terrestrial; woody root climber	Terrestrial; small shrub	Terrestrial; woody (small trees, shrubs, lianas), rarely herbaceous	Epiphytic or terrestrial; herbaceous	Terrestrial (sometimes aquatic); herbaceous
Androecium	Stamens 2, tetrasporangiate	Stamens 4, tetrasporangiate	Stamens 6, tetrasporangiate	Stamens (1–) 2–6 (–10), tetrasporangiate	Stamens 2, bisporangiate	Stamens 3–8, adnate to gynoecium, tetrasporangiate
Gynoecium	Carpels 3–4, ovary unilocular	Carpels 4–5, ovary unilocular	Carpels 3–4, ovary unilocular	Carpels 2–4, ovary unilocular	Carpels 1 (–2), ovary unilocular	Carpels 3–4, ovary unilocular
Number of ovules	1	1	1	1	1	(1)2–many
Number of integuments	1	2	2	2	1	2
Pollen	Inaperturate	Monosulcate	Monosulcate	Monosulcate	Inaperturate	Monosulcate, trichotomosulcate (<i>Houttuynia</i>)
Fruit	Drupe	Drupe	Drupe	Drupe	Drupe	Capsule

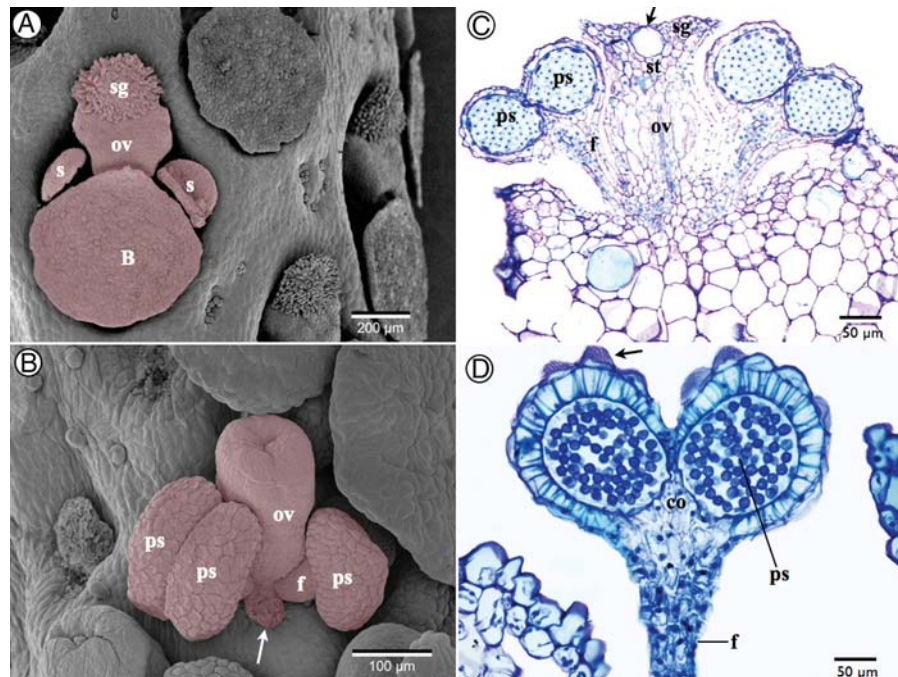


FIG. 13. SEM and LM images of inflorescence and flowers of *Peperomia*. (A) Lateral view of a part of the inflorescence of *P. trifolia* with flowers partially sunken in pits in the spadix rachis. One flower and its subtending bract are coloured pink. The anthers are bisporangiate and the gynoecium has one apical penicillate stigma. (B) Close-up of a flower of *P. wolfgang-krahnii* (pink). The annular ovary wall is still rising. The floral bract has been removed (arrowed). (C) Longitudinal section of a flower of *P. macrostachya*, with two lateral bisporangiate stamens and a cylindric ovary. A conspicuous gland (arrowed) is present near the stigmatic area. (D) Longitudinal section through a stamen of *P. prostrata* showing the two pollen sacs with the pollen grains. The surface of the anther wall is characterized by cuticular ridges (arrow). B, bract; f, filament; g, gynoecium; ov, ovary; ps, pollen sac; s, stamen; sg, stigma.

