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Embryology of *Manekia naranjoana* (Piperaceae) and its Implications for the Origin of the Sixteen-nucleate Female Gametophyte in Piperales

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To the Graduate Council:

I am submitting herewith a thesis written by Tatiana Arias-Garzón entitled "Embryology of *Manekia naranjoana* (Piperaceae) and its Implications for the Origin of the Sixteen-nucleate Female Gametophyte in Piperales." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology and Evolutionary Biology.

Joseph Williams, Major Professor

We have read this thesis and recommend its acceptance:

Edward E. Schilling, Taylor Feild

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for
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Tatiana Arias-Garzón
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ABSTRACT

Piperaceae is unique among Piperales because it is the only tetrasporic group in the order and a great deal of diversity in the ontogenetic trajectories of the female gametophyte is found in its genera. The evolutionary developmental origin of the sixteen-nucleate female gametophyte remains unclear in the family until now. In Piperaceae, *Manekia* has been identified as sister to *Zippelia*, and this clade is sister to core Piperaceae (*Piper, Peperomia*). This research is the first attempt to understand the development of the female gametophyte of *Manekia naranjoana* in order to provide critical data on the origin of tetrasporic development in the family. Several aspects of the floral biology and phenological events taking place in the ovary, the flower and the inflorescence were explored. *Manekia* has a tetrasporic, sixteen nuclei female gametophyte, that is being produced from a single archesporial cell. The egg apparatus is located at the micropylar end of the female gametophyte. It is constituted of three cells, two synergids and an egg. The central cell nuclei consist of two nuclei, one from the micropylar end and the other one from the chalazal one. The eleven remaining nuclei are arranged toward the chalazal pole of the female gametophyte, and sometimes fuse. This description corresponds mostly to the Drusa type. But Penaea type is also occasionally reported for first time in this study for the genus. *Manekia* and *Zippelia* share a similar structure of the female gametophyte with a total of 16 nuclei, and two nuclei in a central cell suggesting a triploid endosperm. The transition from monosporic to tetraporic female gametophyte development can be explained through the theory of modular construction and several kind modifications in the ontogenetic trajectories. Heterochronic and heterotopic changes, additions, and deletions in the development of the female gametophytes reflect evolutionary histories of the particular taxa implicated. A great deal of plasticity in terms of lack of polarity and nuclear fusion of antipodals was found in the chalazal module of the female gametophyte of *Manekia*.

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CHAPTER I
INTRODUCTION

Understanding the origin and early evolution of flowering plants has been a point of interest to many scientists since Darwin (Darwin 1930, Hickey and Taylor 1996, Crepet 2000, Bell et al. 2005). Flowering plants represent around 90% of all land plants and they are by far the most species-rich group of extant seed plants (Endress 2004, Soltis et al. 2005). Flowering plants first appear in the fossil record during the Early Cretaceous (aprox. 145 mya) and today they represent one of the most significant evolutionary radiations since the origin of land plants (Crane et al. 2004).

Elucidating the origin of flowering plants and their evolutionary consequences relies on the interaction of robust phylogenetic analysis, and both comparative and developmental biology of extant plants (Friedman et al. 2004). Impressive progress has been made in the study of angiosperm relationships in the past twenty years (Donoghue and Doyle 1989, Chase et al. 1993, Zimmer et al. 2000, Qiu et al. 2005). It has become clear that *Amborella*, Nymphaeaceae, Hydatellaceae and Austrobaileyales (APG II 2003) represent a basal grade of earliest diverging lineages of extant angiosperms (Mathews and Donoghue 1999, Soltis and Soltis 2004, Saarela et al. 2007). Furthermore, Eumagnoliids (APG II 2003) have been identified as an early-divergent monophyletic group, not among the basal grade, and the relationships among the member orders (Magnoliales, Laurales, Canellales and Piperales) are strongly supported (Qiu et al. 2005).The relationships among the largest clades such as Monocots, Eumagnolids and Eudicotyledons are unsatisfactorily resolved to date (Stevens 2001, Soltis and Soltis 2004, Qiu et al. 2005). These new phylogenies together with the new discoveries in developmental biology have transformed the interpretations about the evolution of many morphological traits in flowering plants. For example, it was once thought that the 7-celled/8-nucleate *Polygonum* type female gametophyte

was the strongest synapomorphy of angiosperms. Based on the new view of angiosperm phylogeny and the careful examination of embryological traits with new technology, this has turned out to be far from true: *Amborella* has an 8-celled/9-nucleate female gametophyte (Friedman 2006), Nymphaeaceae and Austrobaileyales have a 4-celled/ 4-nucleate female gametophyte (Williams and Friedman 2002, Friedman and Williams 2003) and the basal groups of Monocots, Eudicots and Eumagnolids have a 7-celled/8-nucleate *Polygonum* type female gametophyte (Williams and Friedman 2004). Early extant angiosperms, in contrast to angiosperms as a whole, turn out to have a great deal of developmental diversity.

Although the basal grade has become the focus of study for those interested in the origin of unique angiosperm traits, early lineages of monocots, eudicots, eumagnoliids, Chloranthaceae and *Ceratophyllum* are important for offering insight into early evolution of embryological and reproductive traits. Among basal angiosperms, Piperales is an exceptionally diverse clade in female gametophyte morphology and endosperm structure and offers a good example for the study of evolution of embryological and reproductive traits. Piperales and Monocotyledoneae were earlier considered to be sister groups (Burger 1977, Tucker and Douglas 1995). But molecular phylogenetic analyses have all now placed Piperales as sister of Canellales (Wanke et al. 2007) thus removing this taxon as a potential outgroup to the monocots as earlier suggested by Burger (1977). The monophyly of Piperales (Aristolochiaceae, Lactoridaceae, Piperaceae, Saururaceae and Hydnoraceae) is supported by distichous phyllotaxis, a single prophyll and oil cells (Soltis et al. 2005). Hydnoraceae has not been included in the molecular phylogenies yet (it lacks chloroplasts due to its parasitic condition; Nickrent et al. 2002). Thus it now appears that Piperaceae is derived within basal angiosperms (nested within Eumagnoliids).

Piperaceae is known for its remarkable species diversity (Endress 1994, 2004; Crane et al. 2004). This family is distributed pantropically and includes around 2000 species, most of which occur in *Piper* and *Peperomia* (Trealease and Yunker 1950, Callejas 1986, de Figueiredo and Sazima 2000, Jaramillo and Manos 2001, Jaramillo et al. 2004). Piperaceae also is well known for its reproductive diversity that includes different varieties of floral morphology and development, pollinators, seed dispersal mechanisms, and reproductive strategies (Martin and Gregory 1962, Tucker 1980, 1982a-b, 1985, 1993, Callejas 1986, Lei and Liang 1998, de Figuieiredo and Sazima 2000, Wadt and Kageyama 2004). One aspect of Piperaceae reproductive diversity frequently ignored, but of special significance to the question of early angiosperm evolution, is its embryology.

Piperaceae is unique among Piperales because it is the only tetrasporic group in the order and a great deal of diversity in the ontogenetic trajectories of the female gametophyte is found in its genera. The evolutionary developmental origin of the sixteen-nucleate female gametophyte remains unclear in the family. Comparative analyses of female gametophyte ontogenies are necessary to understand the evolutionary pathways in the main evolutionary lines of Piperaceae. In Piperaceae, *Manekia* (Arias et al. 2006) has been identified as sister to *Zippelia* (Liang and Tucker 1995), and this clade is sister to core Piperaceae (*Piper, Peperomia*; Jaramillo and Manos 2001, Jaramillo et al. 2004). Embryological studies in Piperaceae have been focused on *Piper*, *Peperomia* and *Zippelia* (Johnson 1914, Prakash et al. 1994, Lei et al. 2002), but *Manekia* remains completely unknown.

Manekia is a widely distributed genus of perhaps four species (Arias et al. 2006) with a Neotropical distribution (Trelease and Yuncker 1950, Arias et al. 2006). It is a vine with terminal and axillary flowers (see Jaramillo and Callejas 2004) and fruits embedded in the inflorescence rachis; these are two taxonomical

features that differentiate the genus from *Piper, Peperomia* and *Zippelia* (Jaramillo et al. 2004). Little is known about its floral morphology, anatomy and reproductive phenology, because the plant flowers in the high canopy of lowland and montane tropical rain forest, where the flowers are typically inaccessible.

This research is the first attempt to understand the development of the female gametophyte of *Manekia naranjoana* using a combination of pollination experiments and microscopy techniques. Additionally, the studies of development of *Manekia naranjoana* are placed in a comparative context within Piperales. The embryology and other reproductive events such as some aspects of the floral biology in *Manekia naranjoana* (Piperaceae), including morphological characters of evolutionary interest for the order Piperales are presented for the first time in this study. I also combined observations of the embryological events of *Manekia* with several developmental and phenological events taking place in the ovary, the flower and the inflorescence of this species in a timeline framework.

