Ovule-to-seed development in *Dovyalis caffra* (Salicaceae: Flacourtieae) with notes on the taxonomic significance of the extranucellar embryo sac

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ABSTRACT

Dovyalis caffra (Hook.f. & Harv.) Hook.f. is a widespread and horticulturally important southern African endemic. Here described, ovule-to-seed development represents the first embryological information on this genus of the tribe Flacourtieae, Salicaceae *sensu lato*. Results are discussed in the light of data available on the embryology of the order Malpighiales in general and on the tribe Flacourtieae in particular. It is clear that *Dovyalis* E.Mey. ex Arn. shares many characters with other members of the Flacourtieae. Ovules of *D. caffra* are anatropous, bitegmic and crassinucellate with both bisporic *Allium*- and monosporic *Polygonum*-type embryo sacs. One of the most characteristic embryological features of the tribe is the presence of an unusual, extranucellar embryo sac that stretches halfway up into the micropyle. Since this extraordinary-shaped embryo sac also defines the embryo sacs of *Salix* L. and *Populus* L., it is suggested that the character provides strong support for the proposed close phylogenetic link between tribes Flacourtieae and Saliceae of Salicaceae *sensu lato*.

INTRODUCTION

Dovyalis E.Mey. ex Arn. is a relatively small, mostly African genus comprising about 15 species of shrubs or relatively small trees, often thorny, with simple, alternate and exstipulate leaves (Sleumer 1972). All species are dioecious or rarely polygamous (Langenegger 1976) with a tendency towards andromonoecy, i.e. the male plants occasionally bear a few bisexual flowers and fruits (Sleumer 1972). The apetalous flowers are unobtrusive, greenish, nectariferous and borne singly or in few-flowered fascicles. Fruits are indehiscent, fleshy and edible berries in various species. *Dovyalis* is widespread in tropical and subtropical Africa (excluding Madagascar), with one species reported from Sri Lanka (Sleumer 1972). Six species occur in the *Flora of southern Africa* region (Bredenkamp 2003) where they form a common constituent of mixed scrub, riverine bush, open woodland and forest.

When the genus was first validly described, Arnott (1841) regarded the type species, *Dovyalis zizyphoides* E.Mey. ex Arn., as closest to Euphorbiaceae with some resemblance in habit to *Flacourtia* L'Hér. Although Endlicher (1842) consequently listed *Dovyalis* as a dubious genus under his natural order Euphorbiaceae, the association with *Flacourtia* and a placement of *Dovyalis* in Flacourtiaceae DC. were subsequently endorsed by most taxonomists during the 19th and 20th centuries (see Warburg 1893 (as *Doryalis*); Gilg 1925; Hutchinson 1967; Lemke 1988). However, the beginning of the 21st century has seen a radical reshuffling of genera traditionally placed in Flacourtiaceae. Molecular phylogenet-

ic studies, supported by data from several other botanical fields, have indicated that most flacourtiaceous genera fall into two clades, each more closely related to other families within Malpighiales than to one another (Chase *et al.* 2002). Flacourtiaceae were accordingly split into two groups and the flacourtiaceous genera (not *Aphloia* DC.) became part of two recircumscribed families, namely Achariaceae *sensu lato* and Salicaceae *sensu lato*. Consequently, all the southern African flacourtiaceous genera were placed in Salicaceae *sensu lato* with the exception of *Kiggelaria* L., *Rawsonia* Harv. & Sond. and *Xylotheca* Hochst.; these three genera were included in Achariaceae *sensu lato* (Chase *et al.* 2002).

Recent studies on ovule-to-seed development and structure in the three southern African genera of the Achariaceae *sensu stricto* (Steyn *et al.* 2001, 2002a, b, 2003) and in *Kiggelaria* L. (Steyn *et al.* 2003), offer unequivocal embryological support for the proposed close phylogenetic relationship between the herbaceous, highly modified and monotypic genera of Achariaceae *sensu stricto* and the African genus *Kiggelaria* of the woody tribe Pangieae (Flacourtiaceae *sensu stricto*). These taxa share a number of uncommon embryological characters, e.g. sessile ovules with zigzag micropyles, deep-lying embryo sacs covered by an epistase in the ovule and seed, endotestal-exotegmic protective layers in the seed coat, suspensorless embryos and sarcotestal seeds with stomata in the epidermis (Steyn *et al.* 2003).

