

RESEARCH PAPER

Floral ontogeny of Ruteae (Rutaceae) and its systematic implications

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ABSTRACT

Floral development was investigated in *Ruta graveolens* and *Psilopeganum sinense*, representing two genera in the tribe Ruteae. Special attention was paid to the sequence of initiation of organ whorls in the androecium and gynoecium. The antepetalous stamens arise at the same level as the antesealous stamens in both species. The carpels are antepetalous in both taxa, indicating the androecium in both genera is obdiplostemonous. Compared with floral ontogeny of the ancestral genus *Phellodendron* (Toddalioideae), the obdiplostemonous androecium is a derived condition. The floral apex in *P. sinense* is quadrangular before initiation of the two carpels. Additionally, there are four dorsal and four ventral traces in the ovary. Integrated morphological and anatomical evidence indicates that the bicarpellate gynoecium in *Psilopeganum* most likely evolved from a tetracarpellate ancestor. Considering the similarities in morphological, geographical and chromosomal features, the ancestor may be *Ruta*-like. Further molecular phylogenetic and genetic studies are needed to verify this assumption.

INTRODUCTION

The Rutaceae, a large family of mostly trees, shrubs and woody vines, contain 161 genera and 1815 species (Stevens 2001 onwards) of mostly tropical and subtropical distribution (Chase *et al.* 1999). The family is known for its economic importance: edible fruits, timber and aromatic oils (Scott *et al.* 2000). The family has been placed in Rurales (Takhtajan 1987, 1997; Dahlgren 1989; Thorne 1992) or Sapindales (Cronquist 1981; APG 2009). In the only systematic treatment of Rutaceae, Engler (1896, 1931) recognised seven subfamilies: Rhabdodendroideae, Aurantioideae, Flindersioideae, Spanthelioideae, Rutoideae, Dictyolomatoideae and Toddalioideae. The tribe Ruteae is one of 12 tribes established by Engler in subfamily Rutoideae. It differs from other tribes in having two whorls of stamens, bisexual flowers and a herbaceous habit.

Recent molecular systematic studies show that the genus *Ruta*, a representative of Ruteae, is nested within the clade of Aurantioideae rather than in the branch with other Rutoideae (Chase *et al.* 1999; Scott *et al.* 2000; Samuel *et al.* 2001; Poon *et al.* 2007). Unlike other tribes of Rutoideae, flowers of Ruteae have two whorls of stamens and are bisexual, in which especially the two whorls of stamens are also characteristic of many genera of Aurantioideae. Further detailed morphological and ontogenetic investigation will improve our understanding of these taxa. Investigation of the morphology and development of the androecium in Ruteae is one such issue related to the phylogenetic relationship between Ruteae and Aurantioideae.

In addition, genera in Ruteae exhibit abundant variation in carpel number. Genera with four or five carpels, such as *Ruta*, *Haplophyllum* and *Boenninghausenia*, are distributed in Eurasia, while genera with one or two carpels are distributed in Africa and North America, except for *Psilopeganum* in the Chang Jiang (Yangtze) valley in China (Thulin 1999). It would be interesting to know how this geographically disjunct distribution pattern originated and if it reflects an evolutionary trend in carpel number in the tribe. *Psilopeganum* is monotypic. It was treated in a systematic context only by Engler (1896, 1931). It has a narrow distribution in the Three Gorges Reservoir area of Central China (Song *et al.* 2004; Yang *et al.* 2007) and has not been included in recent taxonomic treatments (Townsend 1986; Da Silva *et al.* 1988). Unfortunately, its original habitat was destroyed during construction of the sluice for the Three Gorges Reservoir. Detailed information on its floral morphology and development is of great interest for both systematics and conservation biology.

We selected *Ruta graveolens* and *Psilopeganum sinense* as representatives of the multi-carpellate and few-carpellate genera of Ruteae, respectively. The first aim of our research was to explore the developmental basis for the arrangement and differentiation patterns of floral organs in the two genera to further understand the morphological diversity within Ruteae and between Ruteae and related taxa, especially members of the Aurantioideae. Second, floral development, especially androecial and gynoecial development, was compared between the two genera and with related groups to gain an understanding of the developmental and evolutionary relationship of these genera with their allies.