13A, B; Tucker, 1980, 1982; Lei and Liang, 1998). The development of a flower with three stamens has not been observed, as only one mature flower with three stamens was captured (Fig. 6B). However, it can be assumed that this third adaxial

stamen in the median sagittal plane does not develop exactly at the same time as the two lateral stamens, but slightly later, similar to the third stamen in *Houttuynia* (Saururaceae; Tucker, 1981) and in the dioecious species of *Piper* (Lei and

Liang, 1998), which occupies the same position in the flower. Similar to most *Piper* species, the anthers of *Verhuellia* are tetrasporangiate and dehisce laterally with longitudinal slits, whereas *Peperomia* anthers are bisporangiate and open extrorsely with longitudinal slits (Fig. 7B, C vs. Fig. 13A–D). The laterally extended connective is not present in *Peperomia* (Fig. 11C, D).

In *Verhuellia*, as well as in *Zippelia*, *Piper* and *Saururaceae*, the gynoecium begins development as an annular structure that could be the result of a congenital fusion of several carpels. Another possible explanation could be a complete ontogenetic reorganization resulting in an ontogenetically acarpellate gynoecium primordium. In both interpretations, the meristems from which the stigma branches develop can be considered to be carpel tips. In the second interpretation, the stigma primordia then can be seen as remnants of congenitally fused carpels. The upper part of the carpels can still be seen in the three or four rims on the fruit (Fig. 9A–D). In *Peperomia*, the ovary also originates from an annular primordium, but this can be understood as the ascidiate base of a single carpel (Fig. 13B; Tucker, 1980).

Similar to all other Piperaceae, the gynoecium is superior and unilocular with a single basal ovule (Igersheim and Endress, 1998). As in all other perianthless Piperales studied, the ovule is orthotropous. The ovules of *Verhuellia* and *Peperomia* are characterized by a single integument, in contrast to the other representatives of the family which are bitegmic (Igersheim and Endress, 1998). This can result in the formulation of two hypotheses about the origin and evolution of unitegmy and bitegmy in Piperaceae: (1) the ovule of ancestral Piperaceae was unitegmic and bitegmy evolved in the common ancestor of Zippelioideae and in *Piper* and underwent a reversal to unitegmy in *Peperomia* or (2) ancestral Piperaceae were characterized by bitegmic ovules and unitegmy evolved independently in *Verhuellia* and *Peperomia*. Given that the outgroups to Piperaceae are bitegmic, hypothesis 2, which assumes two origins of unitegmic ovules (two steps), is clearly more parsimonious than hypothesis 1, which requires one origin of unitegmic ovules at the base of the perianthless Piperales, followed by a reversal to bitegmy in the common ancestor of Zippelioideae and Piperioideae, and another reversal to unitegmy in *Peperomia* (three steps).

In *Verhuellia*, a young gynoecium shows some superficial similarities to that of *Zippelia* and *Saururus*, including the presence of four stigma branches and the verrucose surface of the outer wall (Fig. 9A–D). However, the protuberances on the ovary of *Zippelia* at anthesis develop into glochidiate hairs (Liang and Tucker, 1995), whereas the ovary of *Saururus* is characterized by warty ridges (Tucker, 1976), showing no similarity to the multicellular protuberances on the ovary wall of *Verhuellia*. Another character shared by *Verhuellia*, *Zippelia* and *Saururus* is the four recurved free papillate stigmas.

Large multicellular protuberances on the fruit wall of *Verhuellia* were also observed by Schmitz (1872b) and are unique for the genus. The fruit wall of many *Peperomia* species, in contrast, is also provided with protuberances, but these are sticky and unicellular papillae. It can be supposed that the protuberances have a function in epizoochory, but histochemical studies and detailed field observations are required to give conclusive statements about this.