The new phylogenetic hypothesis for Piperales (Jaramillo 2004, Soltis and Soltis 2004) is valuable for comparative analyses that seek to identify evolutionary transitions and key reproductive features in the evolution of the clade. The monosporic, 7-celled/ 8-nucleate female gametophyte (*Polygonum-type*) is a very conserved character among early eumagnoliids, including basal clades in Piperales (Williams and Friedman, 2004). On the other hand, Piperaceae is diverse in both megasporogenesis and female gametophyte development. Megasporogenesis is tetrasporic: cell walls do not form after meiosis so that the female gametophyte initiates from four free megaspore nuclei instead of from one, as is typical in seed plants. Tetrasporic development in Piperaceae is followed by at least three known patterns of female gametophyte development. The *Fritillaria* type (7-celled/8-nucleate) has been reported in *Piper* (Kanta 1962, Nikiticheva et al. 1981, Prakash et al. 1994), the *Peperomia* type

(9-celled/16-nucleate) in *Peperomia* (Johnson 1914, Swamy 1944, Nikiticheva et al. 1981, Plyushch 1982, Smirnov and Grakhantseva 1988), and the *Drusa* type (15-celled/16-nucleate) female gametophyte in *Zippelia* (Lei et al. 2002). The large amount of variation in female gametophyte development in Piperales is difficult to interpret because of the lack of information from critical taxa like *Manekia*. There have been many embryological studies in Piperaceae but some of them are now outdated or lack a comparative and evolutionary context. Lei et al. (2002) provide the only modern study, on *Zippelia*. Establishing ancestral states of morphological features, like female gametophyte development, in the clade comprising *Manekia* and *Zippelia* is critical to understanding the origin of such traits in its sister clade, which comprises the overwhelming majority of the family (Williams and Friedman 2004).

Reproductive ontogenies in flowering plants comprise several dynamic and interacting processes that occur between times of pollination through seed dispersal. These include pollen transfer, stigmatic receptivity, anthesis, pollen tube growth, interaction between male gametophyte and sporophytic tissue, female gametophyte development, double fertilization and finally embryo and fruit development. These reproductive ontogenies are tremendously dependent on each other, but little is known about the relationships among them. Because developmental aspects like ontogenies of pistils, pollen tube, and female gametophyte are often studied separately in angiosperms, the relative timing of these ontogenies is hardly known. Studies of plant reproduction and development that utilize a combination of techniques and observations to understand relationships among these diverse ontogenies would provide a better understanding of angiosperm evolution.

The female gametophyte is implicated in several processes of the life cycle in flowering plants, such as pollen tube guidance (Barrett and Harder 1996, Hiscock et al. 2002, Barrett 2003, Edlund 2004), double fertilization, embryogeny

(Forbis et al. 2002) and the maternal control of seed development (Stephenson and Bertin 1983, Willson and Burley 1983, Floyd and Friedman 2000, Yadegari and Drews 2004). Female gametophyte development in angiosperms takes place in two key phases: megasporogenesis and megagametogenesis (Johansen 1950, Gifford and Foster 1989, Johri et al. 1992). Megasporogenesis refers to the developmental stages through which megaspores (haploid spores) are produced, whereas megagametogenesis refers to the developmental stages through which the female gametophyte is formed from the “functional megaspore” to produce the female gametes, the egg cell and the central cell (Gifford and Foster 1989, Johri et al. 1992). These processes encompass several variations during growth. For example, cell wall formation during megasporogenesis and the number of mitotic divisions during megagametogenesis are factors affecting female gametophyte development (Yadegari and Drews 2004). Additionally, the genetic composition of nuclei and cells varies among developmental pathways. As a consequence, more than fifteen different patterns of female gametophyte ontogeny have been described (Maheshwari 1950, Gifford and Foster 1989, Johri et al. 1992).

Angiosperms undergo three different patterns of megasporogenesis. Monosporic, bisporic or tetrasporic development refers to the process where a single functional megaspore cell is formed containing one, two or four haploid nuclei, respectively. During monosporic megasporogenesis meiosis I and II are each followed by cell wall formation, resulting in four haploid megaspore cells. Three of these degenerate and the chalazal-most one becomes the functional megaspore (Gifford and Foster 1989). Because a single nucleus gives rise to all nuclei in a monosporic female gametophyte, there is more genetic stability and less genetic variation (Haig 1989). With one exception all non-flowering seed plants plus the newly-defined basal grade of angiosperms have monosporic development (Williams and Friedman 2004).

Bisporic megasporogenesis includes cell wall formation after meiosis I but not meiosis II, and the functional megaspore contains two free nuclei. In tetrasporic megasporogenesis there is no cell wall formation after meiosis I or meiosis II resulting in a functional megaspore that contains four free nuclei, each with a different genetic composition (Haig 1989, Johri et al. 1992). In bisporic and tetrasporic development, the functional megaspore cell is a genetic mosaic, and its implications for female gametophyte development and the relationships between embryo and endosperm are poorly understood (Friedman et al. in press, Haig 1990). Bisporic and tetrasporic development have evolved repeatedly in angiosperms and one time in Gnetales.

Megagametogenesis refers to the development of the female gametophyte from the functional megaspore. It takes place from the end of meiosis until a mature female gametophyte is formed and fertilized. During megagametogenesis the functional megaspore enlarges and divides mitotically to form the mature female gametophyte. The mitotic divisions first give rise to a coenocyte, a cell containing four, eight or up to sixteen free nuclei, and are then followed by cell wall formation. At maturity the female gametophyte of all angiosperms has two gametes –the egg cell and the central cell.

Double fertilization occurs when there are two gametic fusion events between the two male sperm cells and the egg cell and central cell of the female gametophyte. This produces a biparental diploid embryo and a biparental triploid (Yadegari and Drews 2004, Williams and Friedman 2004) or diploid endosperm (Friedman 1995, 2001, Williams and Friedman 2002, Friedman and Williams 2003, 2004). Double fertilization and early embryogeny may be key innovations in the radiation of angiosperms (Friedman 2001), but double fertilization in basal angiosperms has rarely been observed, even though it is considered one of the key synapomorphies in angiosperms (Williams and Friedman 2002). It is important to document double fertilization in early angiosperms to understand its

generality in early lineages of flowering plants. This is particularly important in Piperaceae, because endosperm ploidy is quite variable. Furthermore it is unknown the number of cells that participated in the formation of the Piperaceae endosperm, (they could be three, four or fifteen-ploid). Little is known about the number of maternal nuclei that participate in the fusion of central cell with the sperm (Haig 1989) in taxa with high level of polyploidy. Classical studies of anatomical developmental characters using advanced techniques of light and fluorescence microscopy are necessary to interpret all these enigmatic questions (Friedman 2001).

My primary goals were (1) to describe and understand female gametophyte development of *Manekia naranjoana*; (2) to provide an analysis of several reproductive and floral ontogenetic events in the context of the female gametophyte development; and (3) to investigate the evolutionary implications of my findings

CHAPTER II
MATERIALS AND METHODS

Study species

Manekia is a widely distributed genus of perhaps four species (Arias et al. 2006) with a Neotropical distribution, occurring from southern Nicaragua to northern Peru, and the Lesser Antilles to the Atlantic forest of Southern Brazil (Trelease and Yuncker 1950, Arias et al. 2006). It is a vine with terminal and axillary flowers (see Jaramillo and Callejas 2004) and fruits embedded in the inflorescence rachis; these are taxonomical features that differentiate the genus from *Piper, Peperomia*, and *Zippelia* (Jaramillo et al. 2004). Little is known about its floral morphology, anatomy and reproductive phenology, because the plant flowers in the high canopy of lowland and montane tropical rain forest, where the flowers are typically inaccessible. *Manekia* displays a combination of features of early and late successional species, and occurs in both mature and secondary rain forest (pers. observation). *Manekia naranjoana* is distributed in Central America from northern Nicaragua to southern Panama. It bloomed in Costa Rica at the biological station Alberto M. Brenes between the middle of May and until the end of July 2006. While at Tapantí National Park (Costa Rica), this species flowered between the months of March and April 2006 and May and June 2005. Flowering in these places seems annual but variable in a year. At the biological station Alberto M. Brenes two blooming peaks were reported for summer 2006. A large number of inflorescences were produced starting in May, whereas in June there was low production of inflorescences.

Study areas

National Park Tapantí, Costa Rica: located in the province of Cartago, districts of Paraiso, Jimenez and El Guarco. It belongs to “La Amistad Pacifico” conservation area, occupying the Northeast region of the Talamanca Mountain Range. Altitudes range from 700 to 3491 meters above sea level. It is one of the rainiest places in Costa Rica with an average rainfall of 7000 mm. Temperature ranges from 6 to 26°C. It comprises five different life zones: premontane rainforest, premontane forest, low montane rainforest, montane rainforest and sub-andean páramo. *Manekia* is found in premontane rainforest.

Biological Station, Alberto M. Brenes, Costa Rica: it is located in the province of Alajuela, districts of Los Angeles de San Ramon. It belongs to “Arenal” conservation area. Altitudes range from 550-1650 meters above sea level. The annual precipitation range from 3500 to 5300 mm, with a dry season between March and April. The temperature ranges from 17 to 25 °C. The life zones comprise premontane rain forest and low montane rainforest.