On the other hand, available embryological and other structural support for emending the circumscription of Salicaceae *sensu stricto* has been meagre and mainly rests on stamen and pollen characters (Keating 1973; Meeuse 1975; Kaul 1995; Bernhard & Endress 1999). Although Meeuse (1975) listed some ovule characters shared between Salicaceae *sensu stricto* and Flacourtiaceae *sensu stricto*, these characters are not restricted to the two taxa; they are commonly found among related violalean families (Meeuse 1975). Nevertheless, the strong similarity in embryological characters between Achariaceae *sensu*

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stricto and *Kiggelaria* (Steyn *et al.* 2003) suggests that detailed embryological studies of flacourtiaceous species representing the tribes assigned together with Saliceae Rchb. to Salicaceae *sensu lato* might also reveal characters that are diagnostic for Flacourtiaceae *sensu stricto* and Salicaceae *sensu stricto*. Such embryological characters would be useful for testing hypotheses by molecular phylogeneticists for a close evolutionary relationship between these two families.

Here we present data on ovule-to-seed development and structure in *Dovyalis caffra* (Hook.f. & Harv.) Hook.f., a representative of the tribe Flacourtieae DC. and a species that is, like all the other members of *Dovyalis*, embryologically completely unknown. Results are compared with findings recently obtained during a study of seed structure in the polymorphic African willow, *Salix mucronata* Thunb. (Steyn *et al.* 2004) and with information found in classic embryological literature on Salicaceae and Flacourtiaceae. Representatives of Flacourtieae are, according to data collected in multidisciplinary studies, including molecular phylogeny, possibly the closest relatives of *Salix* L. and *Populus* L., both of the tribe Saliceae (Chase *et al.* 2002).

Dovyalis caffra is the only southern African species of the genus that belongs to the section *Aberia* Hochst., the other five species represent section *Dovyalis* E.Mey. ex Arn. (Sleumer 1972). *D. caffra* occurs in the easternmost parts of Western Cape and is widespread in the eastern, subtropical regions of southern Africa, extending from Eastern Cape northwards through KwaZulu-Natal, Swaziland, Mpumalanga and Limpopo into Zimbabwe. Commonly known as the Kei apple, *D. caffra* is also often cultivated for its edible fruit and, because of its formidable thorns, frequently used as a security hedge.

MATERIAL AND METHODS

Floral buds, open flowers and developing fruits were collected from cultivated female trees of *Dovyalis caffra* growing in the Pretoria National Botanical Garden and in a private garden in Murrayfield, Pretoria. All flowering stages were dissected to facilitate penetration of chemicals into ovules and seeds and fixed in a 0.1 M cacodylate buffered solution containing 4% formaldehyde and 2.5% glutaraldehyde. Following the methods of Feder & O'Brien (1968), material was dehydrated in an alcohol series and impregnated with glycol methacrylate (GMA). All material was imbedded in GMA, sectioned transversely or longitudinally at 2–3 µm and subsequently stained with the periodic acid/Schiff reaction and toluidine blue by using the protocols of O'Brien & McCully (1981).

Seed coat terminology follows Corner (1976) as recommended by Schmid (1986).

RESULTS

Placentation, orientation and development of megagametophyte

The gynoecium in the apetalous female flowers (Figure 1A) of *Dovyalis caffra* is usually hexa- or heptacarpellate

and very rarely pentacarpellate. The syncarpous, unilocular and superior ovary is globose and glabrous (Figure 1B) with six or seven styles, each ending in a bifid stigma. Twelve to fourteen ovules, two per carpel, are borne on parietal placentae developing approximately at the same level, ± halfway down the locule (Figure 1A, B). The ovules occur in pairs on the flanks of the fused carpel margins. These thick structures extend deeply into the locule (Figure 1A), limiting the space available to the developing ovules so that they show some spatial adjustment. Nevertheless, longitudinal sections showed that all ovule stages are epitropous with a ventral raphe *sensu* McClean & Ivimey-Cook (1956: 1392) and anatropous or about to become anatropous (Figure 1C).

