Identification and characterization of ten new water gaps in seeds and fruits with physical dormancy and classification of water-gap complexes

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INTRODUCTION

Physical dormancy (PY) is caused by a water-impermeable palisade cell layer(s) in seed or fruit coats (Baskin et al., 2000) along with tightly sealed chalaza and micropyle openings (Gama-Arachchige et al., 2010). PY has been demonstrated or inferred to occur in species of 18 angiosperm plant families, and it is unknown in gymnosperms (Nandi, 1998; Baskin et al., 2000, 2006; Baskin, 2003; Horn, 2004; Koutsovoulo et al., 2005; Angiosperm Phylogeny Group, 2009; Tsang, 2010). Seeds of some species with PY also have physiological dormancy (PD); hence, they are considered to have combinational dormancy (CD). The breaking of PY involves disruption or dislodgement of ‘water-gap’ structures causing the seeds/fruits to become permeable. The water-gap region is a morpho-anatomically specialized area and it differs from the rest of the seed or fruit coat. The location, anatomy, morphology and origin of water gaps can differ between and even within families (Baskin et al., 2000; Jayasuriya et al., 2009). Twelve different water-gap regions in seven families have been characterized previously. However, the water gaps previously have not been characterized in Biebersteiniaceae, Cucurbitaceae, Fabaceae (clade Cладrasti), Lauraceae, Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae.

Water-gap structures in seeds/fruits act as environmental signal detectors for seed germination (Baskin et al., 2000). The ability of water-gap structures to sense environmental conditions allows seeds with PY to become permeable just prior to the commencement of conditions favourable for germination and plant establishment (Taylor, 1996a, b; Jayasuriya et al., 2008a, 2009; Gama-Arachchige et al., 2012). The mechanisms of sensing environmental signals for PY break by water-gap structures differ between species (Jayasuriya et al., 2008a, 2009; Gama-Arachchige et al., 2013). Since the water-gap region plays a major role in maintaining and breaking of PY and thus in plant survival and fitness via timing of seed germination, it is important to characterize the diversity of this structural complex as a basis for understanding how it functions under...
natural conditions. Such a study will also provide information for further investigations into how PY evolved in different plant lineages.

In the literature, the term ‘water gap’ is used interchangeably to define both the opening formed during the PY break and the whole specialized region of the seed or fruit coat (Jayasuriya et al., 2007; Turner et al., 2009; Gama-Arachchige et al., 2010; Karaki et al., 2012; De Paula et al., 2012). Moreover, Gama-Arachchige et al. (2011) studied the development of the water-gap region of Geranium carolinanum and reported that the micropylar water-gap region of G. carolinanum is an anatomically complex structure. They introduced the term ‘water-gap complex’ to define this whole water-gap region of G. carolinanum. However, to date, no attempt has been made to define the different structures involved in early imbibition or to classify water-gap regions.

Our general objectives were to (1) identify the water gaps in seeds/fruits of Cucurbitaceae, Fabaceae (clade Cladastis), Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae; and (2) devise a classification system for water-gap regions based on information from previous studies and from the current study. Such a system would greatly facilitate the evaluation of evolutionary relationships between species with water-impermeable seed/fruit coats with regard to the morpho-anatomy of PY and PY break.

For some of the study species, information on PY break and/or seed/fruit coat anatomy is available. Nandi (1998) studied the seed coat anatomy of eight species of Malvales sensu stricto (including Bixa orellana and Helianthemum nummularium) and compared the anatomical similarities of the specialized chalazal region (Bixoid chalaza). Based on the complex structure of the chalazal region, Baskin et al. (2000) suggested that the Bixoid chalaza may act as the water gap in seeds of Bixaceae and Cistaceae. Razanamiharizaka et al. (2006) and Turner and Dixon (2009) tested the effect of boiling water on PY breaking in the eight species of Adansonia. Two of the species were non-dormant and the other six species had PY. Boiling for 15 s to 5 min (depending on the species) was effective in PY breaking. Physical dormancy of seeds of Ceanothus americanus can be broken by exposing them to hot or boiling water (Table 1; Schratowski, 1962; Conard et al., 1985). Exposing seeds of C. velutina to dry heat resulted in irreversible opening of the hilum (Gama-Arachchige et al., 2012). Based on these observations, the hilar slit can be assumed to be the water gap in Ceanothus spp. Geneve (2009) blocked the whole hilar region of seeds of Ceris canadensis that had been boiled for 1 min and concluded that the hilar area is the water gap. However, the roles of the pseudolens, micropyle and hilar slit in imbibition were not studied. Ohga (1926) and Shaw (1929) studied the fruit anatomy of Nelumbo nucifera and N. lutea, respectively, and characterized a specialized area in the fruit coat known as the protuberance. However, none of these studies focused on identification and characterization of the water-gap regions.