Collections

Female gametophyte development and pollen tube growth: For the study of pollen tube growth and the female gametophyte development of *Manekia naranjoana* flowers and inflorescences in different developmental stages were collected and morphology was described in the field.

Flowers were either fixed for 24 hr in 3:1 (95% ethanol: acetic acid) and stored in 75% ethanol or fixed in FAA (50 ml 95% ETOH: 5ml glacial acetic acid: 10ml 40% formaldehyde: 35 ml dH_2O) and stored in 75% ethanol (Williams and Friedman 2004). Reproductive material was dehydrated through an ethanol series, and was infiltrated and embedded in glycol methacrylate (JB-4 embedding kit; Polysciences, Warrington, Pennsylvania, USA). Serial sections, at 5 µm thick, were obtained and stained with aniline blue (flowers fixed in 3:1),

0.1% tolouidine blue (flowers fixed in FAA) according to the specific requirement (pollen germination, pollen tube growth and/or embryological analysis). Structural features of the pollen, female gametophyte and embryo were characterized using a combination of fluorescence and light microscopy. Images were processed with a Zeiss digital photo system (Carl Zeiss, Oberkochen, Germany) and Adobe Photoshop, version 7.0 (Adobe Systems, San Jose, CA). Camera Lucida drawings were made by tracing structural features of the female gametophyte over images in Photoshop. Vouchers were deposited in the Herbarium of Costa Rica (CR) and The University of Tennessee Herbarium (UT).

Integration of floral ontogeny with female gametophyte development: two different scales of phenology were considered in this study, (a) the flowers and (b) the inflorescences.

(a) Inflorescences: I recorded length, color, orientation, number of flowers in twenty inflorescences.

(b) Flowers: Thirty flowers in five inflorescences for each developmental stage were described more or less according to the size of the inflorescence and its developmental stage. Ten flowers were analyzed at the base, ten at the middle part and ten at the top of the inflorescence. For each flower I recorded its length and width, color, presence of stigmatic secretions, stigmatic receptivity (Peroxtesmo KO peroxidase test paper). Presence of bubbling and change of color on the stigma was recorded. Additionally, flowers in different developmental stages were fixed to observe pollen tube growth in the lab using aniline blue and a fluorescence microscope, number of anthers, sequence of maturation of anthers (bud, in maturation, mature and open), and pollen viability (Kearns and Inouye 1993). Furthermore five inflorescences in early developmental stages were observed in the field and the sequence of maturation of flowers in the inflorescence was tracked until fruit formation, if possible.

Character evolution analyses

Character evolution analysis were conducted using the most recent molecular and morphological phylogenetic trees for Eumagnoliids (Doyle and Endress 2000, citations in Stevens 2001 onwards, Qiu et al. 2005), Piperales (Doyle and Endress 2000, Jaramillo et al. 2004, Wanke et al. 2007) and Piperaceae (Wanke et al. 2007). The families belonging to Piperales are all monophyletic as circumscribed in APG II (2003). Placement of genera within families in Piperales was based on Jaramillo and Manos (2000), and Jaramillo et al. (2004). I determined character states for Piperales from my own work (*Manekia*) and from original sources of embryological and reproductive biology studies. The ancestral states for discrete characters were determined based on parsimony, after mapping the characters on their respective phylogenies to analyze character evolution (MacClade 4.03; Maddison and Maddison 2001). All characters were treated as unpolarized and unordered (all transitions among states are equally probable). Canellales was included as the outgroup.

CHAPTER IV
RESULTS AND DISCUSSION

Floral development

Inflorescence development

The inflorescences of *Manekia naranjoana* are terminal or axillary spikes enclosed by a thin-membranous prophyll (Figs 1 and 2; all figures are located in the appendix). A single individual can have from few inflorescences to >1200, while a single inflorescence bears 10-150 flowers. Flowers in an inflorescence go through approximately the same stages of development simultaneously (Fig. 2). Early in development inflorescences are erect (Fig.1) but they curve to be pendulous when the fruits are formed. Inflorescences also change in color, aroma, and texture, during development. Their color varies from green yellow when the flowers are in bud (Fig. 1), to yellow when the stamens are mature and dehiscent (Fig. 2), to brown after the structural generation of stigma and abscission of stamens, and finally to dark green when in fruit. Inflorescences in anthesis are very aromatic, with an anise-like smell. The texture of an inflorescence changes from smooth when flowers are in bud to granular when anthers start to open (Figs. 1 and 2). In later developmental stages the mature inflorescences appear shrunken and irregular when only few fruits are formed in sectors of the infructescence.

Flower, carpel and ovary development

The *Manekia naranjoana* flower is bisexual, subtended by a single bract (Fig.3) and totally immersed in and fused with the adjacent parenchymatic tissue of the rachis. As a consequence there is no distinction between the external ovary wall and the rachis (Figs.4 to 6). The bract is hypopeltate, persistent, with marginal filamentous muticellular hairs and abaxial oil cells (Fig.3). The ovary is unilocular with a single orthotropus ovule (Figs.4 to 8). The flower appears to be

comprised of four syncarpic carpels, as judged by the occurrence of four vascular strands leading to the four to five stigmatic lobes (Figs.3, 5 and 6). The stigmatic lobes are decurrent, with papillate unicellular protrusions with abundant ethereal oil cells immersed in the tissue (Fig. 4). Sometimes three to five stigmatic lobes were observed in flowers (Fig. 3) but more commonly four lobes were observed. After stigma receptivity the outer stigmatic surface accumulates callose (Figs.3 and 6).

The *Manekia naranjoana* carpel has incomplete postgenital fusion sensu Endress and Igersheim (2000). Early in stylar development there is a short open canal that almost reaches the ovule (Fig. 5). This canal has a single layer of small epidermal cells with dense cytoplasm. The cells immediately adjacent to the walls of the stylar canal are oval, irregular and oriented longitudinally toward the ovule. Later on in floral development this open canal closes through posgenital fusion from the base of the style to its middle part forming a solid transmitting tissue toward the base of the style, and a stylar canal from the middle of the style to the stigma (Figs. 4 and 6). The transmitting tissue has elongated cells oriented longitudinally toward the ovary (Fig. 6 and 23).

Manekia naranjoana has four stamens in each flower at maturity, two are laterally inserted, and one apically inserted (Fig. 3), and one basally inserted. Anthers are rounded with short filaments that slightly raise them above the rachis surface when mature and ready to disperse pollen (Figs. 2 and 3). The flowers are dichogamous with incomplete protandry. When mature, anthers open longitudinally and release pollen, but occasionally they fall off from flowers without opening. First, two lateral stamens are initiated at the same time before the onset of stigmatic receptivity, then after maturation an abscission zone is formed at the base of their short filaments. After stigmatic receptivity a third stamen is initiated on the apical portion of the flower (relative to the inflorescence), and after its maturation an abscission zone is formed at the base

of the filament (Figs. 3 and 6). The fourth basal stamen is the last formed in the flower; it is opposite to the third one and falls off through an abscission zone at the base of the filament.

High rates of ovule abortion before and after fertilization, and embryolessness, were detected in *Manekia naranjoana*. Degenerate ovules were seen alongside with normal ovules at all stages of development from ovary development to fruit formation. Empty ovaries were detected early in the development of the flowers, but fully formed ovaries degenerating after flower maturation were also observed.

Ovule development

The ovule of *Manekia naranjoana* is orthotropous with basal placentation, crassinucellar, and bitegmic (Figs. 5, 7 and 8). The ovule primordium appears at the bottom of the ovarian cavity before postgenital fusion has occurred. The inner integument appears first and forms the micropyle while the outer one initiates growth after the inner but does not participate in the formation of the micropyle (Fig.7 and 8). The micropyle is in contact with the wall of the ovarian cavity at maturity. The inner integument is three cell layers thick (Fig.7). Cells of the inner integument are small and compact with a dense cytoplasm, similar to nucellar cells. The outer integument cells are vacuolated, large, with less dense cytoplasm, and also three cell layers thick (Fig.7 and 8).

A small hypostase is formed in *M. naranjoana* where tannins are accumulated in the nucellar cells at the base of the ovule. The cells are schlerenchymatous, with thick cell walls (Fig. 8). The hypostase is not evident until female gametophyte maturation.

Female gametophyte development

For the study of the female gametophyte in *Manekia naranjoana* two hundred samples were fixed. Each sample has an average of five slides, so more than a thousand slides were observed using a light microscope. Each sample has an average of five flowers each, making an approximate average total of a thousand flowers observed in all the different developmental stages (Table 1).

Megasporogenesis

Female gametophyte development in *Manekia naranjoana* starts at the micropylar end of the ovule when a single first hypodermal cell grows in size and becomes different from the rest of nucellar tissue. This single sporogenous cell gives rise to the megaspore mother cell through mitosis by a single unequal periclinal division (Figs. 9-11). There was no evidence of multiple archesporial cells. The archesporial cell cuts off a parietal cell and more cell divisions occur above the archesporial cell that below it. This parietal tissue pushes the megaspore mother cell down and deep into the nucellus making it crassinucellar (Fig. 11). As a result a four-layered parietal tissue is formed (Figs. 12-13). Once the megaspore mother cell is deep into the tissue it becomes more ovoid. During interphase and before the beginning of meiosis, the genetic material duplicates making the nucleus bigger (Fig 13).

