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Embryology of *Phyllonoma* (Phyllonomaceae, Aquifoliales): characteristics and character evolution

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Abstract *Phyllonoma*, the sole genus of Phyllonomaceae (Aquifoliales) consisting of four Central American species, has not been well-characterized morphologically. Following a previous study of flower and inflorescence morphology, I here report the embryology of the genus based on *P. tenuidens* and compare its characteristics with those of other aquifolialean families, namely, Aquifoliaceae, Cardiopteridaceae, Helwingiaceae, and Stemonuraceae. Comparisons indicate that although *Phyllonoma* resembles all the other families embryologically, it more closely resembles Aquifoliaceae and Helwingiaceae in lacking a vascular bundle in its integument and bearing ab initio Cellular endosperm. The genus especially resembles Helwingiaceae by possessing a tenuinucellate ovule. This result corroborates molecular and floral morphological evidence, supporting the distinctness of *Phyllonoma* as a family and its sister-group relationship to East–Asian Helwingiaceae. However, *Phyllonoma* is clearly distinguished from Helwingiaceae by seed coat structure. In *Phyllonoma*, the seeds (dispersed in berries) have a thick seed coat composed of irregularly enlarged, thick-walled exotestal cells, whereas the seeds (dispersed in drupes) have a thin membranous seed coat in Helwingiaceae. Taken together with earlier information on pollination (entomophily in *Phyllonoma* versus ambophily in Helwingiaceae), embryological evidence shows that distinct evolution has occurred in reproductive characters relating to pollination and seed dispersal in *Phyllonoma*.

Keywords Aquifoliales · Embryology · *Phyllonoma* · Phyllonomaceae

Introduction

Phyllonoma, a genus consisting of four tree or shrub species, occurs from Mexico to Peru (Mori and Kallunki 1977). In the past, it had been placed in various families such as Saxifragaceae (Bentham and Hooker 1865; de Candolle 1873; Engler 1891, 1930; Schulze–Menz 1964), Grossulariaceae (Cronquist 1968, 1981; Mori and Kallunki 1977), Escalloniaceae (Hutchinson 1967, 1973), and Phyllonomaceae (Rusby 1905; Takhtajan 1969, 1997, 2009). However, based on molecular analyses (e.g., Bremer et al. 2002; Kårehed 2001; Olmstead et al. 2000), the Angiosperm Phylogeny Group (2003, 2009) has consistently accepted its own family Phyllonomaceae in the asterid order Aquifoliales (see also Reveal 2012; Stevens 2001 onwards). Within Aquifoliales, Phyllonomaceae are sister to East-Asian Helwingiaceae (*Helwingia* only); a clade of Phyllonomaceae and Helwingiaceae is sister to Aquifoliaceae (*Ilex* only); and a clade consisting of the three families is sister to a clade of Cardiopteridaceae and Stemonuraceae (Soltis et al. 2011; Tank and Donoghue 2010). Until I recently presented a study of its flower and inflorescence morphology (Tobe 2013, 2014), *Phyllonoma* had been poorly understood morphologically (Stevens 2001 onwards). The analyses of flower and inflorescence morphology showed that *Phyllonoma* shares an epiphyllous cymose inflorescence, inferior ovary, and epigynous disc nectary with Helwingiaceae, but differs clearly from

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Helwingiaceae in having glandular trichomes on its sepal margins and a bicarpellate, unilocular gynoecium bearing many ovules on parietal placentae (for more detail see Tobe 2013, 2014).

While examining the flower and inflorescence morphology, I investigated the embryology of *Phyllonoma* to explore evolution in other morphological characters. Embryology has previously provided data for more than 50 characters associated with anther, ovule, and seed development, and thus contributes to a better understanding of the relationships of families (Tobe 1989; for most recent examples, see Yamamoto et al. 2014: Biebersteiniaceae, Sapindales; Ao and Tobe 2015: Helwingiaceae, Aquifoliales). Among the embryological characters, those of seed coat development and structure have often provided evidence for the adaptive evolution of a species or species group (e.g., Tobe et al. 1987: *Oenothera*, Onagraceae). Earlier descriptions of embryological features of *Phyllonoma* are limited to brief descriptions of the ovules and seeds published by Krach (1976), Mauritzon (1933), Takhtajan (2000), and van Tieghem (1898). Van Tieghem (1898, p. 207) described the ovule of *Phyllonoma* as having a thin and ephemeral nucellus with a single integument. Mauritzon (1933, p. 126–127) confirmed this observation in *P. ruscifolia* Willd. ex Schult. Krach (1976) examined mature seed structures of *P. ruscifolia*, describing that the seed coat is composed of a multi-layered outer integument and a two-layered inner integument. However, as I show later, the ovule of *Phyllonoma* is unitegmic and not bitegmic as Krach (1976) observed. Takhtajan (2000), without referring to its development, described the mature seed coat of *P. laticuspis* (Turcz.) Engl. and *P. ruscifolia* as consisting of the exotesta and endotesta with negligible remnants of mesotestal layers. Except for these data, no information is available for embryological characters.

In this paper I present the first description of many embryological characters for *Phyllonoma* or Phyllonomaceae. Based on results obtained, I will compare *Phyllonoma* (Phyllonomaceae) with four other families of Aquifoliales, particularly with Helwingiaceae. Although available information on the other Aquifoliales except for Helwingiaceae is currently insufficient (Ao and Tobe 2015; see also Stevens 2001 onwards), the comparisons seem useful to clarify how *Phyllonoma* is likely related to the four other families embryologically, and to show which characters we should evaluate in studies of the poorly known families (i.e., Aquifoliaceae, Cardiopteridaceae and Stemonuraceae) for a more critical comparison.