The youngest buds examined contained bitegmic, crassinucellate young ovules in the process of curving towards the anatropous position (Figure 1C). The nucellus tissue holds a single megaspore mother cell covered by one or two parietal cell layers. At this early stage the ovule seems to be slightly stalked, but when the curvature is completed at about the time meiosis takes place, the ovule has no funicle (Figure 1D).

Meiosis does not seem to result in a linear tetrad of megaspores. After the first meiotic division, two dyad cells are formed of which the chalazal cell undergoes the second meiotic division, but mostly without the formation of a cell wall between the two megaspore nuclei (Figure 2A); in only one ovule a partitioning wall was seen resulting in two chalazal megaspores (Figure 2C). The micropylar dyad cell degenerates without further division (Figure 2B, C). The starting point of the megagametophyte is therefore usually a bisporic cell with the nuclei situated at opposite poles of the cell (Figure 2B). After two mitotic divisions an eight-nucleate, bisporic embryo sac is formed that conforms to the *Allium*-type of megagametophyte development (Willemse & Van Went 1984). However, the presence of three megaspores in the nucellus (Figure 2C) suggests that monosporic *Polygonum*-type embryo sacs also occur in *Dovyalis caffra*.

Structure of the mature ovule

Ovules are pendant, sessile, ovoid and anatropous structures reaching a size of about 90×60 µm at anthesis (Figure 2D). The two integuments are of equal length so that the micropylar canal is formed by the inner integument only, but in post-fertilization stages the outer integument usually lengthens to form an exostome (compare Figure 2D, E) that is slightly out of line with the endostome. The lower part of both integuments consists of about five parenchymatic cell layers, but in the micropylar region the integuments become thicker (Figure 1D). The outer epidermis of the inner integument is the most prominent of the integumental layers. The lower part of this epidermal layer is uniseriate and consists of meristematic cells with large nuclei and dense protoplasm, whereas the micropylar part is multiseriate and forms most of the cells in the thick micropylar region of this integument (Figure 2D). In the centre of the ovule, the nucellus is an ovoid structure with a slightly attenuate apex consisting of a nucellus cap. The latter is formed by derivatives of the nucellus epidermis and the parietal cell (Figure 2B, C). The nucellus cells are parenchymatic, except at the base of the