Table 1. Number of samples and flowers in different developmental stages analyzed in this study

Developmental stages of inflorescences	***Floral primordium***	***Inflorescence with mature lateral anthers***	***Inflorescence with receptive stigmas***	***Inflorescence with lateral and apical anthers***	***Inflorescence with fertilized flowers***	***Infructescence***
Number of collections	**45**	**35**	**50**	**35**	**20**	**15**
Number of flowers	**225**	**175**	**250**	**175**	**100**	**75**

When the megaspore mother cell is mature, the first meiotic division takes place. The location of the nucleus in the mature megaspore mother cell can be central, micropylar or chalazal; there is not a strong polarization of the nucleus in the cell. The megaspore mother cell becomes larger and more vacuolized, then the nucellar tissue is pushed to the edges of the megaspore mother cell and its cells are crushed, making the edges of the megaspore mother cell appear dark. Starting with the prophase I, condensed chromosomes are found attached to the nuclear membrane (Fig. 14). The spindle of the first nuclear division of the megaspore mother cell is parallel with the vertical axis of the ovule. Once meiosis I has ended an ephemeral dyad is formed. The size of the cell is around 15 µm wide x 35 µm long; its shape is still ovoid. Two nuclei are formed, one toward the micropylar pole and the second one toward the chalazal one. Nuclear envelopes were not found to have formed around the two chromosome complements of the dyad in any of the slides that were examined, suggesting that: (1) nuclear membranes do not form after the meiosis I, and there is a direct skip to metaphase of meiosis II, or (2) telophase I plus prophase II were not detected in the collections because they happen very fast, so the probabilities of finding these stages are very low. Prophase, metaphase and anaphase of meiosis I were detected (Fig. 14 to 15a-b).

During meiosis II the spindle of the micropylar nucleus in the dyad is parallel to the vertical axis of the ovule, while the spindle of the chalazal nucleus is more or less perpendicular to the vertical axis of the ovule (Fig. 16a-b). Metaphase, anaphase, telophase of meiosis II were seen (Fig 16a-b). Following meiosis II nuclear membranes form four free megaspore nuclei within an ovoid coenocyte. Cytokinesis was never observed at this stage.

Variation in megaspore arrangement range from: a tetrapolar arrangement one micropylar, one chalazal and two lateral nuclei (Figs. 17a-b), to the most common pattern we observed, a bipolar arrangement with a micropylar nucleus

and three chalazal nuclei (“1+3” arrangement; Fig. 18). Coenocytes have always had dense cytoplasm and several small vacuoles (Fig.17a-b) or one large vacuole (Fig. 18). The four nuclei are usually of almost equal size (8 µm x 8 µm) but sometimes the chalazal one is smaller.

Megagametogenesis

The four megaspore nuclei in the coenocyte undergo the first mitotic division after the end of meiosis. During prophase of the first mitosis the four nuclei were most commonly distributed in a 1+3 arrangement (Fig. 18a-c, Fig. 30). At the end of the first mitosis eight nuclei are formed and these were usually arranged with two nuclei located at the micropylar pole and six closer to the chalazal pole. Fig. 19 illustrates prophase of mitosis II. The eight nuclei each undergo a second mitosis producing a 16- nucleate immature female gametophyte (Fig.20a-f). A clear polarization of the nuclei forming the sixteen-nucleate female gametophyte was not always observed; but as a general pattern four nuclei were observed toward the micropylar side of the female gametophyte and twelve closer to the chalazal side. Fig. 20 illustrates the less common pattern of quadripolar distribution of nuclei, in which four domains of cellularization are present.

After the second mitosis the nuclei are indistinct and nearly the same size as first. Almost all of them were observed surrounded by dense cytoplasm, with distinct cell membranes and sometimes even cell walls (Fig.20a-f). Several specimens showed 16-nucleate female gametophytes with more than four nuclei at the micropylar end, but when the egg was observed mature just three cells were observed forming the egg apparatus (Figs. 21a-b).

There is a great deal of variation in the arrangement of nuclei in the mature female gametophyte of *Manekia naranjoana* (Fig.20a-f, Fig. 30). Three nuclei are located at the micropylar end forming the egg apparatus, with two

lateral pyriform synergids and a central egg (Fig. 21a-b). When mature the nuclei of the egg apparatus are bigger but their shape and form does not change with respect to the nuclei at the chalazal end. The polar nuclei are usually located closer to the chalazal end of the female gametophyte, where the two nuclei most often fused (Figs. 20a and detail in 22). The central cell nuclei do not have a clear polarization, sometimes they were found closer to the medium axis of the female gametophyte, and sometimes they were located toward the chalazal end. The two nucleoli of the fusion nuclei were always found close to each other but they were not fused. One time the central cell was observed to have four nuclei fusing together before fertilization (see Fig. 27). The eleven remaining nuclei are arranged from the middle zone to the chalazal pole of the female gametophyte, a few times in groups of four (Fig. 20a-f). Their compartmentalization is not very strong toward the chalazal end.

This description corresponds mostly to Drusa type according to the classification of different female gametophytes made by Maheshwari (1950) even though the distribution of the nuclei sometimes resembles a Penaea type of female gametophyte. Sometimes at the chalazal end several antipodal nuclei were observed fusing together.

Pollen tube growth and Fertilization

In collections from natural populations done in summer 2005, pollen tubes were found growing into the stigma and style and penetrating the ovule (Fig. 23 and 24). But in hand crosses using self pollen on a single individual carried out during the summer 2006, no evidence of pollen tube germination or growth was found (adhesion, hydration, or germination of pollen). In the last pollination experiments there was no evidence of viable pollen in any of the samples that were examined. This study shows that stigmas in *Manekia naranjoana* are wet (positive reaction to Peroxtesmo KO peroxidase test paper). According to Kearns

and Inouye (1993) the test paper shows changes in peroxidase level and the test paper will not work in dry stigmas.

In naturally pollinated inflorescences, single pollen tubes were observed growing between cells within the short style (Figs. 23 and 24). Pollen tubes enter the ovarian cavity penetrated the micropyle and then discharged nuclei into the mature female gametophyte. Pollen tubes were observed penetrating the tissues at the stigmatic surface and growing between the cells at the top portion of the style; instead of growing along its epidermal walls. In the middle portion of the style the pollen tube reaches the transmitting tract and grows through cells to reach the micropyle and ovule. The stigmatic surface in self-pollinated stigmas was observed to have callose depositions (Fig. 6); additionally many mature ovules also displayed callose depositions when we stained with Aniline Blue. Pollen grains on the stigmatic surface, generally were in clusters, held together by a mucilaginous substance.

In sections of ovules where pollen tubes were found penetrating the micropyle, then pollen tubes penetrate the female gametophyte and enter one of the degenerating synergids. The contents of the pollen tube (two male gametes and cytoplasm) are released, as indicated by dark staining coloration within the synergid adjacent to the egg wall. In this stain several chromatic material is evident (Fig. 25). In some female gametophytes that had evidence of pollen tube entry a central cell nucleus with two nucleoli was observed close to a second nucleus with a single nucleus (Fig. 26). This endosperm ploidy corresponds to Drusa type. One time the central cell was observed to have four nuclei fusing together before fertilization (Fig. 27), this endosperm ploidy would correspond to Penaea type,upon fertilization. Antipodal nuclei were found fusing occasionally in mature female gametophytes. Early embryo and endosperm were very rare in the collections (Fig. 28).

Integration of floral ontogeny with the female gametophyte development

Flowers of *Manekia naranjoana* were in the same developmental stages throughout the inflorescence. Almost all flowers in the inflorescences are mature and receptive in a short time interval. Stamens mature and open at the same time, stigmatic receptivity is synchronized in the majority of flowers, and the developmental stage of the ovule and female gametophyte within these flowers are almost uniform. Once the inflorescence comes out from the leaf sheath the two lateral stamens mature first and open five to twelve days later, followed by pistil maturation and stigma receptivity from twelve to eighteen days after bud burst.

During anthesis the inflorescences become very aromatic. When the infructescences are formed they become very rigid and hang from the vine. After the opening of anthers and the receptivity of stigmas several kind of floral visitors where found on the inflorescences, among them several species of aphids, ants and two species of spiders making nets among the flowers. Different types of eggs were also found in the inflorescences suggesting some insects complete their life cycle in them. The actual pollinator was not found.

The following sequence of reproductive events in *Manekia naranjoana* was observed in a single plant.

Day 1: Early in its development inflorescences of *Manekia naranjoana* ranges in size from 3 to 5 cm long. They are light green to light yellow. The perianthless flowers are in bud and only bracts are identified on the surface of the inflorescence. Inflorescences are emerging from the leaf sheath (syleptic inflorescences) or the prophyll (proleptic inflorescences), when the first immature stamens begin to develop (Fig. 29).