Thus, the specific objectives of the current study were (1) to describe morpho-anatomically the water gap for the families Fabaceae (clade Cladastis), Cucurbitaceae, Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae; (2) to re-evaluate the water gap...
of Fabaceae (Tribe Cercideae); (3) to confirm (or not) the morpho-anatomy of the water gaps of Bixaceae and Cistaceae; and (4) to devise a classification scheme for water-gap regions and their morpho-anatomical features.

MATERIALS AND METHODS

Seed sources

Mature fruits and seeds for the present study were obtained from commercial and personal seed collections from Australia, China, France, Taiwan and the USA (Table 1). All seeds were stored at room temperature (approx. 23 °C and 50–60 % relative humidity, dry storage) until used. In this study, only the impermeable seeds in the seed lots were used. To select the fraction of impermeable seeds/fruits, seeds/fruits of each species were allowed to imbibe in distilled water in glass beakers for 3 d at ambient room conditions and any imbibed seeds/fruits were discarded prior to experiments.

PY-breaking

Wet and/or dry heat treatments were used to break PY in each study species (Table 1). Dormancy-breaking treatments were selected based on unpublished research of Gama-Arachchige et al. (1) Wet heat: depending on the availability of seeds, five replicates of one, five or 20 seeds were placed in a hot water bath. (2) Wet–dry heat: three replicates of 25 fruits of N. nucifera were subjected to alternating wet and dry heat by immersing them in boiling water for 5 min and then drying in an incubator at 40 °C for 24 h (repeated ten times). (3) Dry heat: seeds of K. paniculata and S. saponaria (five replicates of ten seeds each) were heated in an oven at 60 °C for 7 d and then stored in paper bags under ambient room conditions for 1 month. (4) Open flame: the hilar end of the seed of S. angulatus was held approx. 5 cm over a Bunsen burner flame for 30 s.

Morphological changes in the seed coat after PY-breaking

To study the ultra-morphological changes in the putative water-gap region after PY is broken, impermeable and permeable (heat-treated) seeds were mounted on scanning electron microscope specimen stubs using double-sided carbon tape. Seeds were sputter-coated with gold–palladium (15 nm), scanned with an S-3200 Hitachi scanning electron microscope at an acceleration voltage of 5-0 kV, and micrographs were taken.

Dye tracking

Dye tracking experiments were performed to determine the initial site of water entry into permeable seeds during imbibition. Heat-treated seeds (of all study species) were dipped in concentrated solutions of acid fuchsin or methylene blue. Three seeds each of each species were removed after 30 s, 1 min, 5 min and 10 min intervals and their longitudinal bissections were observed for staining under a dissecting microscope (Zeiss STEMI SVII). The fruits of N. nucifera were examined at 1 d intervals. The pathway of the dye was observed and micrographs were taken using a digital camera (Olympus DP25).

Blocking experiment

To study the role of the pseudolens, the micropyle and hilar slit in the early stages of imbibition of seeds of C. canadensis, seeds were immersed in boiling water for 10 s and then dried overnight at room temperature. One hundred seeds (five replicates of 20) were each blocked with Vaseline® with a sharpened toothpick at the (1) hilar slit and micropyle; (2) pseudolens and micropyle; and (3) pseudolens, micropyle and hilar slit. Non-blocked seeds were used as a control. Seeds were incubated in plastic Petri dishes filled with water for 7 d at 25 °C. The number of imbibed seeds (larger in size and lighter in colour) was counted at intervals of 24 h. The final imbibition percentage data were normalized by arcsine-transformation and analysed by one-way analysis of variance (ANOVA) using SAS ver. 9.2 software to determine significant differences between blocking treatments (P < 0.05).

Light microscopy

To compare the anatomy of the water-gap region and the general seed/fruit coat, microtome sections were used. Seeds/fruits of the study species were manually scarified and allowed to imbibe fully in water at ambient room temperature. Then, these seeds/fruits were glued to wooden blocks by applying Super-Glue® to one side of the specimen. Subsequently, depending on the species, 10–20 μm sections (longitudinal and transverse) were cut using a microtome (Leica RM 2135). Sections were stained with 1 % safranin and/or 2 % fast green solutions when necessary and observed under a light microscope (Olympus BX40) equipped with a digital camera (Olympus DP25), and micrographs were taken and compared.

RESULTS

Morphological changes in the seed coat after PY-breaking

Wet heat treatment caused the chalazal cap and plug to be dislodged, hence opening the chalaza in seeds of B. orellana and H. apenninum (Fig. 1A, B).