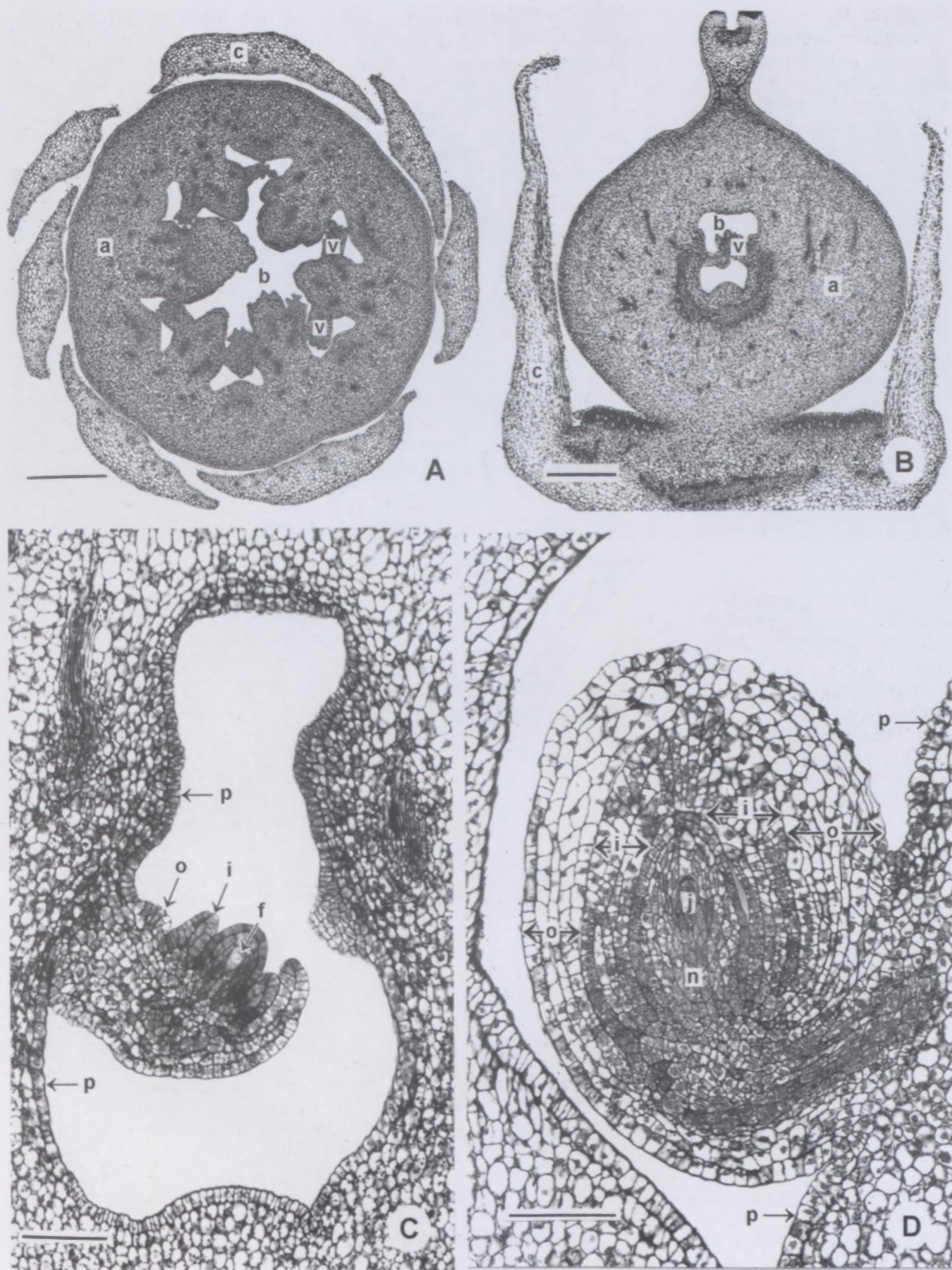
FIGURE 1.—Placentation and orientation of ovules in *Dovyalis caffra*. A, apetalous female flower in t/s showing parietal placentation of developing ovules in unilocular ovary; B, female flower in superficial (tangential) l/s showing position and orientation of young ovules; C, sagittal section of bitegmic, dorsal epitropous ovule primordium with megaspore mother cell; D, anatropous, crassinucellate, sessile ovule in sagittal section during formation of bisporic embryo sac. a, 6-carpellate ovary; b, locule; c, calyx lobe; f, megaspore mother cell; i, inner integument; j, initial stage of bisporic embryo sac; n, nucellus; o, outer integument; p, placenta of locule; v, young ovule. Scale bars: A, B, 50 μm; C, D, 10 μm.

FIGURE 2.—Formation of embryo sac and structure of ovule in *Dovyalis caffra*. A, dyad cells in nucellus with chalazal cell containing two megaspore nuclei; B, nucellus containing bisporic embryo sac in two-nucleate stage and disintegrated micropylar dyad cell; C, nucellus with two chalazal megaspores and disintegrated micropylar dyad cell; D, structure of ovule at anthesis. Note micropyle (curved arrows) formed by inner integument and embryo sac protruding into lower part of endostome; E, embryo sac at fertilization. Note dark-staining filiform apparatus of synergid, extranucellar embryo sac and slightly skewed micropyle (curved arrows) formed by both integuments. e, embryo sac; i, inner integument; j, initial stage of bisporic embryo sac; k, disintegrated micropylar dyad cell; m, chalazal megaspores; n, nucellus; ne, nucellus epidermis; o, outer integument; oi, outer epidermis of inner integument; s, synergid. Scale bars: A, B, C, 5 µm; D, E, 10 µm.

embryo sac where the cell walls are noticeably thicker, darkly stained and possibly lignified (Figure 2D, E). The cells possibly represent a postament as described by Shamrov (1998: 379), i.e. a column-like tissue located below the sporogenous or gametophytic structures, consisting of thin-walled or sometimes lignified cells.