The ovary is closed, and there is no stylar channel in the flower. The ovule is immature; the inner integument is starting to develop. At this stage an archesporial cell produces a megaspore mother cell through mitosis. The archesporial cell size is approximately 5 µm wide x 8 µm long and it shape is round to ovoid (Fig. 29).

Day 5: The inflorescences have grown to 5 - 7 cm in length, they are light yellow. Immature anthers are observed growing over the bracts. The two lateral stamens in a flower are the first to emerge, and later a third apical stamen develops in that same flower. The pistil is immature and covered by the bract. The stylar channel starts to form but its cells are not totally differentiated (Fig. 29).

In the ovule the inner integument is totally developed and it closes to form the micropyle, the outer integument is being formed but degenerates quickly. The ovule has a mature megaspore mother cell deep within the nucellar tissue with an extensive cytoplasm. So the megaspore mother cell is present before the two integuments completely envelop the nucellus. The megaspore mother cell when immature is small (less than 10 x 10 µm long) but comparatively bigger than cells surrounding it (less than 5 x 5 µm long), ovoid and have a very limited cytoplasm and a small nucleus (5 x 5 µm long). Once the megaspore mother cell is deep into the tissue, the cell matures, its cellular area grows (35 x 20 µm long) and its shape becomes more ovoid. During interphase and before the beginning of meiosis, the genetic material duplicates making the nucleus bigger (10 µm wide x 15 µm long), and through vacuolization one or more big vacuoles are produced.

At this stage megasporogenesis takes place where the megaspore mother cell undergoes meiosis to form a coenocityc tetraspore. The size of the four nuclei are usually of almost equal size (8 µm wide x 8 µm long) but sometimes the chalazal one is smaller. When mature the coenocyte’s size is approximately 25 µm wide x 40 µm long.

Day 12: Inflorescences have grown to 10 - 14 cm long. They are yellow to light yellow-brownish. The lateral and apical stamens are mature. The basal stamen from the flower is formed but it is immature. Stigmatic lobes are formed and receptive (positive reaction to test paper Peroxtesmo KO peroxidase). In the stylar channel the cells are highly differentiated. In the ovule, megagametogenesis is taking place, the formation of the female gametophyte from the tetraspore where every cell undergoes two mitotic divisions to form a mature female gametophyte (Fig. 29). The immature female gametophyte is 40 µm wide x 50 µm long, with a circular to ovoid shape. The size of nuclei in the mature female gametophyte ranges from 3 to 5 µm long, while the size of the coenocyte is about 35 to 40 µm wide and 50 to 60 µm long, and its shape is round to ovoid.

Day 18: The inflorescence has grown to 14 - 22 cm long, it is light yellow-brownish with dark brown dots. The basal stamen matures after anthesis and later fall off. The stigma gets oxidized and also the scars from the abscission zone of the stamens that felt off (Fig. 29).

The female gametophyte is mature (Fig. 29). Pollen tubes are growing and discharging sperm nuclei for fertilization with the egg and central cell nuclei.

Day 25: Inflorescences have grown to 22 - 25 cm long, green brownish. Fruit formation and early embryo development is taking place (Fig. 29).

Day 35: Infructescences 25 or more cm long, green brownish. Very few seeds are being developed in the inflorescence in comparison to the number of original flowers.

DISCUSSION

Floral development

Inflorescences

The position of inflorescence (axillary, lateral and/or terminal) is an important taxonomic and architectural character in Piperaceae (Jaramillo and Callejas 2004, Callejas 1986). In the majority of species of *Piper* sensu stricto the inflorescences are lateral and the axes are sympodial (Callejas 1986), while a few groups of *Piper* sensu lato such as *Trianopiper* have axillary inflorescences (Jaramillo and Callejas 2004). The inflorescences in *Manekia* are axillary and terminal; while *Zippelia* has only terminal inflorescences (Lei et al. 2002). The axes in both taxa are monopodial. Two types of plant construction can be identified in Piperaceae, a monopodial type of axis in the *Manekia* and *Zippelia* clade and one composed of sympodial modular units in the vast majority of species in *Piper*.

Piperaceae has an inflorescence with tiny reduced flowers lacking petals or sepals. Among these, Zippelia and Manekia have comparatively large flowers. The sequence of flower development in inflorescences was difficult to interpret for Manekia but the morphology, color, texture and other characters of the inflorescence were good predictors of the developmental stages of the flowers in an inflorescence. Additionally, flowers arrive at the same developmental stage in a short period of time among each other.

Flower, carpel and ovary

Bisexual flowers are found in all Piperaceae except the Asian and South Pacific species of *Piper* belonging to the subgenus *Macropiper* (Callejas 1986, Jaramillo and Manos 2001). Species of *Piper* have been reported as herkogamous and partially dichogamous (Semple 1974, Figueiredo and Sazima

2000) like *Manekia*. This suggests partial reproductive isolation by spatial and temporal separation of mature anthers from receptive pistils. But the significance of apical and basal anthers maturing after stigmatic receptivity for *Manekia* can not be interpreted because of the lack of data in terms of pollen tube growth. If stigmatic receptivity overlaps with the maturation of apical and basal anthers and the species is self-compatible, this could be causing reproductive assurance by self-pollination if cross-pollination does not happen.

High rates of ovule abortion were observed before and after fertilization for *Manekia*. Several authors working in some species of *Piper* and *Peperomia* have also reported high rates of ovule abortion (Kanta 196, Martin and Gregory 1962, Semple 1974, Figueiredo and Sazima 2000). Additionally few fruits are formed for each inflorescence (four to five) in *Manekia*. Apomixis has been reported in a dioecious member of the family (*Macropiper*, Gentry 1955) but it was not observed in the species here studied.

Manekia was interpreted as having four syncarpic carpels based on the presence of independent vascular strands inserting in each of the usually four stigmatic lobes. This case has also being reported by Tucker (1982a, b) for several species of *Piper* and by Han-Xing and Tucker (1995) for *Zippelia*.

Ovule

All members of Piperaceae including *Manekia* have similar ovule structure. They have a single basal orthotropous ovule, with two integuments, they are crassinucellar and there is hypostase formation after the ovule reaches maturity (Gvaladze and Akhalkatsi 1990, Nikiticheva 1981, Igersheim 1998). The micropyle is formed from the inner integument in *Manekia* as also is the case in *Zippelia* (Lei et al. 2002). In some species of *Piper* the micropyle is formed from both integuments (Igersheim 1998).

Pollination biology and floral phenology

There was some evidence that sporophytic or stigmatic self-incompatibility occurs in *Manekia naranjoana*. However the hand pollination experiments to test for self-incompatibility were done in twenty inflorescences of a single individual. Even though many factors suggest self incompatibility (lack of germination of pollen), a larger sample using more than one individual needs to be used to show this. Stigmatic self-incompatibility has been reported in *Saururus cernaus* (Pontieri and Sage 1999).

The sequence of reproductive events in *Manekia* including several aspects of the floral phenology is an important series of data that hardly ever are reported in papers of reproductive biology. The developmental stages of flowers and inflorescences are predictors of the stages of development in the ovule and female gametophyte. Stigmatic receptivity is occurred around day twelve after floral burst; it lasted for approximately six days or more. At the onset of stigmatic receptivity the female gametophyte is still immature, but by day eighteen right when the stigmatic receptivity is ending the female gametophyte is mature.

Female gametophyte

The female gametophyte development for *Manekia naranjoana* is described for the first time in this study. During megasporogenesis a sporogenous cell gives rise to the single megaspore mother cell. The megaspore mother cell undergoes meiosis without cell wall formation. As a result four free megaspore nuclei are formed within an ovoid coenocyte. The four megaspore nuclei in the coenocyte undergo two mitotic divisions resulting in a sixteen-nucleate female gametophyte. Three nuclei were located at the micropylar end forming the egg apparatus, with two lateral pyriform synergids and a central egg. The central cell contains two nuclei, one from the micropylar end and the other one from the chalazal one. The eleven remaining nuclei were arranged toward

the periphery of the chalazal pole of the female gametophyte, and sometimes fused. This description corresponds to the Drusa type. Few times, I found three nuclei located at the micropylar end forming the egg apparatus, a central cell containing four nuclei, one from the micropylar end, one from the chalazal end and two coming from each lateral pole. The eleven remaining nuclei were arranged three toward the chalazal pole, and three at each lateral pole. This description correspond the Penaea type. There was considerable variation arrangement of nuclei at both early and mature stages, but the extremes reported here were found at all stages (Fig. 30).