Materials and methods

Flower buds and fruits of *Phyllonoma tenuidens* Pittier in various stages of development were collected from Provincia

Fig. 1 Development of anthers and microspores in *Phyllonoma tenuidens*. **a** Flowers on leaf. **b** *Longitudinal section* of flower bud. **c** *Transverse section* (TS) of anther in flower bud. **d** TS of young anther wall. Note that the middle layer has a common histogenetic origin with the endothecium. **e** TS of young anther wall showing binucleate tapetal cells and simultaneous cytokinesis in meiosis of microspore mother cells. **f** TS of older anther showing microspores arranged tetrahedrally. **g** TS of mature anther. *Arrowheads* indicate positions of longitudinal slit. **h** TS of mature anther wall enlarged. **i** Two-celled mature pollen grains. *ent* endothecium, *ep* epidermis, *gc* generative cell, *ml* middle layer, *mmc* pollen mother cell, *ov* ovule, *pe* petal, *se* sepal, *st* stamen, *t* tapetum, *v* nucleus of vegetative cell. *Asterisks* in **c** indicate two styles. *Scale bars* are 1 mm in **a**, 200 μm in **b** and **c**, 100 μm in **g**, 20 μm in **e**, **f** and **h**, and 10 μm in **d** and **i**

Puntarenas, Costa Rica (voucher: *William A. Haber 12765*, MO). They were fixed in FAA (5 parts stock formalin, 5 parts glacial acetic acid, 90 parts 70 % ethanol). Flower buds and seeds were dehydrated via an ethanol series, and then embedded in Technovit 7100 (Kulzer, Wehrheim, Germany) for microtoming. Serial resin sections cut at a thickness of 5–7 μm were stained with Heidenhain's hematoxylin and mounted in Entellan. All the microtome sections and acetocarmine-stained pollen grains were observed with an Olympus BX-51 microscope (Tokyo, Japan).

Results

Anthers and microspores

Flowers are bisexual and borne on a small epiphyllous cymose inflorescence (Fig. 1a). Each flower bears five sepals, five petals, five stamens, and a bicarpellate gynoecium with an inferior ovary and two short styles (Fig. 1b, c). The anther of a stamen is tetrasporangiate (Fig. 1b, c). Prior to maturation, its wall is composed of four cell-layers: an epidermis, an endothecium, one middle layer, and a tapetum (Fig. 1d). The middle layer has a common histogenetic origin with the endothecium (Fig. 1d). Therefore, wall formation conforms to the "Dicotyledonous" type (Davis 1966, p. 10). The tapetum is glandular (Fig. 1e, f). Its cells are initially uninucleate and later become binucleate (Fig. 1e). During maturation, the middle layer degenerates, and cells of both the epidermis and endothecium become enlarged (Fig. 1e). By the time of anther wall dehiscence, the cells of the endothecium develop fibrous thickenings (Fig. 1g, h). Although the cells of the epidermis are unspecialized, they are persistent. Anther dehiscence takes place by longitudinal slits, with each slit common to two microsporangia of the theca (Fig. 2g).