Pollen of *Verhuellia* resembles *Peperomia* pollen in being inaperturate and very small. However, *Verhuellia* pollen is distinctively characterized by its ornamentation: evenly distributed, uniquely formed exine elements consisting of more-or-less hemispherical microechinate aggregates (Fig. 11A–D, resembling mace-heads, the medieval cutting weapons). Pollen of *Peperomia* is characterized by a less conspicuous ornamentation with unevenly shaped verrucae with few, tiny microechini (Fig. 14A, B). The inaperturate pollen of *Verhuellia* and *Peperomia* contrasts with the sulcate pollen in other perianthless Piperales (see Table 1). Remarkably, pollen of the genus *Aristolochia*, another member of the Piperales, is also inaperturate, whereas the pollen of its closest relatives are (mono)sulcate (Asaroideae, Hydnoraceae and *Lactoris*). Hence, parallel to the origin and evolution of unitegmy in Piperaceae, two hypotheses about the occurrence of inaperturate pollen in Piperales can be made: (1) ancestral Piperales pollen was inaperturate and the various aperturate morphologies have evolved in the different groups and (2) ancestral Piperales had a particular type of aperturate pollen and inaperturate pollen and the various aperturate pollen types developed in the different Piperales lineages. The interpretation of their evolution is somewhat uncertain because of uncertainty on the positions of *Lactoris* and Hydnoraceae, but, given the sulcate condition in Canellales,

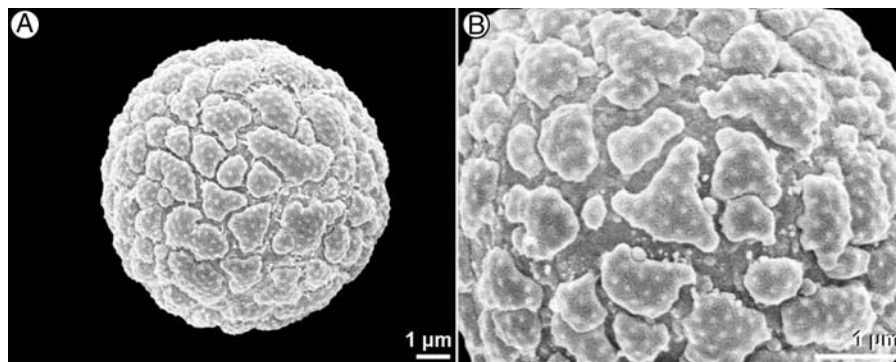


FIG. 14. SEM images of pollen morphology of *Peperomia rubella*. (A) Pollen grain with verrucate ornamentation; the verrucae are covered with few, tiny microechini. (B) Close-up of the pollen surface. Note the different size of verrucae.

Saururaceae and most Piperaceae, hypothesis 2 is probably much more parsimonious than loss of the sulcus at the base of Piperales followed by several reappearances within the order. In basal magnoliids inaperturate pollen is infrequent, however not rare; beside the mentioned examples it is found also in, for example, Laurales (Furness *et al.*, 2002). Apart from the difference in pollen ornamentation, *Verhuellia* also contrasts with *Peperomia* in the presence of orbicules. Very inconspicuous orbicules have been observed on the tapetum of *Verhuellia* whereas orbicules are not known from *Peperomia*.

Finally, the very distinct ornamentation of *Verhuellia* pollen allows comparison with presumed fossil members of Piperales. According to Friis *et al.* (2006), *Appomattoxia ancistrophora* is most closely related to members of Piperales. However, Doyle and Endress (2010) state in a phylogenetic study of a morphological data set for extant basal angiosperms to assess the relative parsimony of placements of early angiosperm fossils that *Appomattoxia* is more parsimoniously placed near Chloranthaceae or *Amborella* than in Piperales. Smith and Stockey (2007) already showed that *Appomattoxia* pollen differs from Saururaceae pollen. Pollen of *A. ancistrophora* also differs from *Verhuellia* pollen in dimension, aperture condition, pollen wall stratification and pollen ornamentation (Friis *et al.*, 1995). It is much larger (16–19 µm), it is sulcate (*Verhuellia* pollen is inaperturate), it has a thick foot layer and a continuous tectum (*Verhuellia* shows a thin foot layer and a strictly discontinuous tectum) and its ornamentation is verrucate–rugulate and microechinate.