All the members of Piperaceae are reported as tetrasporic in previous studies but the development of the female gametophytes is different for several of its groups. For all the genera of Piperaceae (including data reported in this study) except for *Peperomia* there is no cell wall formation after meiosis I and II. However, Johnson (1914) described formation of rudimentary cell walls in the tetraspore of *Peperomia*. *Manekia* and *Zippelia* have a single archesporial cell and a similar ontogenetic trajectory for the female gametophyte (Lei et al. 2002), while *Piper* (Fritillaria type) and *Peperomia* (Peperomia type) each has a different type of female gametophyte. *Peperomia* has occasionally more than a single archesporial cell (Johnson 1914). Lack of clear polarization in the four nuclei of the tetraspore was sometimes observed in this study for *Manekia* (Fig. 30). Additionally the female gametophyte structure was variable sometimes according to the shape of the coenocyte. More pear-like female gametophytes produce tetrapolar arrangement of nuclei, while in more spheroid-like coenocytes the distribution of the nuclei was bipolar (Fig. 30). This change in structure in the female gametophyte with the shape of the coenocyte has also been reported in *Peperomia* (Maheshwari 1963).

Lack of polarization was also frequently observed. The antipodal nuclei in the mature female gametophyte of *Manekia* have been observed having a

spontaneous pattern of fusion without any particular organization (Fig. 27); this was also reported by Lei et al. (2002) for *Zippelia*.

Tetraspory and the origin of the sixteen nuclei female gametophyte in Piperales

The tetrasporic development of the female gametophyte is a derived and homoplastic character in the phylogeny of flowering plants, but little is known about its specific developmental origins and its evolutionary significance. Piperales represents an excellent group to examine the consequences of the origin of tetraspory because of the high variation among its groups, in terms of female gametophyte development (Fig. 31) and because of strong evidence for its origin within Piperaceae.

The phylogeny of Piperales, and a simple parsimony-based character analysis, suggests that monosporic development is ancestral in the order (Fig. 31). Monosporic development of the female gametophyte has been found in its sister group Saururaceae (Quibell 1941, Raju 1961, Murty 1960, Yoshida 1961), and even though bisporic development has been suggested in *Saururus*, the evidence supporting this type of development is weak (Nikiticheva 1981). Aristolochiaceae and Lactoridaceae are also monosporic (Johri and Bhatnagar 1955, Wyatt 1955, Nair and Narayanan 1961, Tobe et al. 1993). In contrast, Piperaceae at least three different types of tetrasporic development are found (Fig. 31), and a fourth, Penaea-type was found as a variant in *Manekia.* This suggests that the ancestor of Piperaceae was monosporic and once the tetrasporic condition was reached in Piperaceae variation in developmental pathways was easy to develop (Fig. 31).

In Piperaceae the tetrasporic sixteen-nucleate female gametophyte could be considered an ancestral stage of the tetrasporic development in the family (Fig. 31). Two different parsimony arguments emerge when analyzing the phylogeny of Piperales in terms of female gametophyte development: (1) the sixteen nuclei female gametophyte of *Manekia*, *Zippelia* and *Peperomia* arose one time in Piperaceae, and the 7-celled/8-nucleate Fritillaria-type arose one time in *Piper*. Or (2) the sixteen-nucleate female gametophyte arose two times: once in *Manekia* and *Zippelia* and once in *Peperomia*; and the 7-celled/8-nucleate Fritillaria-type arose one time in *Piper*.

These two scenarios can be interpreted in terms of three basic traits of the female gametophyte: (1) the number of nuclei, (2) their origin (monosporic or tetraporic), and (3) the endosperm ploidy. The tetrasporic sixteen nuclei female gametophyte of *Manekia*, *Zippelia* and *Peperomia* originates once from a monosporic 7-celled/8-nucleate female gametophyte and the tetrasporic 7-celled/8-nucleate female gametophyte of *Piper* originates once in the family from a tetrasporic sixteen nuclei female gametophyte. Or the tetrasporic sixteen nuclei female gametophyte originates twice first from a monosporic 7-celled/8-nucleate female gametophyte in *Manekia* and *Zippelia*, and then from the tetrasporic 7-celled/8-nucleate female gametophyte of *Piper* in *Peperomia*. In terms of the ploidy of the endosperm the tetrasporic triploid (*Manekia* and *Zippelia*), nonaploid and dodecaploid (*Peperomia*) endosperms originates once from the monosporic triploid endosperm and the tetrasporic pentaploid endosperm in *Piper* originates once from a tetrasporic triploid (*Manekia* and *Zippelia*), nonaploid and dodecaploid (*Peperomia*) endosperms. Or the tetrasporic triploid (*Manekia* and *Zippelia*), nonaploid and dodecaploid (*Peperomia*) endosperms originates twice. The tetrasporic triploid endosperm in *Manekia* and *Zippelia* originates once from a monosporic triploid endosperm, while the nonaploid and dodecaploid endosperms of *Peperomia* originates once from the tetrasporic pentaploid endosperm in *Piper*.

In terms of developmental biology many cellular events have to take place in the female gametophyte to switch from a monosporic to a tetrasporic condition. Heterochronic and heterotopic changes, additions and deletions early in the development of the female gametophytes influenced evolutionary stories of the particular taxa implicated (Fig. 31). Early in megasporogenesis several ontogenetic steps differ and ultimately determine the female gametophyte configuration. In monosporic taxa of Piperales the cell walls are persistent after meiosis I and II, additionally the three upper haploid cells degenerate. On the contrary the failure of cell wall formation (*Manekia* or *Zippelia*) or degeneration of the cell walls (*Peperomia*) after meiosis I and II, and the loss of megaspore nucleus degeneration determines the tetrasporic development (Fig. 31).

Heterotopic changes (phyletic changes in location from which one organ differentiates in ontogeny; Gould 1977) took place in the nuclei of the coenocytic tetraspore after the end of meiosis II. The ancestral arrangement of the four independent megaspores in monosporic taxa of Piperales is linear or T-shaped. I suggest that through migration of nuclei to two or four domains were established in the megaspore of tetrasporic Piperaceae after the failure of cell wall formation(heterotopy). In comparison to Saururaceae with a linear distribution of megaspores, in *Manekia* and *Zippelia* heterotopy occurs when three nuclei from the coenocytic tetraspore migrate to the chalazal end (Fig. 31).

The origin of tetrasporic development started with the failure in cell wall formation during meiosis. Four genetically different nuclei are being conserved in a single coenocyte while in monosporic development three of those nuclei are discarded and the genetic variation is limited to one single nucleus. As a result the nuclei of a monosporic female gametophyte are genetically homogeneous, while in tetrasporic taxa the nuclei that compound the female gametophyte are not all the same. This genetic mosaic in the coenocyte of tetraspores could

potentially have been an important event in the evolutionary history of Piperaceae (see Haig 1987, 1990).

Many other changes are followed after this initial formation of the megaspore. While in monosporic groups of Piperales polarization of the nuclei starts after the first mitosis of the remaining megaspore, in tetrasporic groups two poles or four poles are established after meiosis II. This suggests acceleration in polarization of nuclei during the ontogenetic trajectory (Fig. 31). Acceleration of tetrasporic ontogenetic trajectories in Piperaceae is explain by the fact that more nuclei are present in the coenocyte after meiosis II, in comparison to the number of nuclei in the monosporic groups of Piperales (Fig. 31). In this way the mature female gametophyte in tetrasporic taxa is complete after two mitotic divisions but it has a higher number of nuclei (sixteen) in comparison to monosporic taxa were after three mitotic divisions the female gametophyte has a total of eight nuclei.

During megagametogenesis of tetrasporic taxa a deletion on the tail of the developmental trajectory is suggested because mitosis III is not taking place as in the ancestral monosporic condition (Fig. 31). In any case the nuclei of a monosporic or tetrasporic coenocyte stop dividing mitotically when a set of four nuclei has been formed at the micropylar pole (Friedman and Williams 2003). The four nuclei at the micropylar pole contribute to the formation of the egg, synergids and the central cell nuclei. This set can be completed with a different number of cells and ploidy at the chalazal pole (Fig. 31).

The mature female gametophyte in monosporic and tetrasporic taxa varies in the number of nuclei implicated in its construction, the polarity of such cells, the number of nuclei participating in the central cell nuclei, and the ploidy of such nuclei (Fig. 31). In tetrasporic development two developmental stages are fundamental in the determination of nature of the female gametophyte: (1) the arrangement of nuclei in the tetraspore which ultimately establish separate

cytoplasmic domains, and (2) the number of nuclei contributed to the central cell and hence to the endosperm. But as shown in this study there is a high amount of variation and/or plasticity in key steps of the ontogenetic trajectory for *Manekia naranjoana*. *Peperomia*, *Manekia* and *Zippelia* have developmentally unstable endosperm.

Genetic diversity in the endosperm of *Manekia* and *Zippelia* is higher than in monosporic taxa of Piperales. In these two taxa the three nuclei participating in its formation are genetically different while in monosporic taxa two cells of the endosperm are genetically identical and just the male nucleus is genetically different (Fig. 31). In *Piper* and *Peperomia* the endosperm is considerably more genetically diverse than in monosporic taxa and tetrasporic *Manekia* and *Zippelia*, because of the participation of a high number of cells in the central cell nuclei with different genetical composition (Friedman et al. in press).