MATERIALS AND METHODS

Ruta graveolens was collected in the Beijing Botanic Garden (Wei 07041), Chinese Academy of Sciences, *Psilopeganum sinense* was collected in the Three Gorges Reservoir Botanic Garden (Wei 06011), Wanzhou, Chongqing. Voucher specimens were deposited in the Chinese National Herbarium (PE).

The plant material was fixed in formalin–acetic acid–alcohol (FAA). The FAA-fixed materials were dehydrated in an ethanol series. Flower buds were dissected and studied in 95% ethanol using a dissecting microscope, then transferred through an ethanol–iso-amyl acetate series, critical-point dried, mounted on a metal stub and sputter-coated with gold/palladium. The flower buds were observed and micrographs taken with a Hitachi S-4800 scanning electron microscope (SEM) (Hitachi, Tokyo, Japan) at 10 kV. For light microscopy, material in FAA was dehydrated in an ethanol series and embedded in paraffin. Transverse serial sections of 10 µm were made using a microtome and stained with toluidine blue.

RESULTS

Floral initiation and development in *Ruta graveolens*

The actinomorphic flowers are grouped in terminal thyrsoids made up of a large number of cymes (Fig. 1A). Floral merism is variable, with tetramerous lateral flowers and pentamerous terminal flowers. The androecium of eight or ten stamens is arranged in two whorls and is apparently obdiplostemonous, with the antepetalous stamens adnate to the base of the petals.

The flower primordia and related bracts and bracteoles arise helically and acropetally on the main inflorescence branch (Fig. 2A). The first sepal primordium emerges on the abaxial side after the two lateral bracteoles become visible (Fig. 2C), followed by others in rapid sequence, either clockwise or counter-clockwise (Fig. 2D and E). Differences in size are evident soon after production of the sepals and are related to the order of initiation, with the first sepal obviously larger than the others. For the terminal pentamerous flowers, the initiation of the calyx follows a classical quincun-

cial spiral sequence. During development of the calyx, the floral apex increases in height and flattens. In lateral tetramerous flowers, the first petal primordium appears abaxially between the first two sepals (Fig. 2F). The other three petal primordia arise in very close succession on the floral apex inside the sepal whorl (Fig. 2F and G).

Immediately after initiation of the petals, the stamen primordia begin to emerge. The two whorls of stamens are nested with each other and arise at the same level of the floral apex inside the petal whorl. The abaxial antesepalous stamen arises first (Fig. 2H), followed by the abaxial antepetalous stamens (Fig. 2I). The four antesepalous and four antepetalous stamen primordia emerge in rapid succession (Fig. 2J and K). The antesepalous stamens are larger than the antepetalous stamens throughout development (Fig. 2L and M). In late development, each stamen enlarges laterally and differentiates into anther and filament, and the median and transverse furrows divide the anther into two thecae and four pollen sacs (Fig. 2O). After stamen initiation, the floral apex becomes flat, with four corners (Fig. 2M). Four independent carpel primordia emerge simultaneously in the antepetalous positions (Fig. 2N–P). The four carpels with four bitegmic ovules per ovary locule become post-genitally united (Fig. 2Q and R) and further differentiate to form the style and stigma.

Floral initiation and development in *Psilopeganum sinense*

Flowers appear to be solitary (Fig. 1B) or grouped in loose cymes, but the inflorescence structure was not studied in detail. The flowers are tetramerous and actinomorphic, with stamens in two whorls, in which four antepetalous stamens are adnate to the base of four petals. However, the gynoecium is composed of two carpels.

The inflorescence apex turns into a floral primordium (Fig. 3A). The two lateral sepal primordia arise first, followed by the abaxial and adaxial sepal primordia (Fig. 3B and C). After initiation, slight zonal growth takes place at the base of the sepals. Four petal primordia arise almost simultaneously at the periphery of the flattened floral apex inside the sepal