The mature embryo sac is a narrow-elliptical structure that occupies an unusual position in the ovule—it lies partly inside and partly outside the nucellar tissue (Figure 2D). In its growth towards maturity, the base of the embryo sac does not succeed in penetrating the chalazal nucellus, the thick-walled cells of the postament possibly acting as a barrier tissue to stop the encroachment of the embryo sac into the chalaza (McLean & Ivimey-Cook 1956; Bouman 1984). However, in the micropylar region the apical part of the embryo sac has broken through the nucellus epidermis and, flanked by the inner integument, stretches up halfway into the endostomium (Figure 2D, E). The detailed structure of the embryo sac elements could not be determined. At fertilization the antipodals have disappeared, the central cell nucleus lies near the egg apparatus in the extranucellar apical region and the synergids show the unmistakable presence of a filiform apparatus (Figure 2E).

Seed development

Embryo: fertilized ovules take about three months to develop into mature, woolly seeds of ± 10 × 5 mm, embedded in the fleshy pulp of the fruit, which is a berry. Embryo development was not studied in detail and the embryo could not be typified. The youngest embryo found was ± eight weeks old. Against the wall of the embryo sac the first layers of endosperm cells have been formed. At eight weeks, the embryo is in an early globular stage with a long, uniseriate suspensor stretching up into the micropyle (Figure 3A). The cells of the suspensor contain starch grains and extend past the nucellus epidermis into the region originally occupied by the extranucellar part of the embryo sac. It seems possible that, by elongating, the suspensor has pushed the embryo proper into the developing endosperm. At seed maturity the embryo is without chlorophyll, erect and spathulate with thin, expanded cotyledons and lies imbedded in oily endosperm.

Seed coat: in *Dovyalis caffra* the seed coat is not multiplicative, i.e. periclinal divisions do not occur during seed development to add extra layers to the seed coat. Since the only mechanical layer in the mature seed coat develops from the outer epidermis of the inner integument, the seed is exotegmic.

In the early stages of seed coat development (Figure 3B, D), the testa comprises about four to five cell layers, except in the raphal region where the mesophyll is multilayered (Figure 3B). The mesophyll and inner epidermis of the testa consist of thin-walled parenchyma. The outer epidermis of the testa possibly plays an important part in the protection of the seed before the mechanical layer of the tegmen matures. After fertilization, numerous thin-walled, unicellular epidermal hairs are formed which cover the young seed (Figure 3B, D) at the early globular stage of the embryo (Figure 3A). The hairs grow perpendicular to the seed surface (instead of becoming depressed) and eventually permeate the space between the seeds and the pericarp. The hairs possibly guard against desiccation of the inner seed tissues by preventing the loss of water vapour through the numerous stomata in the epidermis of the testa (Figure 3D). Apart from the hairs, the outer epidermis also contains large numbers of tanniniferous cells with walls impregnated and lumina filled with phenolic compounds. These polyphenols increase the rigidity of the thin epidermal cell walls and contribute towards the hardness of the seed coat (Werker 1997: 100). Phenolic-containing cells offer resistance to biodegradation and help to protect against attacks by herbivores, insects and micro-organisms (Boesewinkel & Bouman 1984). In the ripe fruit the outer epidermis with its hairs persists, but the phenolic substances have disappeared and the cells of the mesophyll and inner epidermis collapse (Figure 3C).