Meiosis in a microspore mother cell is accompanied by simultaneous cytokinesis (Fig. 1e), and the resultant microspore tetrads are predominantly tetrahedral (Fig. 1f). Pollen grains are two-celled when shed (Fig. 1i).
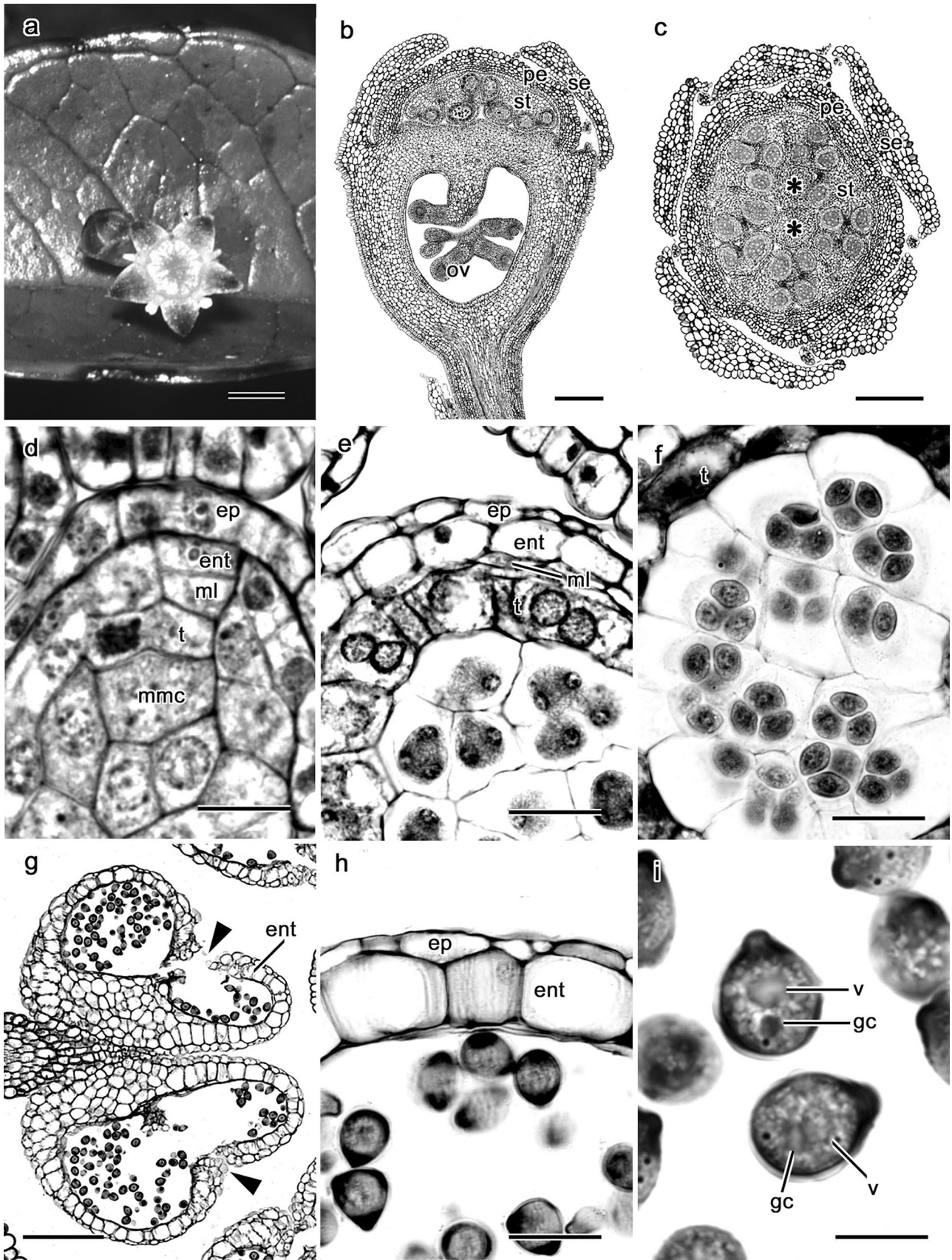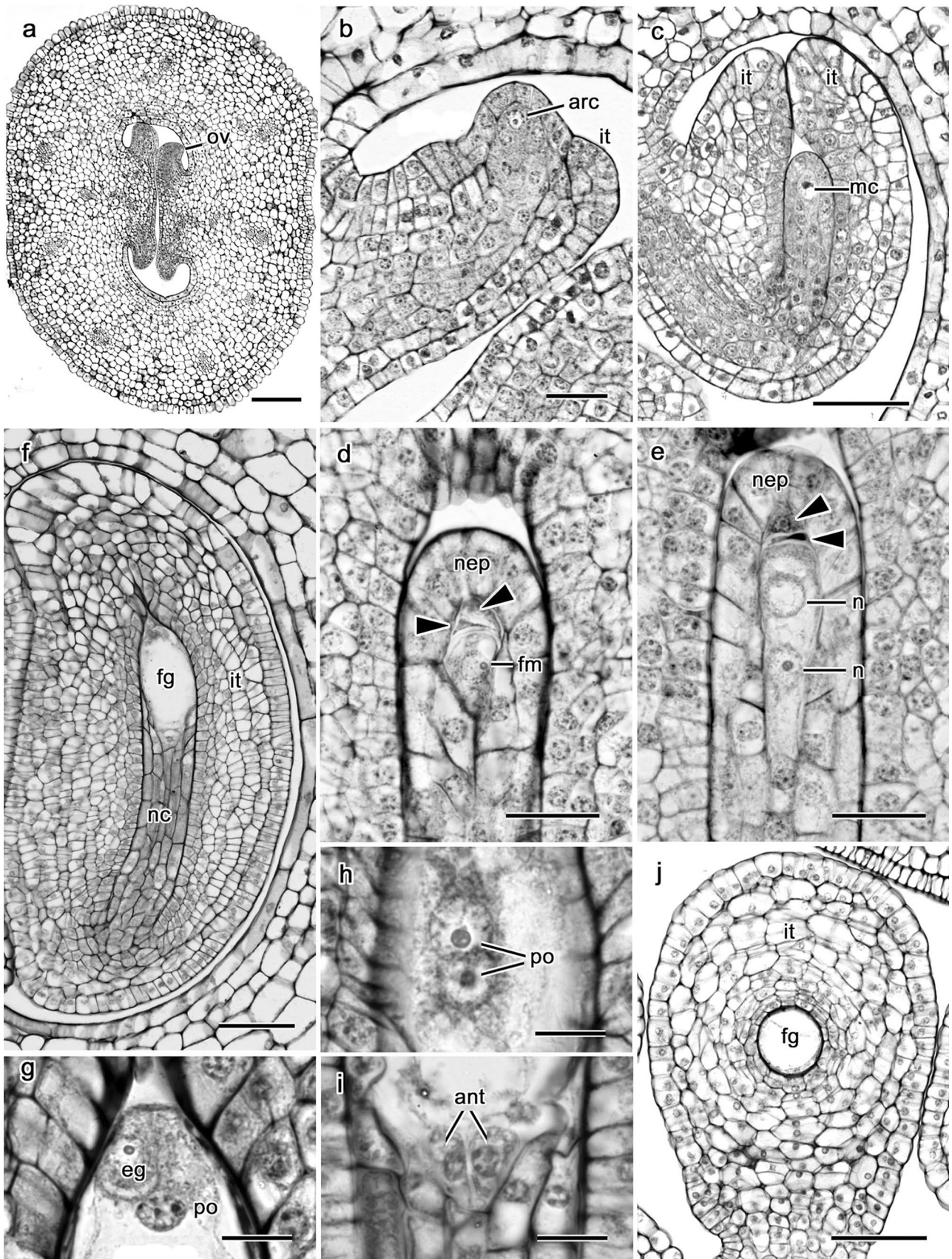
◀ **Fig. 2** Development of ovules and female gametophytes in *Phyllonoma tenuidens*. **a** *Transverse section* (TS) of gynoecium with ovule primordia. **b** *Longitudinal section* (LS) of ovule primordium with an archesporial cell. **c** LS of ovule with megaspore mother cell. **d** LS of ovule with functioning megaspore on chalazal side. Note two degenerating megaspores (*arrowheads*) on the micropylar side. **e** LS of ovule with two-nucleate female gametophyte and two degenerating megaspores (*arrowheads*). **f** LS of ovule with mature female gametophyte. **g** LS of mature female gametophyte, showing an egg cell and one polar nucleus (shortly moving to the middle of the central cell) on the micropylar side. Two synergids appear in the adjacent sections. **h** LS of mature female gametophyte, showing two polar nuclei in the middle of the central cell. **i** LS of mature female gametophyte, showing two antipodal cells. Another antipodal appears in adjacent section. **j** TS of mature ovule. *ant* antipodal cell, *arc* archesporial cell, *eg* egg cell, *fg* female gametophyte, *it* integument, *fm* functioning megaspore, *mc* megaspore mother cell, *n* nucleus in female gametophyte, *nc* nucellar tissue, *nep* nucellar epidermis, *ov* ovule primordium, *po* polar nucleus. Scale bars are 100 μm in **a**, 50 μm in **c–f** and **j**, 20 μm in **b**, and 10 μm in **g–i**