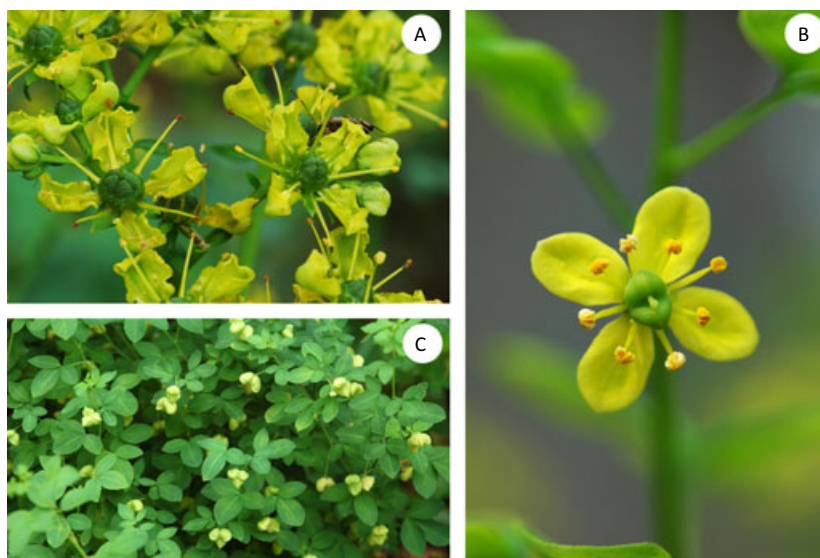


Fig. 1. *Ruta graveolens* and *Psilopeganum sinense*. A: Part of inflorescence of *R. graveolens*. B: Solitary flower of *P. sinense*. C: Fruiting plants of *P. sinense*.