The hilar slit is tightly closed in PY seeds of S. angulatus and, after the open flame heat treatment, it forms a concave slit-like opening (Fig. 1C). No other opening or morphological change was observed between impermeable and permeable seeds.

The micropyle and hilar slit are located perpendicular to each other in the hilar end of the seeds of C. canadensis. After 10 s in boiling water, a blister (pseudolens) formed immediately adjacent to the micropyle (Fig. 1D). Moreover, the micropylar and hilar openings widened. Unlike C. canadensis, only the pseudolens popped open in B. acuminata seeds after the PY-breaking treatment (Fig. 1E). In C. kentukea seeds, the lens split and formed a narrow slit after the wet heat treatment (Fig. 1F).

After the wet heat treatments, a circular opening formed in the chalaza of A. digitata and G. ulmifolia, while a narrow oval-shaped opening formed in the chalaza of B. cordifolia (Fig. 1G–I).

The protuberance of impermeable N. nucifera was visible as a slightly raised area in the fruit wall near the persistent style (not shown). After the heat treatment, the outer epidermal layer and palisade layer in the protuberance became dislodged and a
Fig. 1. Scanning electron micrographs of water-gap regions of non-dormant (heat-treated) seeds or fruits. (A) Chalazal region of *Bixa orellana* without outer permeable cell layers. (B) Chalazal region of seeds of *Helianthemum apenninum* without outer permeable cell layers. (C) Hilar region of *Sicyos angulatus*. (D) Hilar region of *Cercis canadensis*. (E) Pseudolens (open) of *Bauhinia acuminata*. (F) Lens (open) of *Cladrastis kentukea*. (G) Chalazal region of *Adansonia digitata* without outer permeable cell layers. (H) Chalazal region of *Guazuma ulmifolia* without outer permeable cell layers. (I) Chalazal region of *Berrya cordifolia* without outer permeable cell layers. (J) Protuberance (open) of fruit of *Nelumbo nucifera* without outer permeable cell layers. (K) Hilar region of *Ceanothus americanus*. (L) Micropylar region...
circular gap was formed, revealing the sclerenchyma cell layer and a narrow opening to the cavity of the protuberance (Fig. 1J).

In wet heat-treated seeds of *C. americanus*, the hilar slit ruptured and widened (Fig. 1K). No other visible changes on the seed coat were observed. The outermost layer of the heart-shaped aril region of *C. halicacabum* consists of spongy mesophyll cells, whereas palisade cells form the outermost layer of the other part of the seed (Fig. 1L). After wet heat treatment, a slit was formed along the margin of the aril region starting at the micropyle (Fig. 1L). In seeds of *K. paniculata*, the hilar plug separated from the palisade cells after wet heat treatment, thus widening the hilar rim (Fig. 1M). The dry heat treatment caused the palisade cells to crack, and blisters formed all over the seed (Fig. 1N, O); however, no changes were observed on the hilar region. The hilar slit opened in seeds of *S. saponaria* after the PY break (Fig. 1P). Moreover, perpendicular splits in the palisade layer formed in the hilar region. These splits extended up to the sclerenchyma layer beneath the palisade layer. After the wet heat treatment, a suture originated at the carpellary hilum of *S. spathulatum* and continued around the radicle side of the endocarp (Fig. 1Q). The suture did not form on the vascular bundle side of the endocarp.

**Dye tracking**

The time taken for entry of the dye into seeds varied among species. In *H. apenninum* and *G. ulmifolia*, dye was first observed in the chalazal opening after 30 s (Figs 2A, B and 3A, B), while in *B. cordifolia*, dye was first observed in the chalazal opening after 1, 1 and 10 min, respectively (Figs 2C–E and 3C–E). In *C. americanus*, *S. angulatus* and *S. saponaria*, the dye entered into seeds via the hilar slit and was observed in adjacent tissues after 30 s (Figs 2F–H and 3F–H). In fruits of *S. spathulatum*, the dye entered into the endocarp through the carpellary slit and carpellary–hilar suture, and reached the embryo after 1 min (Figs 2I and 3I). The dye did not enter through the vascular bundle side of the endocarp. *Nelumbo nucifera* fruits imbied very slowly and the dye was first observed in the cavity of the protuberance after 48 h (Figs 2J and 3J). In *C. halicacabum* seeds, the dye was first observed in the micropylar slit after 1 min (Figs 2K and 3K). Dye entered through the hilar rim in wet heat-treated seeds of *K. paniculata* and was observed in inner tissues after 3 min (Figs 2L and 3L), while in dry heat-treated seeds the dye entered through blisters (figures not shown).