At the onset of seed coat development, the tegmen consists of about five layers (Figure 2E). The outer epidermis of the tegmen (exotegmen) show frequent anticlinal divisions to form a single layer of thin-walled, radially flattened cells (Figure 3D) that rapidly stretch in a direction parallel to the longitudinal axis of the seed (Figure 3B). The inner epidermis of the tegmen (endotegmen) also divides anticlinally and forms a layer of small, tightly packed cells containing large amounts of dark-staining phenolic substances (Figure 3B, D). In the ripe fruit the exotegmen forms the mechanical layer of the seed and comprises thick-walled, radially flattened cells (Figure 3C) with cell walls impregnated with phenolic compounds. The mesotegmen has disintegrated and the endotegmen remains as small, strangely formed flask-shaped cells adjacent to the persistent nucellus epidermis that separates the endotegmen from the endosperm (Figure 3C).

DISCUSSION

A detailed comparison of ovule and seed characters in the 36 families placed by Savolainen *et al.* (2000) in Malpighiales—the clade previously termed Violales (Chase *et al.* 2002)—is hampered by a lack of comparable data for many of the families, as given in compendia dealing with comparative embryology. Nevertheless, according to such works (Davis 1966; Corner 1976; Johri *et al.* 1992; Nandi *et al.* 1998) similarities include bitegmy, anatropy and crassinucelli with the nucellus epidermis participating in the formation of the nucellus cap (tenuinucellate in Clusiaceae, Dichapetalaceae, Linaceae, Ochnaceae, Trigoniaceae); both integuments usually form the zigzag micropyle canal (inner integument only in Dichapetalaceae, Erythroxylaceae, Rhizophoraceae, outer integument only in Salicaceae *sensu stricto*); a *Polygonum*-type embryo sac usually develops, but *Allium*- and *Adoxa*-types also occur; endosperm formation is nuclear, it later becomes cellular and is usually copious (exalbuminous seeds in Caryocaraceae, Clusiaceae, Ochnaceae, Salicaceae *sensu stricto*); the embryogeny varies considerably, but the mature embryo is typically straight, medium-sized to large and protected by a fibrous exotegmen in the seed coat (mesotestal seed in Clusiaceae, exotestal in Dichapetalaceae, Salicaceae).

As far as embryology is concerned, *Dovyalis* seems to fit comfortably into the framework of Malpighiales (com-

FIGURE 3.—Seed and seed coat formation in *Dovyalis caffra*. A, micropylar region of young seed with developing embryo, note long suspensor in micropyle; B, l/s developing seed coat; C, t/s seed coat of seed in ripe fruit; D, t/s developing seed coat. i, inner integument (tegmen); ii, inner epidermis of tegmen; mi, mesophyll of tegmen; mo, mesophyll of testa; n, nucellus; ne, nucellus epidermis; o, outer integument (testa); oi, outer epidermis of tegmen; oo, epidermis of testa; o, testa; t, epidermal hair; u, suspensor; w, endosperm; x, guard cell of stoma; y, embryo. All scale bars 10 μm.

TABLE 1.—Comparison of selected ovule and seed characters in *Dovyalis* and *Salix*