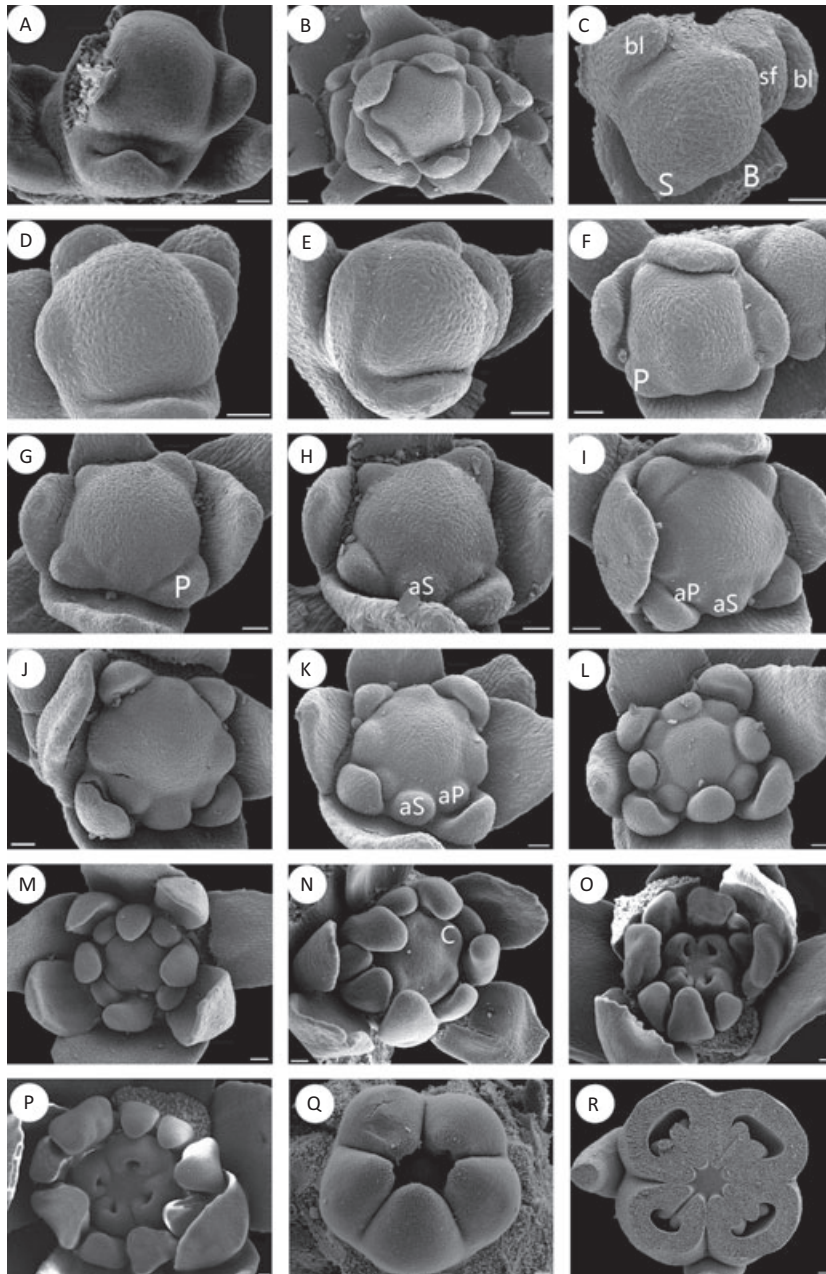


Fig. 2. Floral development in *Ruta graveolens*. A: Apical view of young inflorescence with lateral subunits. B: Later stage, flower primordia arise in the axils of each bract of the lateral subunits. C: Flower primordium with a bract and two bracteoles. In the axil of one of the bracteoles, there is a secondary flower primordium. The first sepal primordium arises in abaxial position. D, E: Sequential initiation of sepals. F: Initiation of petals. The first petal primordium arises on the abaxial side. G: All petals initiated. The different sizes of petals indicate the initiation sequence. H–K: Sequential initiation of stamens. L: Fully initiated androecium, showing different sizes between antesepalous stamens and antepetalous stamens. M, N: Early gynoecium development. The four carpels arise in antepetalous positions. O, P: Later stage of flower bud, showing the four carpels. Q: Later stage of gynoecium development, showing post-genital union of carpels. R: Transverse section of ovaries of mature carpels. B, bract; bl, bracteole; sf, secondary flower; S, sepal; P, petal; aS, antesepalous stamen; aP, antepetalous stamen; C, carpel. Scale bars = 40 μm .

whorl (Fig. 3C–E), with the abaxial petal slightly earlier (Fig. 3F). At a later stage, as the petals grow upward, they curve inward and enclose the centre of the flower apex in an imbricate aestivation.

After petal initiation, the first whorl of stamen primordia arises inside the petal whorl in antesepalous position (Fig. 3G and H). The antepetalous stamens arise after the antesepalous stamens (Fig. 3I and J), but at the same level as those, similar to *R. graveolens*. The antesepalous stamens are larger than the antepetalous stamens throughout development (Fig. 3K and L). In late development, each stamen differentiates into anther and filament. Carpel initiation begins when the floral apex flattens to become quadrangular. At first, seemingly four carpel primordia arise in antepetalous position (Fig. 3K–M). Later, the quadrangular floral apex

begins to elongate in a transverse direction and the four corners become blurred so that the gynoecium outline becomes ellipsoid (Fig. 3N) and two shallow furrows become visible in transverse direction (Fig. 3O). Two carpels soon rise above the floral apex in antesepalous position (Fig. 3P and Q). In the mature gynoecium, the two carpels form a bilocular ovary with axile placentation. The two styles and capitate stigmas are post-genitally united (Fig. 3R). However, if viewed in transverse section, the situation is more complex. At the base of the ovary there are four locules in antepetalous positions (Fig. 4F–H). Higher up, two of the four septa disappear and the four locules merge into two (Fig. 4I–L). In the upper half of the ovary the two axile placentae with numerous bitegmic ovules become visible (Fig. 4M and N). In the uppermost part of the ovary