During the dye-tracking experiment, the pseudolens of *C. canadensis* swelled but was not dislodged (Figs 2M and 3M). Dye was observed in the palisade cells of the pseudolens and in the upper portion of the hilar and micropylar palisade cells, but not in the pseudolens opening even after 4-5 h (Figs 2M and 3M). However, after 4-5 h, moisture was observed in the sclerenchyma cells under the pseudolens near the micropyle. Dye entered into the seeds of *B. acuminata* and *C. kentukea* through the pseudolens gap and lens slit, respectively, and it was observed in these regions after 30 s (Figs 2N, O and 3N, O).

**Blocking experiment**

Blocking the pseudolens + micropyle + hilar slit of *C. canadensis* completely inhibited the water uptake during the incubation period of 120 h, while 97 % of the non-blocked seeds imbied within the first 24 h (Fig. 4). Imbition in seeds with the hilar slit + micropyle or the pseudolens + micropyle blocked was slower than it was in non-blocked seeds, with the former category imbibing more rapidly (90 % by 48 h) than the latter (10 % by 48 h). The rank order of the imbition rate was non-blocked > hilar slit + micropyle blocked > pseudolens + micropyle blocked > pseudolens + micropyle + hilar slit blocked.

**Light microscopy**

An epidermal layer, multiple sub-epidermal layers, a palisade layer and several layers of sclerenchyma cells comprise the seed coat of *S. angulatus* (Fig. 5A). The embryo is enased by the nucellar–endosperm casing. Palisade cells have narrow lumens that branch near the upper and lower periclinal walls (Fig. 5B). The light line runs very close to the upper periclinal wall. Near the hilar slit, palisade cells gradually become shorter (Fig. 5C). Furthermore, several layers of flattened sub-epidermal parenchyma cells are present near the hilar slit (Fig. 5D). Two bulges, each with two lobes, are located at the hilar end (Fig. 5E). These bulges are formed by loosely arranged thick-walled sclerenchyma cells (Fig. 5F).

The seed coat of *C. canadensis* consists of a palisade layer and several layers of sclerenchyma cells (Fig. 6A). Near the hilar slit, the palisade cells are slightly longer than those elsewhere in the seed coat. At the hilar slit, palisade cells are slightly shorter and curved (Fig. 6A, C). The light line runs through the upper quarter of the palisade cells and near the hilar slit, and ascends to the lower quarter of the cell. The palisade cells of the pseudolens are similar in length to those of hilar palisades (Fig. 6A, B, D). A thick layer of sclerenchyma cells is present at the hilar end of the seed, and vascular bundles penetrate through this layer (Fig. 6A). The sclerenchyma cells are tightly arranged on the pseudolens side and loosely arranged on the vascular bundle side (Fig. 6A, B, D).

Seed coats of both *A. digitata* and *B. cordifolia* consist of an exotegmic palisade layer that varies little throughout the seed coat (not shown). However, this layer is discontinued at the chalazal opening. Palisade cells at the margin of the chalazal opening are slightly shorter and more curved than those in the rest of the seed coat. The chalazal plug is located below the palisade layer of the chalazal region. Tightly packed, dark-coloured, thin-walled sclerenchyma cells of the upper portion of the chalazal plug fill the gap in the chalazal opening.