Ovules and female gametophytes

Approximately 13–14 ovules are borne from two parietal placentae in the single locule of the inferior ovary (Fig. 2a). The ovules are oriented variously, depending on their position in the locule as I documented previously (Tobe 2013). They tend to be erect with a micropyle oriented downward in the upper part of the locule, horizontal in the middle part of the locule, and erect with a micropyle oriented upward in the lower part of the locule. Irrespective of its position, the ovule becomes anatropous at maturity.

Early in development, the ovule has a one-celled archesporium differentiated beneath the apical dermal layer of the nucellus (Fig. 2b). The archesporial cell differentiates directly into a megaspore mother cell without producing a parietal cell (Fig. 2c). Thus, the ovule is tenuinucellate. Meiosis in the megaspore mother cell results in a linear triad of megaspores. Although I did not observe the first division, the second division occurs only in a lower cell of a dyad of megaspores. While the megaspore at the chalazal side is functional, the two remaining megaspores degenerate (Fig. 2d), so that two degenerating megaspores are always observed above a developing female gametophyte. Indeed, in each of the 13 ovules at the two-nucleate female gametophyte stage, I observed two degenerating megaspores (Fig. 2e). One might consider the possibility of a bisporic development of the female gametophyte. However, since no case has ever been reported in which the second division occurs only in an upper cell of a dyad of megaspores resulting in a triad of megaspores as in Fig. 2d, the female gametophyte of *Phyllonoma* is monosporic (for review of suppression of the second division in the upper cell that results in the triad, see Bouman 1984, p 132). A functional megaspore successively develops into the two-nucleate (Fig. 2e), four-nucleate, and eventually

eight-nucleate female gametophyte. A mature female gametophyte is ellipsoidal in shape (Fig. 2f). However, because the female gametophyte, such as the ovule itself, is slightly curved, it is often sectioned obliquely. Based on such oblique-longitudinal sections, I confirmed that the mature female gametophyte has an egg cell, two synergids, two polar nuclei, and three antipodal cells (Fig. 2g–i). Thus, the mode of development of the female gametophyte conforms to the Polygonum type. The antipodal cells are ephemeral.

Throughout the development of the female gametophyte, the apical dermal cells of the nucellus do not divide periclinally to form a nucellar cap (Fig. 2d, e). The apical dermal cells of the nucellus are destroyed by the developing female gametophyte and disappear, so that the upper half of the mature female gametophyte directly borders the integument (Fig. 2f, g, j).

The nucellus is small in the early stages of development. At the megaspore mother cell stage, the nucellus has only a few cells below the archesporial cell (Fig. 2b). However, as the ovule develops, cell divisions rapidly proceed below the megaspore mother cell or developing female gametophyte, forming a long, narrow cylindrical nucellar tissue on the chalazal side (Fig. 2c, f). No hypostase is formed (see chalazal region of a seed in Fig. 2f). The funicle is thick; however, no obturator develops from the funicle.

The ovule is unitegmic (Fig. 2b, c, f, j), as described by van Tieghem (1898). The integument is multiplicative and is initially four cell layers thick. As the ovule develops, cells other than those of the outer epidermis further divide periclinally, so that the integument becomes more than eight cell layers thick in the nearly mature ovule (Fig. 2f, j). A median longitudinal section of the young ovule shows that the integument develops not only on the antiraphal (dorsal) side but also on the raphal (ventral) side (Fig. 2c). The thick integument forms a micropyle above the female gametophyte. No vascular bundles differentiate in the integument (Fig. 2j). Cells of the inner epidermis are not specialized; thus, they do not develop into an integumentary tapetum or endothelium (Fig. 2f, j).