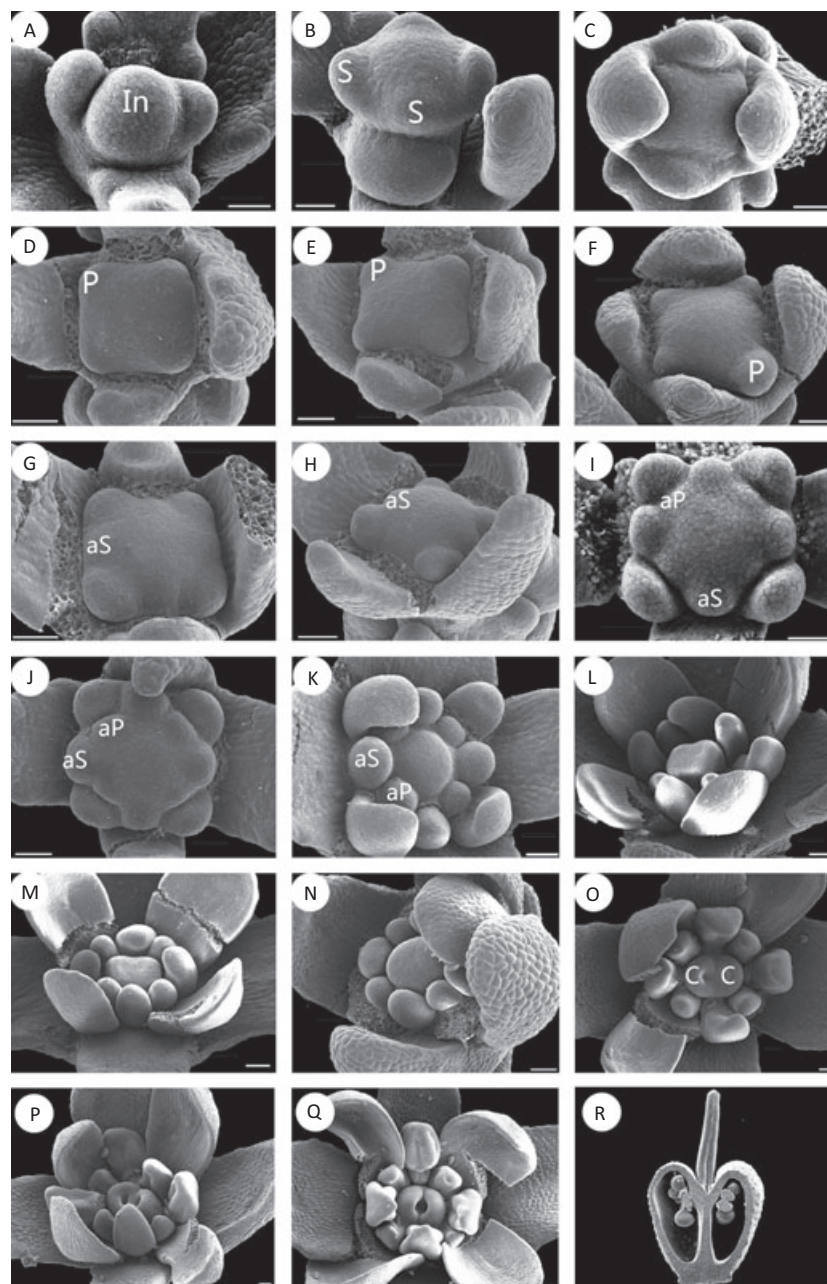


Fig. 3. Floral development in *Psilopeganum sinense*. A: Apical view of young inflorescence. B, C: Apical view of young flowers located terminally on the inflorescence apex, showing initiation sequence of sepals. D–F: Initiation of petals. The different petal sizes indicate initiation sequence. G, H: Initiation of the antesepalous stamens. I, J: Initiation of the antepetalous stamens. K: Fully initiated androecium, showing different sizes between antesepalous and antepetalous stamens. L: Young gynoecium showing four corners in antepetalous positions. M, N: Young gynoecium expanding in transverse direction. O: Development of the two carpels in antesepalous position. P, Q: Later stages of carpel development. R: Longitudinal section of the two mature carpels. Abbreviation: S, sepal; P, petal; aS, antesepalous stamen; aP, antepetalous stamen; C, carpel. Scale bars = 40 μ m.

the two carpels bulge upwards and are only post-genitally united (Figs 3R and 4O).

Floral anatomy

The pedicel contains a eustele with many collateral bundles separated by considerable interfascicular parenchyma. Higher up in the pedicel, one of the bundles diverges to the periphery. The next three or four bundles follow a few micrometers higher; the four or five bundles ramify after their divergence yielding the median and lateral traces of the sepals (Fig. 4A). At a higher level, the remaining bundles alternating with the sepal median traces organise into a quadrangular ring. The angles of this ring branch to form the vascular traces of the petals in the same radii (Fig. 4A); they represent the petal

median and lateral traces (Fig. 4B). The antesepalous and antepetalous stamen traces leave the central vascular cylinder at the same level (Fig. 4C). These bundles do not split and go directly into the stamens. There is no fusion between stamen traces and other traces, similar to *Ruta*, but differing from other genera in the Ruteae, such as *Dictamnus* and *Thamnosma* (Saunders 1934).

After the successive departure of the bundles of the sepals, petals and stamens, the remaining carpellary vascular tissue is arranged in a continuous vascular cylinder. Just under the ovary, four main carpel bundles (*i.e.*, dorsal bundles) diverge in antepetalous positions from the cylinder at the same level (Fig. 4D). The remaining ventral vascular tissue is arranged into four groups alternating with the main carpel bundles (Fig. 4E and G).