of Cardiospermum halicacabum. (M) Hilary region of wet heat-treated Koelreuteria paniculata. (N) Hilary region of dry heat-treated Koelreuteria paniculata. (O) Seed coat away from the hilar region of dry heat-treated Koelreuteria paniculata. (P) Hilary region of Segundus saponaria. (Q) Endocarp circumlinear suture of Stylotus spathulatum without outer permeable mesocarp and exocarp cell layers. Abbreviations: Ar, aril; Bg, bulges of the hilar region; Bl, blisters formed in the palisade layer; Cho, chalazal oculus; Chp, chalazal plug; Chs, chalazal slit; Crh, carpellary hilar slit; Crm, crystalline cells of the mesocarp; Ecs, endocarp circumlinear suture; Em, epidermal mesophyll; Ep, epidermal cell layers; Hc, hilar cracks; Hp, hilar plug; Hr, hilar rim; Hs, hilar slit; Hsr, ruptured hilar region; Ls, lens slit; Mi, micropyle; Ms, micropylar slit; Pl, pseudolens; Plg, pseudolens gap; Pm, mouth of protuberance; Pr, protuberance gap.
Fig. 2. Light micrographs of longitudinal sections of water-gap regions of seeds or fruits imbibed in acid fuchsin/methylene blue for different periods of time. (A–E) Chalazal region of *Helianthemum apenninum*, *Guazuma ulmifolia*, *Bixa orellana*, *Berrya cordifolia* and *Adansonia digitata*, respectively. (F–H) Hilar region of *Ceanothus americanus*, *Sicyos angulatus* and *Sapindus saponaria*, respectively. (I) Carpellary hilar region of the endocarp of *Stylobasium spathulatum*. (J) Protuberance region of fruit of *Nelumbo nucifera*. (K) Aril–micropylar region of *Cardiospermum halicacabum*. (L) Hilar region of *Koelreuteria paniculata*. (M and N) Hilar region of *Cercis canadensis* and *Bauhinia aemumina*. (O) Hilar–lens region of *Cladrastis kentukea*. Abbreviations: Ar, aril; Bg, bulges of the hilar region; Cho, chalazal oculus; Chp, chalazal plug; Chs, chalazal slit; Crh, carpellary hilar slit; Dy, dye; Em, embryo; En, endosperm; Ep, epidermal cell layers; Hc, hilar cracks; Hp, hilar plug; Hs, hilar slit; L, lens; ll, light line; Mi, micropyle; Nu, nucellus; Pa, palisade cells; Pb, protuberance cavity; Pl, pseudolens; Plg, pseudolens gap; Ra, radicle; Sc, sclerenchyma; St, seed coat; Sy, style; Vb, vascular bundle. The time given in each figure is the duration of imbibition in the dye; * indicates regions which have imbibed.
FIG. 3. Light micrographs of the surface view of water-gap regions of seeds or fruits imbibed in acid fuchsin/methylene blue for different periods of time. (A, B) Chalazal regions of *Helianthemum apenninum* and *Guazuma ulmifolia*, respectively, with outer permeable layers removed to expose the chalaza. (C–E) Chalazal regions of *Bixa orellana*, *Berrya cordifolia* and *Adansonia digitata*, respectively, with outer permeable and impermeable palisade layers removed to expose the chalazal plug. (F–H) Hilar regions of *Ceanothus americanus*, *Sicyos angulatus* and *Sapindus saponaria*, respectively. (I) Carpellary hilar region of the endocarp of *Stylosanthes spathulata*. (J) Protuberance region of fruit of *Nelumbo nucifera*. (K) Aril–micropylar region of *Cardiospermum halicacabum*, with the impermeable palisade layer removed to expose the radicle. (L) Hilar region of *Koelreuteria paniculata*. (M, N) Hilar region of *Cercis canadensis* and *Bauhinia acuminata*, respectively. (O) Hilar–lens region of *Cladrastis kentukea*. Abbreviations: Ar, aril; Bg, bulges of hilar region; Cho, chalazal oculus; Chp, chalazal plug; Crh, carpellary hilar slit; Dy, dye; Ecs, endocarp circumlinear suture; Ep, epidermal cell layers; Hc, hilar cracks; Hp, hilar plug; Hs, hilar slit; L, lens; Mi, micropyle; Pa, palisade cells; Pl, pseudolens; Plg, pseudolens gap; Po, protuberance gap; Ra, radicle. The time given in each figure is the duration of imbibition in the dye.
The pericarp of *N. nucifera* is composed of an epidermal layer, a sub-epidermal palisade layer, a wide layer of sclerenchyma and several layers of parenchyma cells (Fig. 7A). The protuberance organ is located in the sclerenchyma layer near the stylar end of the fruit (Fig. 7B). It is outlined by modified sclerenchyma cells and crystalliferous cells (Fig. 7B–E). The cavity of the protuberance organ is formed by degeneration of crystalliferous cells (Fig. 7B, E). Its mouth is occluded by slightly shorter palisade cells and elongated sclerenchyma cells (Fig. 7C). Vascular bundles run through the parenchyma cell layer, and one of them ends at the protuberance organ (Fig. 7B, F).

In *C. americanus*, a palisade layer and several layers of crushed mesophyll cells located below the palisade cells comprise the seed coat (Fig. 8A). Palisade cells near the hilar slit are approx. 1.5 times longer than those located elsewhere in the seed coat (Fig. 8B). In palisade cells away from the hilum, the light line runs very close to the upper periclinal wall, and near the hilum it gradually descends to one-third of the length of the cell.

The seed coat of *C. halicacabum* is composed of a single layer of palisade cells and multiple layers of sclerenchyma cells (Fig. 9A, B). The palisade cells of the aril region differ from those of the micropylar region (Fig. 9C, D). Palisade cells of the aril region are irregular in size, slightly convex, lack a light line, are hyaline and have small lumens containing rhomboidal oxalate crystals (Fig. 9C). Moreover, several layers of parenchyma cells can be seen attached to the outer periclinal walls of the palisade cells of this region. The micropylar palisade cells are columnar, uniform, with large lumens containing a brownish material, and possess a light line (Fig. 9D). The sclerenchyma layer is thinner near the micropyle due to the presence of the radicle (Fig. 9E). Near the micropyle, a slightly discernible marking (rupture line) separates the micropylar sclerenchyma cells from hilar sclerenchyma (Fig. 9F). Blisters were observed on imbibing seeds and were formed due to rupturing of palisade cells at the light line (Fig. 9G).