To conclude, observations on the morphology, anatomy and development of inflorescence, flower, pollen and fruit of *V. lunaria* confirm that the genus is a clearly distinct lineage within Piperaceae. It does not show any intermediate characters between Saururaceae and Piperaceae and there are only superficial similarities with any other specific group within perianthless Piperales. The unitegmatic ovule and the inaperturate pollen of *Verhuellia* and *Peperomia* appear to be either an independent parallel evolution or a reversal to the ancestral state. *Appomattoxia ancistrophora* pollen is different from the pollen of *Verhuellia*.

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LITERATURE CITED

- Arias T, Williams JH. 2008. Embryology of *Manekia naranjoana* (Piperaceae) and the origin of the tetrasporic, 16-nucleate female gametophytes in Piperales. *American Journal of Botany* 95: 272–285.
- Blume KL. 1830. *Zippelia*. In: Roemer JJ, Schultes JA, eds. *Systema Vegetabilium* 7. Stuttgart: Cottae, 1614, 1651.
- de Candolle C. 1866. Mémoire sur la famille des Piperacées. *Mémoires de la Société de Physique et d'Histoire Naturelle de Genève* 18: 219–248.
- Doyle JA, Endress PK. 2010. Integrating early Cretaceous fossils into the phylogeny of living angiosperms: Magnoliidae and eudicots. *Journal of Systematics and Evolution* 48: 1–35.
- Friis EM, Pedersen KR, Crane PR. 1995. *Appomattoxia ancistrophora* gen. et sp. nov., a new Early Cretaceous plant with similarities to *Circaeaster* and extant Magnoliidae. *American Journal of Botany* 82: 933–943.
- Friis EM, Pedersen KR, Crane PR. 2006. Cretaceous angiosperm flowers: innovation and evolution in plant reproduction. *Palaeogeography, Palaeoclimatology, Palaeoecology* 232: 251–293.
- Furness CA, Rudall PJ, Sampson FB. 2002. Evolution of microsporogenesis in angiosperms. *International Journal of Plant Sciences* 163: 235–260.
- Heywood V. 1993. *Flowering plants of the world*, 2nd edn. Oxford: Oxford University Press.
- Igersheim A, Endress PK. 1998. Gynoecium diversity and systematics of paleoherbs. *Botanical Journal of the Linnean Society* 127: 289–370.
- Jaramillo MA, Kramer EM. 2007. Molecular evolution of the petal and stamen identity genes, APETALA3 and PISTILLATA, after petal loss in the Piperales. *Molecular Phylogenetics and Evolution* 44: 598–609.
- Jaramillo MA, Manos PS, Zimmer EA. 2004. Phylogenetic relationships of the perianthless Piperales: reconstructing the evolution of floral development. *International Journal of Plant Sciences* 165: 403–416.
- Lei LG, Liang HX. 1998. Floral development of dioecious species and trends of floral evolution in *Piper* sensu lato. *Botanical Journal of the Linnean Society* 127: 225–237.
- Lei LG, Liang HX. 1999. Variations in floral development in *Peperomia* (Piperaceae) and their taxonomic implications. *Botanical Journal of the Linnean Society* 131: 423–431.
- Liang HX, Tucker SC. 1989. Floral development in *Gymnotheca chinensis* (Saururaceae). *American Journal of Botany* 76: 806–819.
- Liang HX, Tucker SC. 1990. Comparative study of floral vasculature in Saururaceae. *American Journal of Botany* 77: 607–623.
- Liang HX, Tucker SC. 1995. Floral ontogeny of *Zippelia begoniaefolia* and its familial affinities: Saururaceae or Piperaceae? *American Journal of Botany* 82: 681–689.
- Madrid EN, Friedman WE. 2009. The developmental basis of an evolutionary diversification of female gametophyte structure in *Piper* and Piperaceae. *Annals of Botany* 103: 869–884.
- Samain MS. 2008. *Tackling Pandora's Box. Order out of chaos in the giant genus Peperomia* (Piperaceae). PhD thesis, Ghent University, Belgium.
- Samain MS, Wanke S, Mathieu G, Neinhuis C, Goetghebeur P. 2008. *Verhuellia* revisited – unravelling an intricate taxonomical history and a new subfamilial classification of Piperaceae. *Taxon* 57: 583–587.
- Schmitz F. 1872a. Der morphologische Aufbau von *Verhuellia* Miq. *Flora* 26: 402–415.
- Schmitz F. 1872b. Der morphologische Aufbau von *Verhuellia* Miq. *Flora* 27: 417–424.
- Smith SY, Stockey RA. 2007. Pollen morphology and ultrastructure of Saururaceae. *Grana* 46: 250–267.
- Tebbs MC. 1993. Piperaceae. In: Kubitzki K, Rohrer JG, Bittrich V, eds. *The families and genera of vascular plants*, vol. 2. Berlin: Springer-Verlag, 516–520.
- Tucker SC. 1975. Floral development in *Saururus cernuus* (Saururaceae): 1. Floral initiation and stamen development. *American Journal of Botany* 62: 993–1007.
- Tucker SC. 1976. Ontogeny of the inflorescence of *Saururus cernuus* (Saururaceae). 2. Carpel initiation and floral vasculature. *American Journal of Botany* 63: 289–301.
- Tucker SC. 1979. Ontogeny of the inflorescence of *Saururus cernuus* (Saururaceae). *American Journal of Botany* 66: 227–236.
- Tucker SC. 1980. Inflorescence and flower development in the Piperaceae. I. *Peperomia*. *American Journal of Botany* 67: 686–702.
- Tucker SC. 1981. Inflorescence and floral development in *Houttuynia cordata* (Saururaceae). *American Journal of Botany* 68: 1017–1032.