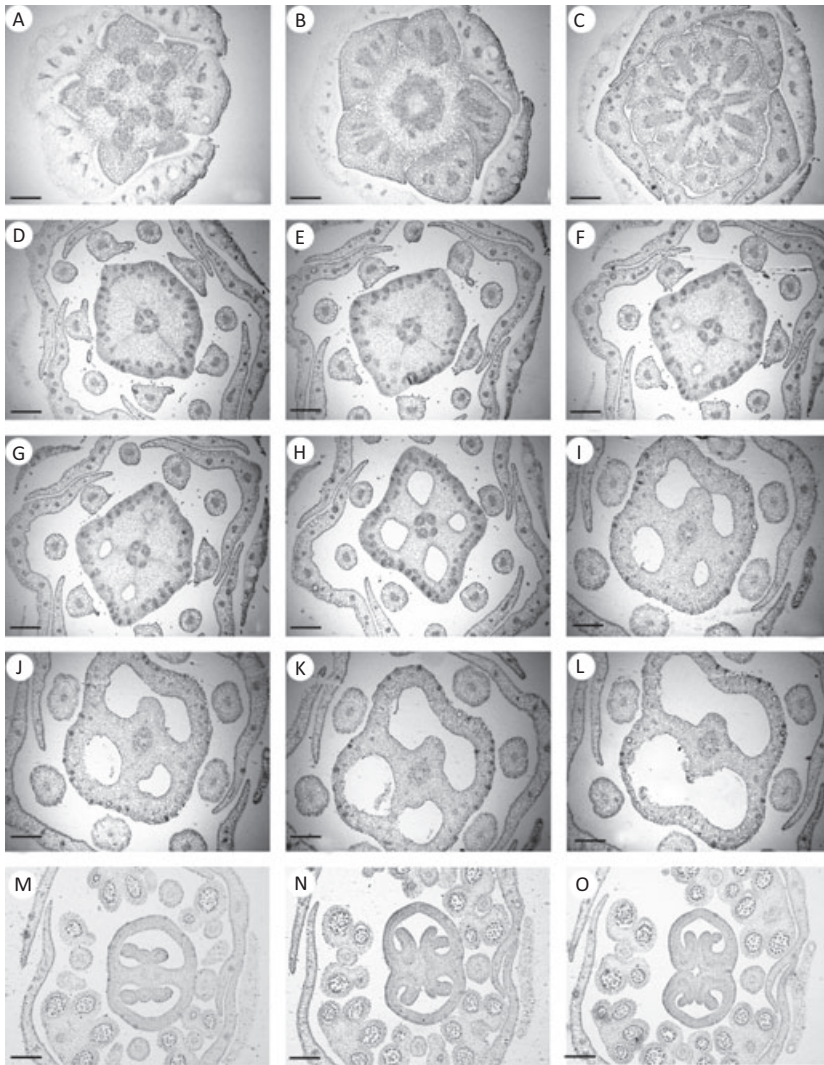


Fig. 4. Floral anatomy of *Psilopeganum sinense*, transverse section series of floral bud. A: Base of flower bud, showing petal bundles departing from central cylinder. B: Petal mother bundles split and reach the petal bases, representing the petal median and lateral traces. C: Departure of the stamen traces at the same level. D–G: Departure of the four carpel dorsal traces in antepetalous positions. H: The four locules in the ovary base and the four carpel ventral traces. I–K: The septa between the two locules within a carpel begin to disappear. L: The two locules at mid-length of ovary. M: Placentae become visible. N: The two placentae at mid-length. O: Uppermost part of ovary, the two carpels only post-genitally united at this level. Scale bars = 200 μm .

DISCUSSION

The terminal apical meristem of the thyrsoid of *R. graveolens* successively produces cymose subunits helically and acropetally. Each cymose subunit usually consists of two or three developing flowers with subtending bracts and lateral bracteoles. The few-flowered cymes in *P. sinense* are similar to the cymose subunits of *R. graveolens* in structure and development. The four sepals of a flower in *R. graveolens* are initiated with the abaxial sepal first, and in a quincuncial helical sequence in the terminal pentamerous flowers. The four sepals of a flower in *P. sinense*, however, are initiated in the order of two lateral sepals emerging first followed by the abaxial and adaxial sepals. Petal initiation shares a similar initiation sequence between the two genera, with abaxial petals initiating slightly ahead of the others.