The seed coat of *K. paniculata* consists of a palisade cell layer and multiple layers of sclerenchyma cells, except at the hilar region (Fig. 10A–D). The light line of the palisade cells runs through the outer one-third of the cell (Fig. 10D). The hilar–micropylar region is closed with a hilar plug formed by sclerenchyma cells and mesophyll cells (Fig. 10A–C). A part of the plug protrudes outwards out of the palisade layer. The plug is formed by a mass of sclerenchyma cells of which the region embedded in...
the seed coat is lined by an outer annulus of several layers of spongy mesophyll cells. The bottom of the plug is lined by crushed remnant cells of the endosperm (Fig. 10A). An opaque spongy mesophyll cell mass is located at the micropylar region, closer to the bottom of the hilar plug (Fig. 10B, C). A vascular bundle runs through the hilar plug and continues beneath the sclerenchyma layer, diverging from the micropylar side. Blisters on the seed coat were observed in dry heat-treated seeds (Fig. 10E, F). Microtome sections through these blisters showed that they are formed at the light line of the palisade layers due to the formation of cracks (Fig. 10E); these cracks continued to form ruptured areas in the palisade cells (Fig. 10F).

The seed coat of *S. saponaria* is composed of a palisade cell layer and multiple layers of sclerenchyma cells (Fig. 11A). At the hilar slit, absence of these cell layers results in a dome-shaped cavity (Fig. 11B). The palisade cells near the hilum are almost twice the length of those away from the hilum (Fig. 11B, C). They gradually become shorter towards the hilar slit (Fig. 11B). The light line of the palisade cells runs near the centre of the cells in the hilar region but closer to the upper periclinal wall elsewhere (Fig. 11A, B). Blisters similar to those in seeds of *K. paniculata* were observed in seeds of *S. saponaria* after dry heat treatment (Fig. 11D, E).

The pericarp of *S. spathulatum* consists of two discernible areas: (1) the outer mesocarp with spongy mesophyll cells bound on the outside by a thin exocarp (not shown in figures) and on the inside by crystalliferous cells; and (2) the inner endocarp with a single palisade layer that has a thick light line and a broad layer of irregularly oriented hyaline sclerenchyma cells (Fig. 8C). The inner endocarp epidermis is bound by radially elongated, brownish parenchyma cells (Fig. 8D, E). The endocarp discontinues at the carpellary–hilar opening. The sclerenchyma cells of the endocarp suture are brownish in colour. The circumlinear endocarp suture is formed by rupturing of palisade cells and by slightly yellowish coloured sclerenchyma cells of the endocarp (Fig. 8E).

**DISCUSSION**

In the present study, ten new water gaps were identified and characterized morphologically and anatomically in seven families, and two water gaps in Bixaceae and Cistaceae previously hypothesized to exist by Baskin et al. (2000) were confirmed. Information on all water gaps known to occur in angiosperms is summarized on Table 2. Based on the location, anatomy and morphology, there are 24 different kinds of water-gap regions in 16 families; water gaps in fruits of Bibersteiniaeaceae and Lauraceae remain to be characterized (Table 2). New names were assigned to certain water gaps that had been previously reported in the literature for clarity and to avoid ambiguity. Circular water gaps with plug-like structures occluding the opening were given the name ‘oculus’ (eye), circular or narrow linear water gaps occluded by lid-like structures formed from palisade cells were named ‘gap’ and narrow linear water gaps were named ‘slit’. Jayasuriya et al. (2009) characterized the water-gap region of Convolvulaceae and called it the bulge gap adjacent to the micropyle, except for *Cuscuta*, in which the water gap is the hilar slit. However, the two bulges are located on the opposite ends of the hilar slit. Thus they are more closely located to the hilum than to the micropyle and could possibly be an extension of the hilum itself. For these reasons, the water gap was renamed ‘bulge gap adjacent to the hilum’.

The lens gap acts as the water gap in most PY species in Fabaceae. The lens and micropyle in this family are usually located on the opposite side of the hilar slit (Lersten et al., 1992). However, in the case of seeds of subfamily...
Caesalpinioideae tribe Cercideae, the lens is located next to the micropyle on the same side of the hilar slit; thus, this structure is called the ‘pseudolens’ (Lersten et al., 1992). Based on the results of dye tracking and blocking experiments, it was shown that the pseudolens acts as the water gap in seeds of *C. canadensis* and *B. acuminata*. However, in *C. canadensis*, the micropyle and hilar slit are also responsible for initial water imbibition. Therefore, all three structures are involved in initial water uptake. This is the first report on the role of the pseudolens as the water gap, thus adding a new kind of water gap to the family Fabaceae. Moreover, similar to other clades in Papilionoideae except Genistoids *sensu lato* (where the water gap is the hilar slit), the lens slit acts as the water gap in the clade Cladrastis.