Embryological plasticity

The female gametophyte of *Manekia naranjoana* is highly variable at the key steps of the ontogenetic trajectory that will determine the identity of the female gametophyte. The arrangement of the four nuclei in the tetraspores observed in this study was variable (Fig. 30). In mature tetraspores a tetrapolar arrangement was rarely evident while a weak bipolar arrangement of four nuclei was more frequent, one nucleus at the micropylar end and three at the chalazal (Fig. 30). Strong polarity of nuclei in the coenocyte was seldom observed. This weak polarity of the nuclei of the coenocyte was also reflected at mature stages of the female gametophyte (Fig. 30). In the mature coenocyte we observed a bipolar distribution of nuclei as a general pattern (Fig. 30). However few mature coenocytes possessed a tetrapolar distribution of nuclei in the female gametophyte instead of a bipolar. Some other times any kind of pattern of distribution was not even evident (Fig. 30). Polarity could be interpreted as a

plastic character in *Manekia* tetrasporic female gametophyte. As an alternative the determination of structure in its female gametophyte has to be more associated with the origin of the megaspore (monosporic or tetrasporic) and the number of nuclei participating in the female gametophyte.

Fusion of nuclei is a recurrent characteristic in tetrasporic female gametophytes of Piperales, and also seems a plastic character in the order. In monosporic female gametophytes the identity of individual nuclei is conserved. Fusion of antipodal nuclei with the nuclei in the endosperm has been reported for *Zippelia* (Lei et al. 2002). In any of these two cases this appears to be a factor influencing the structure of the female gametophyte as a whole. Also, this is not a process implicated and fixed in the ontogenetic trajectory of these groups like it is the case of the fusion of nuclei forming a triploid antipodal nucleus in the tetraspore of *Piper*. The central cell nuclei in *Peperomia* show a dramatic example of nuclear fusion. Johnson (1914) found that between four to fourteen nuclei group together in the center of the coenocyte forming a highly polyploid endosperm. In taxa with a high ploidy of the endosperm like *Piper* and *Peperomia* it is difficult to establish if double fertilization is taking place when the sperm is discharged in the coenocyte.

The variability and/or plasticity in the structure of female gametophyte in Piperaceae is taking place at the chalazal pole of the female gametophyte. But the micropylar pole has been conserved in terms of structure and form. It is in this way that the theory of modular construction of the female gametophyte (Friedman and Williams 2003) lacks coherence in terms of cytoplasmically autonomous domains and even static terminal ontogenetic stages (see Friedman et al in press). Additionally the variability observed in this study shows how even though the embryological types proposed by Maheshwari (1950) are useful to illustrate the embryological trajectories in flowering plants, they have to be carefully interpreted. Future studies in embryology should concentrate on

describing such variation in the developmental pathways instead of trying to accommodate data to embryological types that do not reflect the plasticity of such characters.

Modularity in tetrasporic Piperaceae

I interpreted my findings from *Manekia* in the context of female gametophyte diversity within Piperales. Placing these data into an evolutionary framework could explain evolution of flowering plant endosperm. There are two sets of hypotheses that could be plausible: (a) the evolution of the female gametophyte through modular duplication, resulting in increases of endosperm ploidy, and (b) the evolution of the female gametophyte through gradual reduction, resulting in ploidy reduction of endosperm (Battaglia 1951). The first hypothesis deals with the concepts of cell modularity and duplication. Modular developmental subunits constructed through duplication have been proposed to explain the early evolution of the angiosperm female gametophyte. An ancestral four celled female gametophyte could be duplicated to form the 7celled/8nucleate female gametophyte (Williams and Friedman 2002, Friedman and Williams 2003, 2004), which has a triploid biparental endosperm. The 7 -celled/8-nucleate female gametophyte is also known as the Polygonum type and it was largely considered the ancestral type of female gametophyte (Palser 1975). Virtually all early angiosperm female gametophytes consist of one or two modules, but the female gametophytes of some Piperaceae have not yet been interpreted in this context. The hypothesis of modular duplication suggests that the chalazal module of the Polygonum type (7celled/8nucleate female gametophyte) is a developmental novelty; this means that the second polar nucleus and the antipodal cells are angiosperm innovations (Williams and Friedman 2004). Friedman and Williams (2003) explain that key innovations between different types of female gametophyte lie in the modification of early development either

due to heterochronic or heterotopic changes of groups of nuclei in different cytoplasmic domains (micropylar and chalazal) of the developing female gametophyte. The developmental origin of bisporic and tetrasporic female gametophyte could also be explained using the hypothesis of modular duplication. In these cases, acceleration of module initiation result in additional modules being form.

The second hypothesis deals with the evolution of female gametophyte through gradual reduction. Ancestral female gametophytes have thousands of cells (i. e. gymnosperms), while more derived ones have a drastic reduction in the number of cells (varying from sixteen to eight to four). For example, Piperaceae was once thought to be a model for understanding evolutionary patterns among the earliest flowering plants (Arber and Parkin 1907, Burger 1977, Donogue and Doyle 1989, Qiu et al. 2000, 2005). The female gametophyte of *Peperomia* was once considered to represent an intermediate stage in the origin of the angiosperm female gametophyte from a gymnosperm-like female gametophyte. This was because of the members of Piperaceae had female gametophytes with high numbers of cells and nuclei (i. e. 9-celled/16-nucleate and 15-celled/16-nucleate female gametophytes.) This morphological arrangement is more similar in appearance to the gymnosperm female gametophyte, with an even higher number of cells (Gifford and Foster 1989). In contrast, the majority of angiosperms present a more reduced female gametophyte (i. e. 4 cell and 7celled/8nucleate female gametophytes), which was believed to be a derived condition (Johnson 1914, Gvaladze and Akhalkatsi 1990), relative to Piperaceae.

My findings from *Manekia* agree with the hypothesis of the evolution of the female gametophyte through modular duplication, resulting in increases of modules form a 7celled-8 nucleate Poygonum type with two modules. The effect of module increase through heterochrony is to increase endosperm genetic

variation. The second hypothesis about the evolution of the female gametophyte through gradual reduction resulting in ploidy reduction of endosperm (Battaglia 1951) lacks support because of the phylogenetically derived placement of Piperaceae in all recent molecular phylogenies of flowering plants (Donoghue and Doyle 1989, Chase et al. 1993, Zimmer et al. 2000, Qiu et al. 2005).

In terms of modularity (sensu Friedman et al. in press, Friedman and Williams 2003) the tetrasporic female gametophytes in Piperaceae can be interpreted as two modular or four modular according to the taxa and its ontogenetic trajectory (Fig. 31). Furthermore, the final number of nuclei in each module of a tetrasporic female gametophyte is always complete after two nuclear mitoses. Without exception the micropylar module is highly conserved in the number of nuclei. The number of final nuclei will be four and at least one of them always migrates to the central cell. This module receives the pollen tube, participates in the reproduction, and fertilization of the egg. The lateral modules and/or the chalazal module are highly variable in tetrasporic female gametophytes of Piperaceae, they can have four to twelve nuclei, and always participate with at least one nucleus in the central cell. These modules are implicated in the nutrition of the embryo. The more cells present in the supplementary modules, the higher the nutrients coming from the nucellar tissue to feed the embryo (Willemse 1981).

The female gametophyte of *Manekia* and *Zippelia* with sixteen nuclei establishes two modules in the tetraspore after meiosis. The initial micropylar module has one nucleus while the chalazal has three nuclei. At maturity the micropylar module has four cells and the chalazal module twelve cells (Fig. 31). So the differences between modules are based on the number of cells but the cytoplasmic domains still indicate two modules like the monosporic 7-celled/8nucleate female gametophyte. The Penaea type rarely observed in this study has a quadripolar distribution of the sixteen nuclei in the female

gametophyte. Each of the four nuclei undergoes two mitoses, forming a female gametophyte with sixteen nuclei and four modules.

The female gametophyte of *Piper* represents a highly organized and derived type of development. During the tetraspore development additionally to the formation of a micropylar domain with one nucleus and a chalazal domain with three nuclei, where there is a fusion of the three nuclei to form a triploid nucleus. In this case two modules are formed based again in cellular domains but the chalazal pole is genetically highly variable. At maturity the female gametophyte in *Piper* resembles the 7-celled/ 8-nucleate female gametophyte of the majority of flowering plants but in this case the chalazal domain has four cells that are each triploid (Fig. 31).

Peperomia represents a special case in which four different domains are identified in the tetraspore after meiosis. In this case four different modules with four cells each are taking place in the construction of the female gametophyte in the genus. There is an addition of two lateral modules with four cells each in the ontogenetic trajectory (Fig. 31), in comparison to the rest of the monosporic and tetrasporic groups analyzed here. However and at maturity the cells in the female gametophyte of *Peperomia* do not show a very strong polarity to each domain. This makes the theory of modularity difficult to interpret in terms of cytoplasmic domains for this species. Or structural modules must be differentiated in such a small space that they are hard to distinguish.