Endosperm and embryo

By the time of fertilization, the ovules grow and increase their sizes within the locule (Fig. 3a). Fertilization is porogamous. The pollen tube enters from the micropyle above the nucellar apex (Fig. 3b). Division of the primary endosperm nucleus occurs at nearly the middle of the central cell, and is accompanied by cell wall formation. Figure 3c shows a longitudinal section of a seed in the earliest stage of endosperm development. Thus, endosperm formation is of ab initio Cellular type. The seed shown in Fig. 3c appears to have a two-celled endosperm plus a zygote. At
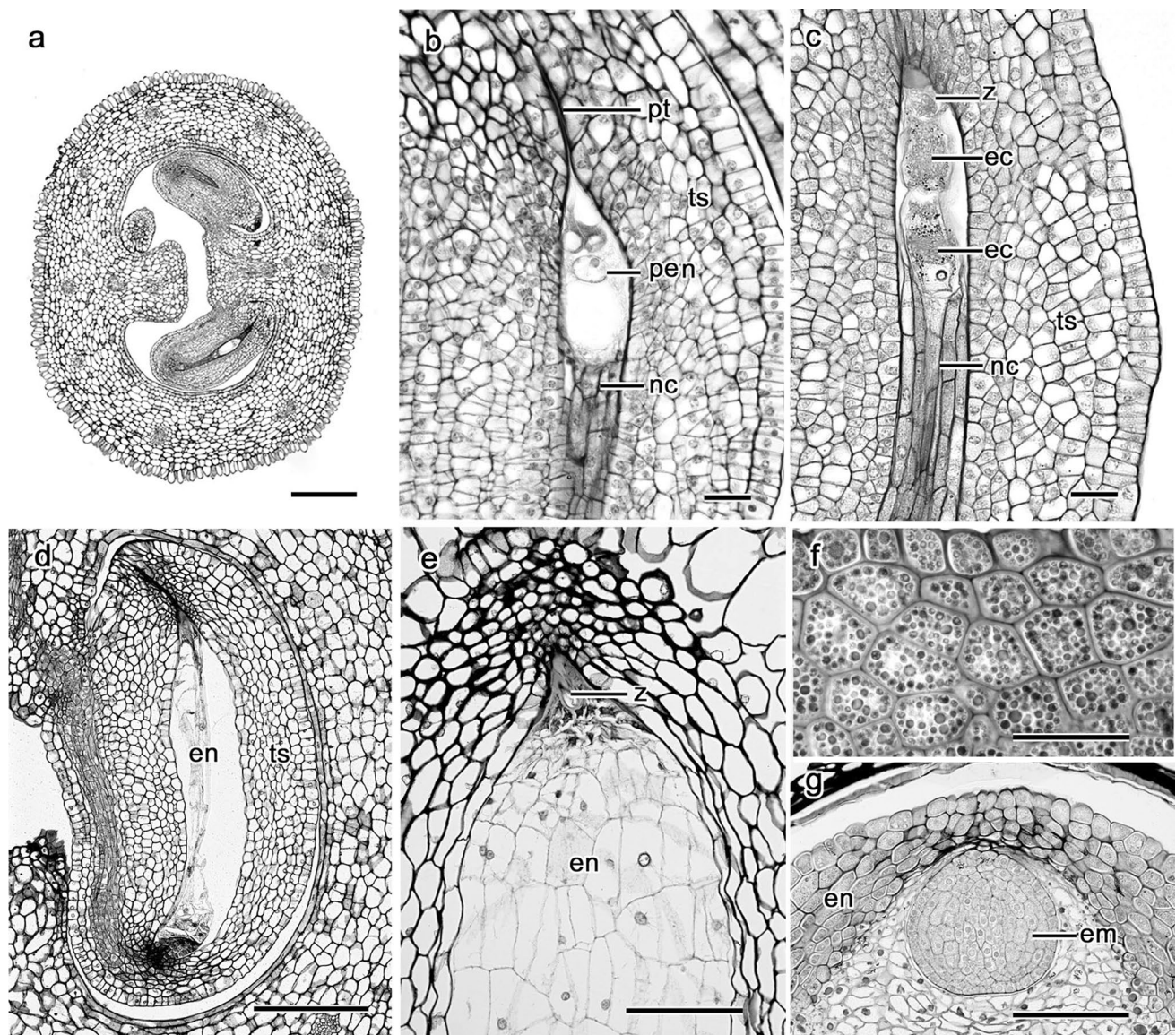
Fig. 3 Development of endosperm and embryo in *Phyllonoma tenuidens*. **a** *Transverse sections* of young fruit. **b–f** *Longitudinal sections* of seeds. **a** and **b** Young seed with the primary endosperm nucleus. **b** Young seed with a zygote and a primary endosperm nucleus. **c** Young seed with a zygote and two endosperm cells. **d** Young seed with a few endosperm cells. **e** Older seed with many endosperm cells. **f** Endosperm in mature seed. **g** Embryo in mature seed. *ec* endosperm cell, *em* embryo, *en* endosperm, *nc* nucellar tissue, *pen* primary endosperm cell, *pt* pollen tube, *ts* testa, *z* zygote. *Scale bars* are 200 μm in **a** and **d**, 100 μm in **e** and **f**, 50 μm in **f**, and 20 μm in **b** and **c**

this developmental stage, the nucellar tissue still remains in the lower half. However, as the seed develops, the whole tissue of the nucellus is replaced by a developing cellular endosperm (Fig. 3d). No prominent structure of endosperm like a micropylar or chalazal haustorium is observed. The mature seed has a copious endosperm, whose cells appear to have accumulated abundant lipids (Fig. 3f).