In the present study, three new water gaps were identified in the subfamily Sapindoideae (Sapindaceae). Three of the four kinds of water-gap regions in this family are associated with the hilum, and only the water gap of tribe Paullinieae is associated with the micropyle (Turner et al., 2009; Table 2). In seeds of *K. paniculata*, dry and wet heat treatments act differently on PY break, causing the formation of two different openings in the seeds. Wet heat dislodged the hilar plug, allowing the seeds to imbibe water through the hilaroculus. After dry heat treatment, on the other hand, blisters formed all over the seed coat, especially near the hilar region, and they functioned as the water gap. This indicates that the formation of water gaps can differ depending on the PY-breaking treatment. However, seeds collected in the spring of 2012 under the trees (possibly dispersed the previous autumn) on the campus of University of Kentucky had dislodged hilar plugs, indicating that the hilar oculus acts as the water gap under natural conditions (N. S. Gama-Arachchige, pers. obs.). The water-gap region of *K. paniculata* is similar in several ways to the chalazal oculus of Bixaceae, Cistaceae, Malvaceae, Sarcolaenaceae and Sphaerosepalaceae. In seeds of all these species, the water gap is occluded by a plug-like structure formed by water-impermeable sclerenchyma cells. During PY break, this plug is pushed slightly into the seed and forms a circular opening (oculus) through which the seeds imbibe water.

In the family Surianaceae, only the genus *Stylobasium* has been shown to contain a water-impermeable endocarp (Baskin et al., 2006). The water gap of *Stylobasium* is rather different from all the other water-gap regions. Unlike water-gap regions in other taxa, a suture (endocarp circumlinear suture) is formed all around the endocarp as PY is broken. Moreover, compared with other species the water-gap complex of *Stylobasium* is morpho-anatomically simple.

Ohga (1926) studied the pericarp anatomy of *N. nucifera* and reported a specialized structure known as the protuberance organ near the stylar end in the fruit. In the present study, dye-tracking
Fig. 8. Longitudinal sections of hilar and non-hilar regions of the seed coat of Ceanothus americanus and endocarp of Stylobasium spathulatum. (A) Seed coat of the non-hilar region of C. americanus. (B) Hilar region of C. americanus. (C) Endocarp of the non-carpellary hilar region of S. spathulatum. (D) Carpellary hilar region of S. spathulatum. (E) Inner part of the endocarp of the non-carpellary hilar region of S. spathulatum. Abbreviations: Crm, crystalliferous cells of mesocarp; Crh, carpellary hilar slit; Eip, elongated parenchyma cells of inner wall of endocarp; En, endosperm; Hpa, hilar palisade cells; Hs, hilar slit; Ip, parenchyma cells of inner wall of endocarp; ll, light line; Mms, multiple layers of mesophyll cells of seed coat; Pa, palisade cells; Sc, sclerenchyma; Sc*, yellowish sclerenchyma cells of endocarp.

Fig. 9. Longitudinal sections of the micropylar and non-micropylar regions of the seed coat of Cardiospermum halicacabum. (A) Seed coat of the micropylar region. (B) Seed coat of the aril region. (C) Close-up of the seed coat of the aril region. (D) Close-up of the seed coat of the micropylar region. (E) Seed coat of the micropylar region. (F) Close-up of the micropylar region. (G) Seed coat of imbibing seeds with blisters. Abbreviations: Bl, blister; Cs, crystals; Em, epidermal mesophylls; ll, light line; Mi, micropyle; Pa', palisade cells of aril region; Pa'', palisade cells of micropylar region; Ra, radicle; Sc, sclerenchyma.
FIG. 10. Longitudinal sections of hilar and non-hilar regions of the seed coat of *Koelreuteria paniculata*. (A) Hilar region. (B) Close-up of the micropylar side of the hilar region. (C) Close-up of the vascular bundle side of the hilar region. (D) Seed coat (dry heat treated) of the non-hilar region without blisters. (E) Initial stage of blister formation of palisade cells of the seed coat (dry heat treated) of the non-hilar region. (F) Seed coat (dry heat treated) of the non-hilar region with blisters. Abbreviations: Bl, blisters; Cr, crack of palisade cells at light line; En, endosperm; Hp, hilar plug; ll, light line; Mi, micropyle; Pa, palisade cells; Sc, sclerenchyma; Sp, spongy mesophyll cells; Sp*, spongy mesophyll cell mass of hilar plug; Vb, vascular bundle.