- Tucker SC. 1982a.** Inflorescence and flower development in the Piperaceae. II. Inflorescence development of *Piper*. *American Journal of Botany* **69**: 743–752.
- Tucker SC. 1982b.** Inflorescence and flower development in the Piperaceae. III. Floral ontogeny of *Piper*. *American Journal of Botany* **69**: 1389–1401.
- Tucker SC. 1985.** Initiation and development of inflorescence and flower in *Anemopsis californica* (Saururaceae). *American Journal of Botany* **72**: 20–31.
- Tucker SC, Douglas AW, Liang HX. 1993.** Utility of ontogenetic and conventional characters in determining phylogenetic relationships of Saururaceae and Piperaceae (Piperales). *Systematic Botany* **18**: 614–641.
- Vogel S. 1998.** Remarkable nectaries: structure, ecology, organophyletic perspectives. II. Nectariales. *Flora* **193**: 1–29.
- Wanke S, Jaramillo MA, Borsch T, Samain MS, Quandt D, Neinhuis C. 2007a.** Evolution of the Piperales – *matK* and *trnK* intron sequence data reveal lineage specific resolution contrast. *Molecular Phylogenetics and Evolution* **42**: 477–497.
- Wanke S, Vanderschaeve L, Mathieu G, Neinhuis C, Goetghebeur P, Samain MS. 2007b.** From forgotten taxon to a missing link? The position of the genus *Verhuellia* (Piperaceae) revealed by molecules. *Annals of Botany* **99**: 1231–1238.
- Wu CY, Wang WT. 1957.** Preliminary study of the flora of subtropical areas of Yunnan. *Acta Phytotaxonomica Sinica* **6**: 183–254.