Embryogenesis proceeds very slowly compared to the development of endosperm (Fig. 3e). I did not examine

Fig. 4 Development of seeds, seed coats, and fruits in *Phyllonoma* ▶ *tenuidens*. **a** Mature fruit. **b** Mature seeds obtained from **a**. **c** Scanning electron micrograph of mature seed. Its longitudinal section is presented at *lower left*. *Arrowhead* indicates the position of a hilum. **d** *Longitudinal section* (LS) of young seed. **e** Magnified view of the seed enclosed by a *rectangle* in **d**. **f** LS of immature seed coat. *An arrow* indicates degenerating cells of testa. **g** LS of mature seed coat. **h** *Transverse section* (TS) of mature seed. **i** TS of mature seed coat. *en* endosperm, *exts* exotesta, *fw* fruit wall, *rap* raphe, *ts* testa. Scale bars are 6 mm in **a**, 2 mm in **b**, 500 μm in **c**, **d,** lower left in **c**, and **h**, 200 μm in **e**, 100 μm in **f**, and 50 μm in **g** and **i**
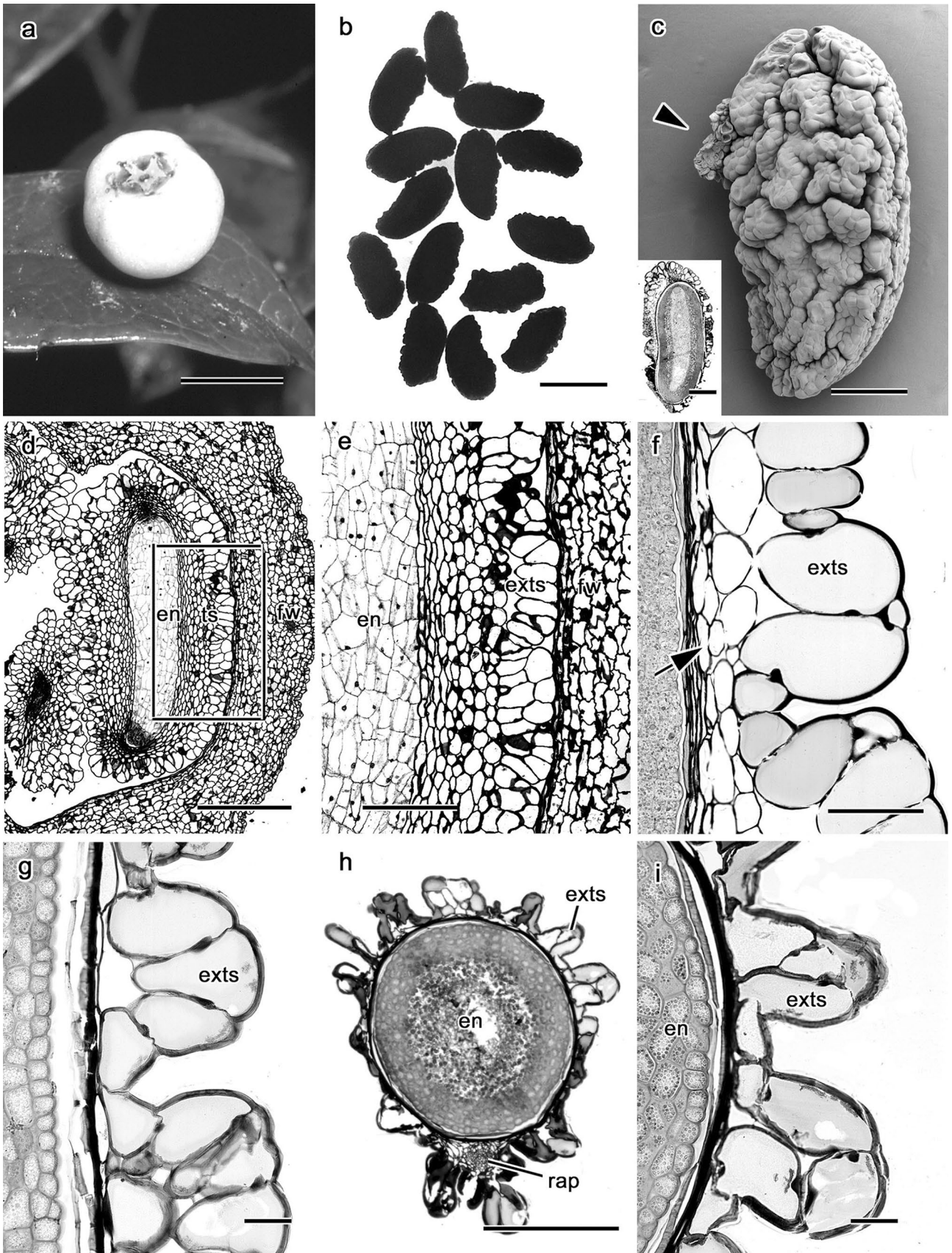
embryogenesis in detail; however, fragmentary data on early and late embryogenesis indicated that it proceeds normally to form a globular embryo (Fig. 3g). The embryo in mature seeds is small, approximately 1/17–1/18 of the length of the endosperm. It shows the initiation of two cotyledons with a very short suspensor. Such a small embryo in mature seeds is also observed in *P. laticuspis* and *P. ruscifolia* (Krach 1976; Takhtajan 2000).