FIG. 11. Longitudinal sections of hilar and non-hilar regions of the seed coat of *Sapindus saponaria*. (A) Seed coat of the non-hilar region. (B) Hilar region. (C) Palisade cells of the hilar region. (D) Initial stage of blister formation of palisade cells of the seed coat (dry heat treated) of the non-hilar region. (E) Seed coat (dry heat treated) of the non-hilar region with blisters. Abbreviations: Bl, blister; Cr, crack of palisade cells at light line; Hs, hilar slit; ll, light line; Pa, palisade cells; Pa*, elongated palisade cells of hilar region; Sc, sclerenchyma.
<table>
<thead>
<tr>
<th>Taxa</th>
<th>Species</th>
<th>Water gap</th>
<th>Secondary opening(s)</th>
<th>Type of water-gap complex</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1 Anacardiaceae</td>
<td>Rhus glabra</td>
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<td>3 Biebersteinia heterotemont</td>
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<td>8 Dipterocarpaceae</td>
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<td>Christiansen and Moore (1959)</td>
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### Table 2. Continued

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<tr>
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<td><em>Cladodiscia floribunda</em></td>
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<td>Type-I simple</td>
<td><em>Turner et al.</em> (2009)</td>
</tr>
<tr>
<td><em>Dodonaea petiolaris</em></td>
<td>Gap to hilum</td>
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<td>Type-I compound</td>
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<td><em>Koelreuteria paniculata</em></td>
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<td>This study</td>
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<td><em>Cardiospermum halicacabum</em></td>
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<td>23</td>
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<td><em>Leptolaena pauciflora</em></td>
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<td>4*</td>
<td>Type-III simple</td>
<td><em>Nandi</em> (1998)</td>
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<td><em>Dialyceras parvifolium</em></td>
<td>Circumlinear endocarp suture</td>
<td>24</td>
<td>Carpellary hilar slit</td>
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</table>

Water-gap complexes in physically dormant species


Based on the current data, there are six different kinds of water-gap regions associated with the chalaza, present in those six aforementioned families (Table 2).

#### Water-gap complex

*Gama-Arachchige et al.* (2011) introduced the term ‘water-gap complex’ to describe the water-gap region of *G. carolinianum* (Geraniaceae). In general, the water-gap complex in all species with PY is a morpho-anatomically complex structure and is composed of (1) an opening formed after PY break; (2) specialized structures that occlude the gap; and (3) associated specialized tissues. Therefore, the term ‘water-gap complex’ is an appropriate term to define the whole water-gap region, and the term ‘water gap’ is suitable when referring to the actual opening. Based on the morphology of the water-gap opening and the anatomy of the occluding structures, the water-gap complexes can be divided into three types: Type-I, Type-II and Type-III (Fig. 12). Type-I water-gap complexes are the water gaps with narrow linear openings usually occluded by modified elongated palisade cells. Type-II water-gap complexes are circular or narrow linear openings occluded by lid-like structures formed by the palisade cells, and Type-III water-gap complexes are either narrow linear or circular openings occluded by plug-like structures usually formed by water-impermeable sclerenchyma cells.

In some species, more than one opening is involved in the early stages of imbibition after PY is broken (Table 2). In genera such as *Canna, Cercis, Geranium, Koelreuteria, Kosteletzya, Rhus, Sida* and *Stylobasium*, the water gap along with other closely-located structures such as micropyle, chalaza, hilum, carpellary hilar slit or carpellary micropyle are involved in initial water uptake. In the case of *Ipomoea* spp., two identical water-gap openings are involved in early imbibition. Therefore, water-gap
complexes can be divided into two groups based on the number of openings involved in early imbibition: (1) a simple water-gap complex, where only one opening is involved in initial water imbibition; and (2) a compound water-gap complex, where two or more openings are involved in initial imbibition.

The current study is the most recent and most detailed analysis of different water-gap complexes in seeds and fruits of PY/CD species. In this study, for the first time: (1) ten new water gaps were morpho-anatomically characterized in seven families with PY/CD; and (2) a scheme was proposed for classifying water-gap complexes in 16 of the 18 angiosperm families known to have PY. The classification recognizes three basic types (I, II and III) which are further sub-divided into simple and compound water-gap complexes based on the number of openings involved in the initial water uptake. Moreover, the outcomes of this study provide a basis for developing an identification key for different kinds of water-gap complexes in PY seeds/fruits which will be discussed in a future article.

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LITERATURE CITED


Gama-Arachchige et al. — Water-gap complexes in physically dormant species


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