Androecium

There are two types of androecium in Rutaceae, with stamens arranged in one or two whorls (Cronquist 1981). In the androecium with the stamens arranged in two whorls, ante-

petalous stamens are adnate to the base of the petals, such as in *Ruta* and *Psilopeganum*. Since the orientation of the carpels is antepetalous when they are isomerous, this type of androecium is usually considered obdiplostemonous (Matthews *et al.* 2001).

The term obdiplostemony was introduced by Chatin (1855) to describe an androecial configuration of two stamen whorls, with the outer stamens opposite the petals and the inner opposite the sepals. However, Endress (2010) argues that it is sometimes not possible to distinguish whether antepetalous stamens are inside antepetalous stamens because the dimensions of the attachment area of the antepetalous and antepetalous stamens can be very different. In the two species studied here, initiation of both antepetalous and antepetalous stamens is at the same level and without a conspicuous plastochron between them. Therefore, in mature flowers, the eight stamens are inserted approximately in one whorl, as suggested by Endress (2010) for other taxa. In addition, the initiation sites of the carpels alternate with the antepetalous stamens rather than with the antepetalous stamens in *R. graveolens*, which is a clear indicator for obdiplostemony. There is a strong correlation between obdiplostemony and

antepetalous carpel position. This may correspond to a maximum use of space, as the antepetalous stamens are usually displaced to the periphery and delayed in development after initiation, and there is more space in petal sectors when carpels initiate (Ronse De Craene *et al.* 1998; Endress 2010).

Obdiplostemony evolved repeatedly in eudicots (Ronse De Craene & Smets 1995) and in some lineages has been regarded as an important synapomorphy (Ronse De Craene 2010). In some families the occurrence of obdiplostemony tends to co-occur with haplostemony, indicating that obdiplostemony is related to androecium evolution (Ronse De Craene & Smets 1995). Obdiplostemony may represent either a prelude to the loss of the antepetalous stamens or a secondary increase in number of antepetalous stamens (Ronse De Craene & Smets 1995). In genera with a haplostemonous androecium in Rutaceae, such as *Phellodendron amurense* in subfamily Toddalioidae and *Tetradium daniellii* in subfamily Rutoideae, the sequence of floral organ initiation follows an alternate pattern, from sepals, petals, stamens to carpels, with successive formation of each whorl (Zhou *et al.* 2002, 2006). In general, floral organ reduction may occur by failure of organ initiation or by suppression of development after organ initiation (Tucker 1988). In *Phellodendron*, no sign of primordial suppression and no initial gap indicating failure of initiation are observed during floral development. From chemosystematics, *Phellodendron*, *Zanthoxylum* and *Tetradium* have been considered ancestral, because they have retained the primitive 1-benzyltetrahydroisoquinoline (1-BTIQ) alkaloid pathway and produce 1-BTIQ alkaloids (Da Silva *et al.* 1988). Price (1963) and Waterman (1975) further suggest the placement of these genera in a 'proto-Rutaceae' group, which is supported by molecular phylogenetic studies, where these genera group together in a strongly supported clade (Poon *et al.* 2007). Interestingly, all genera of 'proto-Rutaceae' have a haplostemonous androecium. This indicates that haplostemony with antepetalous stamens is likely the ancestral condition in Rutaceae, while the additional presence of antepetalous stamens in Ruteae is a derived condition.

Molecular phylogenies provide a framework for understanding the significance of obdiplostemony on a large scale. According to recent molecular phylogenies (APG 2009), Rutaceae belongs to Sapindales, which is grouped together with Malvales, Brassicales and other orders to form Malvids. Obdiplostemony has been frequently reported in Malvids, *e.g.*, Burseraceae and Anacardiaceae (Bachelier & Endress 2009), Nitrariaceae (Bachelier *et al.* 2011) and Malvaceae (von Balthazar *et al.* 2006). The morphological evolution of the androecium in Rutaceae, especially among Ruteae and members of the Aurantioideae, deserves further study, with broader sampling of floral development and anatomy, together with molecular phylogenetics.

Gynoecium

There is abundant variation in carpel number in Rutaceae, especially in the tribe Ruteae. Based on carpel number, Engler (1931) divided Ruteae into three groups, one with carpel number ranging from three to five, one with two carpels, and the third with one carpel.