No. Character	*Dovyalis* (according to present study)	*Salix* (according to literature)
1. Ovule position and number	Parietal, multi-ovular	Parietal, multi-ovular (Chamberlain 1897; Judd *et al.* 2002: 281; Steyn *et al.* 2004)
2. Ovule type	Anatropous, bitegmic, crassinucellate	Anatropous, unitegmic, crassinucellate (Chamberlain 1897; Meeuse 1975; Johri *et al.* 1992; Steyn *et al.* 2004)
3. Outer integument	4- or 5-layered, parenchymatic, forms exostome in seed	3- or 4-layered, parenchymatic, forms micropyle canal (Steyn *et al* 2004)
4. Inner integument	4- or 5-layered, parenchymatic, forms endostome	Absent (Corner 1976; Judd *et al.* 2002; Steyn *et al.* 2004)
5. Funicle	Absent, ovule and seed sessile	Present, ovule and seed stalked, (Chamberlain 1897; Steyn *et al.* 2004), or without funicle (Corner 1976) (not confirmed)
6. Nucellus cap	Comprises derivatives of both nucellus epidermis and parietal cell	Comprises derivatives of both nucellus epidermis and parietal cell (Chamberlain 1897)
7. Embryo sac type	Usually bisporic *Allium*-type, rarely *Polygonum*-type, extranucellar	Monosporic *Polygonum*-type (Maheshwari & Roy 1951); *Allium*-type, extranucellar (Chamberlain 1897)
8. Seed type	Anatropous, albuminous exarillate	Anatropous, exalbuminous (Corner 1976), arillate (Steyn *et al.* 2004)
9. Endosperm	Nuclear becoming cellular, oily, copious in seed	Nuclear becoming cellular, oily, absent in seed (Martin 1946; Corner 1976)
10. Embryo	Large, erect, spathulate; cotyledons thin, non-chlorophyllous	Large, erect, spathulate (Martin 1946); cotyledons thick, chlorophyllous (Steyn *et al.* 2004)
11. Embryogeny	Type unknown; suspensor present	Asterad-type (Davis 1966); suspensor present (Chamberlain 1897)
12. Seed coat	Exotegmic, consists of longitudinal fibres; stomata and hairs in epidermis of testa; exarillate	Exotestal; epidermis of testa with thickened walls, glabrous without stomata; hairy aril present (Steyn *et al.* 2004)
13. Dispersal mechanism	Endozoochory	Anemochory, hydrochory, epizoochory (Ridley 1930: 554; Steyn *et al.* 2004)

pare Table 1: Nos 2, 3, 4, 6, 7, 8, 9, 10 & 12). Reports in classical literature (Davis 1966; Corner 1976; Johri *et al.* 1992) indicate that the genus also shares many of the above-mentioned characters with other representatives of the Flacourtieae, e.g. *Arechavaletaia uruguayensis* Speg., *Idesia polycarpa* Maxim., *Flacourtia indica* (Burm.f.) Merr. However, features that seem to stand out as particular to Flacourtieae include the following: an embryo sac that breaks through the nucellus and protrudes into the endostome, a variation in the functional behaviour of the megaspores and a fibrous exotegmen in the seed (Johri *et al.* 1992). These are also characters that proved to be definite for *Dovyalis* in the present study.

Embryological studies on *Salix* and *Populus* L. (Chamberlain 1897; Maheshwari & Roy 1951; Nagaraj 1952; Steyn *et al.* 2004) partly support the placement of Salicaceae *sensu stricto* in Malpighiales (see Table 1: Nos 1, 2 (partly), 3, 6, 7, 8 (partly), 9 (partly), 10 (partly), 11). It is noteworthy that two other embryological characters of diagnostic value for Flacourtieae, namely the unusual extranucellar embryo sac and the inconsistent behaviour of the megaspores, are also characteristic for both *Salix* and *Populus* (Chamberlain 1897; Nagaraj 1952). Furthermore, the hairy seed coat in *Dovyalis* may be taxonomically significant. Although the seed itself is glabrous in *Salix*, the genus is characterized by the presence of long, unicellular, intra-ovarian hairs as well as seed with a hairy aril (Steyn *et al.* 2004). This propensity to produce epidermal hairs in the ovary and its associated structures such as ovules, seed and arils may well reflect a close phylogenetic link between Flacourtieae and Saliceae.

Despite all the similarities between *Salix* and *Dovyalis*, significant deviations of the basic flacourtiaceous pattern also occur. In *Salix* the endosperm is ephemeral (Håkansson 1954), the seed exalbuminous and exotestal and the inner integument absent. Unitegmy results in the lack of an endostome and the absence of a tegmen with its characteristic fibrous exotegmen. Steyn *et al.* (2004) suggested that the marked differences in seed coats between *Salix* and animal-dispersed flacourtiaceous taxa like *Dovyalis* may possibly be linked to seed adaptations for different dispersal strategies—*Salix* seeds are mostly dispersed by wind and water, mechanical layers would only unnecessarily increase seed weight, whereas *Dovyalis* seed occur inside edible berries and need the protection of exotegmic fibres against the onslaught of animal feeders.

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