Seed and seed coat

When ripened, fruits become white spherical berries approximately 8.0–8.4 mm in diameter (Fig. 4a). The endocarp is collapsed without being specialized (see Fig. 4e). All the ovules in the ovary are fertilized and develop into mature seeds (Fig. 4b). Mature seeds are exarillate, obovoid, but slightly curved, 2.6–3.2 mm long and 1.1–1.3 mm wide (measured from raphe to antiraphe; Fig. 4c, see also the longitudinal section of the mature seed at lower left). Each seed has a hilum at the position approximately 1/3 from the micropylar top of the seed (Fig. 4c). The surface is rugged with many irregularly shaped and sized warts (Fig. 4c).

The seed coat is not multiplicative, and is approximately eight to ten cell layers thick in early stages of development (Fig. 4d, e). During seed development, cells of an outer epidermis (exotesta) enlarge, particularly to the radial direction (Fig. 4e) (terminology of the unitegmic seeds of *Phyllonoma* followed Schmid [1986]). In immature seeds, cells of the seed coat are mostly collapsed, except for the exotestal cells which enlarge further into irregular shaped cells (Fig. 4f). The seed coat is hard and thick at maturity, although it only consists of thick-walled exotestal cells. Many (but not all) of the exotestal cells are enlarged and 150–200 μm high; however, the exotesta does not show a palisadal layer as Takhtajan (2000) described for seeds of *P. laticuspis* and *P. ruscifolia*. A group of approximately 10 or more exotestal cells form individual warts on the seed surface (Fig. 4c). This structure looks similar to the seed coat structures in *P. laticuspis* and *P. ruscifolia* which are illustrated by Takhtajan (2000: Figs. 4a, 5a, b, 9a–c).

Discussion

Summary of embryological features of *Phyllonoma*

Prior to my analyses, information on the development of the anther, ovule, and seed of *Phyllonoma* was fragmentary, although a few embryological characters were described by Krach (1976), Mauritzon (1933), Takhtajan (2000), and van Tieghem (1898). My work based on *P. tenuidens* provides most of the missing information and updates current

concepts for a few characters. The overall information on the embryological features of *Phyllonoma* can be summarized as follows.

Anther tetrasporangiate; anther wall four cell layers thick, its formation of the Dicotyledonous type; anther epidermis persistent, and its cells enlarged; endothecium fibrous; middle layer degenerating; and tapetum glandular and its cells binucleate. Cytokinesis in the microspore mother cell simultaneous; microspore tetrads predominantly tetrahedral; and pollen grains two-celled when shed.

Ovule borne from parietal placenta, anatropous, and tenuinucellate; archesporium hypodermal and one-celled. An archesporial cell differentiating directly into a megaspore mother cell, which undergoes meiosis resulting in a linear triad of megaspores (tetrad not formed due to the failure of the second division in the upper cell of a dyad of megaspores); chalazal megaspore functional, developing into an eight-nucleate Polygonum type female gametophyte; and antipodal cells ephemeral. Apical nucellar epidermal cells degenerating, and thus the upper half of mature female gametophyte directly bordering on the integument; hypostase not differentiating; and obturator absent.

Ovule unitegmic; integument multiplicative, thickening to more than eight cell layers thick, having no vascular bundles; micropyle formed by thick integument; and cells of inner epidermis unspecialized (not differentiating into endothelium).

Fertilization porogamous; endosperm formation of ab initio Cellular type; and mature seeds having lipids in copious endosperm. Embryogenesis uncertain and embryo in mature seed small, approximately 1/16–1/18 in endosperm length.

Seed obovoidal, but slightly curved, exarillate, with a hilum at the position 1/3 from the top and its surface rugged with many irregularly shaped and sized warts. Seed coat not multiplicative; mature seed coat exotestal, consisting only of irregularly enlarged, thick-walled exotestal cells; and a group of approximately10 or more exotestal cells forming individual warts.

Comparisons with other Aquifoliales

Embryological features of *Phyllonoma* are compared with those available for the four other families of Aquifoliales (Aquifoliaceae, Cardiopteridaceae, Helwingiaceae, and Stemonuraceae) in Table S1 (supplementary Table). Except for Helwingiaceae whose embryological features have just been published by Ao and Tobe (2015), the embryology of the three other families remain poorly understood. As previously described (Ao and Tobe 2015), I obtained data from Brewbaker (1967), Copeland (1963), Corner (1976), Herr (1959, 1961), Schürhoff (1921), and van Tieghem (1898) for Aquifoliaceae; from Fagerlind (1945) and Mauritzon (1936) for Cardiopteridaceae; and from Fagerlind (1945), Mauritzon

(1936), and Padmanabhan (1961) for Stemonuraceae. Some data are published for four [*Cardiopteris*, *Citronella* (=*Villarestia*), *Gonocaryum*, and *Leptaulus*] of the five genera constituting Cardiopteridaceae. As performed in a previous paper (Ao and Tobe 2015), I removed data on *Cardiopteris* reported by Kong et al. (2002) from Table S1. According to Kong et al. (2002), *C. platycarya* Gagnep. has orthotropous, ategmic ovules with a monosporic eight-nucleate female gametophyte, in which an egg apparatus is positioned on the chalazal side (see also Kong et al. 2014). These traits are very likely autapomorphies for the genus; therefore, they are not useful for extensive comparisons among the families.