The genus *Ruta* belongs to the clade with three to five carpels according to Engler (1931). The flowers of *Ruta* are usu-

ally characterised by four carpels, except the terminal flower with often five carpels. *Psilopeganum* belongs to the clade with two carpels within Ruteae according to Engler (1931). Its other floral organs, however, are in tetramerous whorls, alternating as in *R. graveolens*. Even though the mature flowers of *Psilopeganum* have two carpels, the gynoecium primordium is quadrangular, with the four angles antepetalous, as in *R. graveolens*, and in the developed ovary each of the two locules is subdivided into two at the base. In addition, four carpel dorsal traces and four ventral traces are present. Thus the gynoecium appears to begin development as tetramerous but later appears to consist of only two carpels. The additional rudimentary septum has been frequently used as evidence for the evolutionary trend in carpel reduction. For instance, a rudimentary septum in the lower third of the unilocular ovary of *Theligonum cynocrambe* (Rubiaceae) is interpreted as reminiscent of a bilocular gynoecium, a common feature in Rubiaceae (Rutishauser *et al.* 1998). Additionally, reduction in carpel number is frequently correlated with fertile carpels accompanied by additional carpellary bundles, dorsal traces or fused dorsal and ventral traces (Wilson 1982; Weberling 1989). Ontogenetic and anatomical data are often useful in solving problems of relationships because they may suggest homologies, and are helpful in interpreting the direction of evolution (Stuessy 2009). Integrating the morphogenetic and anatomical evidence, we suggest that the bicarpellate gynoecium in *Psilopeganum* might have evolved from a tetracarpellate ancestor.

Morphologically, except for the bicarpellate gynoecium, *Psilopeganum* shares more common features with *Ruta* than with any other genus within the tribe Ruteae. They both have yellowish tetramerous flowers, as well as verrucose protuberances on their pericarps. In the gynoecium, they have the same type of placentation and a similar post-genitally united part. Flowers of both genera have spreading petals with antepetalous stamens adnate to the base of the petals and lack a gynophore. *Psilopeganum* and *Ruta* also have more complicated secretory cavities than other genera, such as *Boenninghausenia* (Liu & Hu 1998), a genus shown to be closely related to *Ruta* in a molecular phylogenetic study (Salvo *et al.* 2008).

In addition, the base chromosome number of *Psilopeganum* is $n = 9$ (Huang 1997) while *Ruta* shows an infrageneric dysploidy of chromosome numbers with $n = 9$ or 10 (Stace *et al.* 1993; Stace 1995; Bacchetta *et al.* 2006), indicating a close relationship between the two genera in chromosome number. The present areas of distribution of both *Psilopeganum* and *Ruta* belong to the coast of the Tethys Sea in the Mesozoic. The present discontinuous distribution of the two genera may have resulted from the up-thrust of the Himalaya, and subsequent isolation by mountains and deserts (Wu *et al.* 2003). The data from morphology and geography as well as chromosome evidence all suggest *Psilopeganum* has a closer relationship with *Ruta* than with other genera in the tribe. Since a tetracarpellate gynoecium is common in Ruteae, the bicarpellate gynoecium of *P. sinense* might have evolved from a *Ruta*-like tetracarpellate ancestor.

CONCLUSIONS

We here report that in *Ruta* and *Psilopeganum*, the antepetalous stamens in the obdiplostemonous androecium are

additionally initiated and further displaced to the periphery during development to be adnate to petals in mature flowers. Along with previous data from floral development, chemosystematics and molecular phylogenies, our results indicate that the haplostemonous androecium with antesealous stamens is likely the ancestral condition, while the obdiplostemonous androecium with additional presence of antepetalous stamens in Ruteae is a derived one in Rutaceae.

We further find a quadrangular floral apex before initiation of the two carpels in *Psilopeganum*, which might reflect a residual condition from a four-carpellate ancestor, supported by a remnant septum in each ovarian locule and four carpellary dorsal and ventral traces in its bicarpellary gynoecium. We suggest that the bicarpellary gynoecium of *Psilo-*

peganum might have evolved from a *Ruta*-like tetracarpellary ancestor. Further studies using a combination of floral ontogeny, anatomy and molecular phylogeny would provide a better understanding of the evolutionary relations in Rutaceae.

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