Comparisons based on the available information showed that *Phyllonoma* has many embryological features in common with the other Aquifoliales. Features shared by all the five families of Aquifoliales include the following: ovule anatropous, unitegmic; arrangement of megaspores linear; female gametophyte formation conforming to Polygonum type; antipodals ephemeral; nucellar cap not formed; nucellar tissue in mature ovule lacking in the upper half of the nucellus; and chalaza not specialized. Furthermore, most features relating to the development of anthers and microspores are common to Phyllonomaceae, Helwingiaceae, and Stemonuraceae (data not available for Aquifoliaceae and Cardiopteridaceae), including the following: anther tetrasporangiate; anther wall four to five cell layers thick; endothecium fibrous; middle layers ephemeral; tapetum glandular; cytokinesis in the microspore mother cell simultaneous; and microspore tetrads predominantly tetrahedral. However, most of these common embryological features can be found in many other families of asterids. To discuss more critically how Aquifoliales are characterized by one or more embryological synapomorphies, we must know missing information on Aquifoliaceae, Cardiopteridaceae, and Stemonuraceae, which remain poorly known embryologically (see Table S1).

Despite the inadequate level of our knowledge on the embryology of Aquifoliales, *Phyllonoma* (Phyllonomaceae) likely conforms with Aquifoliaceae and Helwingiaceae in lacking a vascular bundle in the integument (present in Cardiopteridaceae and Stemonuraceae) and having ab initio Cellular endosperm (not Nuclear type endosperm as in Cardiopteridaceae and Stemonuraceae). It is uncertain whether either one or both of these features are apomorphic or not; however, the lack of a vascular bundle in the integument and/or the Cellular type endosperm supports either a clade of Aquifoliaceae, Helwingiaceae, and Phyllonomaceae or a clade of Cardiopteridaceae and Stemonuraceae. Within a clade of Aquifoliaceae, Helwingiaceae and Phyllonomaceae, Helwingiaceae and Phyllonomaceae likely share the tenuinucellate ovule as a synapomorphy (for discussion of this character in Aquifoliaceae see Ao and Tobe 2015, pp. 173–174). Therefore, in agreement with evidence from

molecular analyses (Soltis et al. 2011; Tank and Donoghue 2010) as well as from flower and inflorescence morphology (i.e., an inferior ovary and epiphyllous inflorescence; Tobe 2013), embryological evidence supports a sister-group relationship between Phyllonomaceae and Helwingiaceae. A few differences exist between Phyllonomaceae and Helwingiaceae. For instance, the endothelium is not differentiated in *Phyllonoma* but is differentiated in Helwingiaceae as in Aquifoliaceae; the testa is not multiplicative in Phyllonomaceae as in Aquifoliaceae, but is multiplicative in Helwingiaceae; and the mature seed coat is exotestal in Phyllonomaceae (as in Aquifoliaceae), but no particular cell layers develop to form a permanent mechanical structure in Helwingiaceae. Among these differences, the lack of endothelium is likely an autapomorphy of Phyllonomaceae, whereas both the non-multiplicative testa and the lack of a permanent cell layer(s) in the mature seed coat are likely autapomorphies of Helwingiaceae. However, this must be confirmed by additional studies of Aquifoliaceae, Cardiopteridaceae, and Stemonuraceae.

Thus, like molecular and floral-morphological evidence, the available embryological evidence supports the distinctness of *Phyllonoma* as a family and its sister-group relationship to Helwingiaceae in the Aquifoliales. Differences between *Phyllonoma* and Helwingiaceae are found mainly in seed coat structure. The seeds of *Phyllonoma* have a thick seed coat and are dispersed in berries, whereas those of Helwingiaceae have a thin membranous seed coat and are dispersed in drupes as in Aquifoliaceae (for data of seed coat in Helwingiaceae and Aquifoliaceae see Ao and Tobe [2015] and Corner [1976]). Birds are most likely to play a role in seed dispersal in both families. In their digestive system, the seed entity must be protected by the thick seed coat in *Phyllonoma* and by the thick endocarp or pyrene in Helwingiaceae. Ao and Tobe (2015) showed that, in contrast to anemophily (insect-pollination) in *Phyllonoma*, ambophily (pollination by insect and wind) is characteristic of Helwingiaceae. A series of morphological and developmental analyses (Ao and Tobe 2015; Tobe 2013, 2014; present study) have shown the morphological diversification of flowers and seeds in Central-American *Phyllonoma* and East-Asian Helwingiaceae. Embryological information thus shows that in *Phyllonoma*, distinct character evolution has occurred in reproductive characters relating to pollination and seed dispersal.

The present study has further shown that embryological studies in the three other families Aquifoliaceae (*Ilex* only), Cardiopteridaceae (five genera), and Stemonuraceae (12 genera) are required for critical comparison. In particular, the unique development and structure of the female gametophyte in *Cardiopteris*, which was documented by Kong et al. (2002, 2014), must be confirmed. Likewise, an intensive developmental study of the female

gametophyte is required in each of the four other genera (*Citronella*, *Gonocaryum*, *Leptaulus*, and *Pseudobotrys*) of Cardiopteridaceae.

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