In terms of modularity the numbers of cells from each module migrating to the central region are essential. The contribution of nuclei and ratio of paternal vs. maternal genomes in the endosperm are highly variable in tetrasporic female gametophytes. Selection favors endosperms with higher ploidy (Stebbins 1974), higher heterozygosity (Brink and Cooper 1947), and lower maternal vs. paternal conflict (Friedman et al. in press). The higher the levels of heterozygosity and ploidy the better nourished the embryo is expected to be (Friedman et al. in

press). The ancestral type of tetrasporic female gametophyte in *Manekia* and *Zippelia* receives in the central cell one nucleus from the micropylar module, one from the chalazal, but both of them are genetically different to each other (Fig.30). In comparison to Saururaceae which has a Polygonum type female gametophyte where two genetically identical haploid cells are participating in the central cell, and lately the endosperm (Fig. 31).

The female gametophyte of *Manekia* and *Zippelia* has a higher heterosis and higher ploidy in comparison to Saururaeae, and as a consequence a more vigorous endosperm is formed (Fig. 31). In *Piper* the central cell is composed of one haploid nucleus coming from the micropylar domain and a triploid cell with three different nuclei coming from the chalazal domain. So in this case the proportions of micropylar vs. chalazal contribution are unequal, and the chalazal domain has a higher contribution in terms of genetic diversity (Fig. 31). *Peperomia* represents an extreme case where heterozygosity and levels of ploidy are the highest among flowering plants; each of the four domains contributes with differently to the genetics to the central cell (Johnson 1914).

Embryology and its implications in systematics of Piperaceae

The molecular phylogenies for Piperaceae place *Manekia* and *Zippelia* as sister groups (Jaramillo et al. 2004, Wanke 2007). In this study we found a pattern of female gametophyte development for *Manekia* similar what was found in a previous study of *Zippelia* (Lei et al. 2002). In both genera the female gametophyte has a similar structure with sixteen nuclei, and two nuclei in a central cell where a triploid endosperm is formed. The female gametophyte development is substantially different in the two more species-rich genera of the family *Piper* and *Peperomia,* not just between them but also in comparison to *Manekia* and *Zippelia*. The genus *Piper* has a 7-celled/8-nucleate female

gametophyte, a central cell with four nuclei, and as a result a pentaploid endosperm (Swamy 1945, 1944; Kanta 1961,1962; Prakash 1994), while *Peperomia* has 9-celled/16-nucleate female gametophyte, and a central cell in which four to fourteen nuclei could participate (Johnson 1900, 1914; Campbell 1901; Plyushch 1982; Smirnov and Grakhantseva 1988). This evidence suggest that a least three different ontogenetic pathways of tetrasporic development are taking place in Piperaceae.

Manekia was largely considered based on morphological characters to be a part of *Piper* (De Candolle 1923, Jaramillo and Manos 2001). But contrasting morphological characters (Jaramillo et al. 2004, Arias 2006), molecular phylogenies (Jaramillo and Manos 2001, Wanke et al. 2007) and the developmental evidence found in this study suggest *Manekia* as more closely related to *Zippelia*. Some of the synapomorphies that *Manekia* and *Zippelia* share are: a sixteen nucleate female gametophyte known as Drusa type, and triploid endosperm. Developmental evidence is offered in this study that indicates molecular analyses are congruent with the embryology of genera in Piperaceae.

CHAPTER V
CONCLUSIONS

My analysis of female gametophyte development in *Manekia naranjoana* suggests a similar pattern of female gametophyte development for *Manekia* as was observed in a previous study of *Zippelia*. This evidence shows that both groups share similar evolutionary stories in terms of female gametophyte structure and they are different from the overwhelming majority of species in the family. At least three different ontogenetic pathways of tetrasporic development occurs place in Piperaceae. Penaea type is being reported for first time in this study for the family. Their evolutionary significance relies on the assumption that the increase in ploidy of the endosperm promotes vigor in the embryo. The genetic diversity in the endosperm of *Manekia* and *Zippelia* is higher than in monosporic taxa of Piperales. Additionally, in *Piper* and *Peperomia* the endosperm is considerably more genetically diverse than in monosporic taxa and *Manekia* and *Zippelia*, because of the participation of a high number of cells in the central cell nuclei with different genetical composition.

The tetrasporic development of the female gametophyte is a derived character in Piperales. Heterochronic and heterotopic changes, additions and deletions have to take place in the female gametophyte ontogenies to switch from a monoporic to a tetrasporic condition. Female gametophytes in Piperaceae can be interpreted as two modular (*Manekia*, *Zippelia* and *Piper*) or four modular (*Peperomia*). With the increase in the number of modules there is an increase in the genetic variation in the central cells and ultimately the endosperm. The micropylar module is highly conserved in the number of nuclei, while the chalazal pole is highly variable. The variability and/or plasticity in the structure of female gametophyte in Piperaceae are taking place at the chalazal pole of the female

gametophyte. *Manekia naranjoana* is highly variable at the key steps of the ontogenetic trajectory that will determine the identity of the female gametophyte. Fusion of nuclei and lack of polarity are recurrent events in the ontogenies of tetrasporic clades, while in monosporic female gametophytes the identity of individual nuclei and the polarity are highly conserved.

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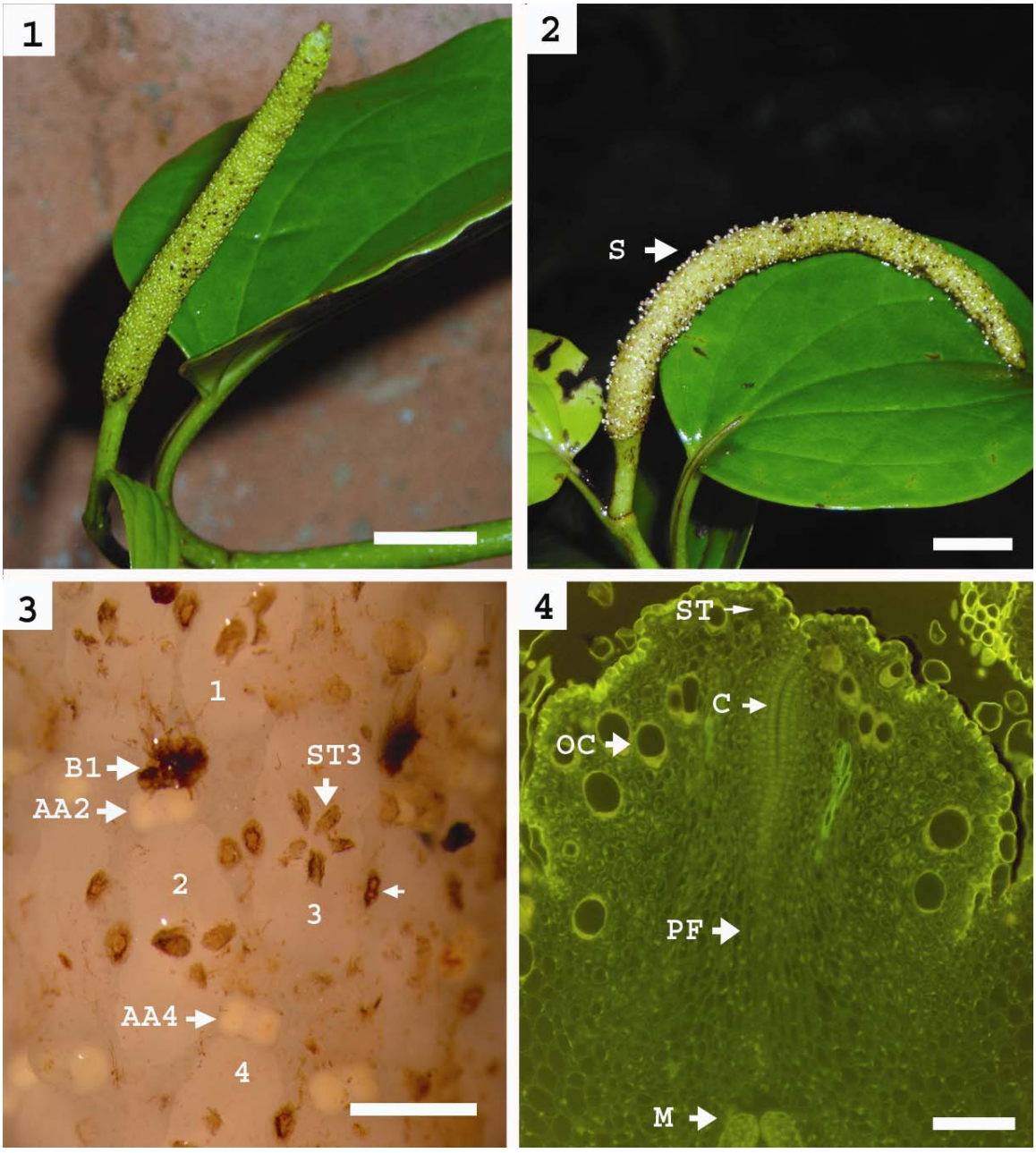
APPENDIX



Figures 1-4. Inflorescences and floral morphology of *Manekia naranjoana* (Piperaceae).

Scale Bars in Fig 1 = 1cm; Fig.2 = cm; Fig. 3 = 0.2cm, Fig. 4 = 50µm.2

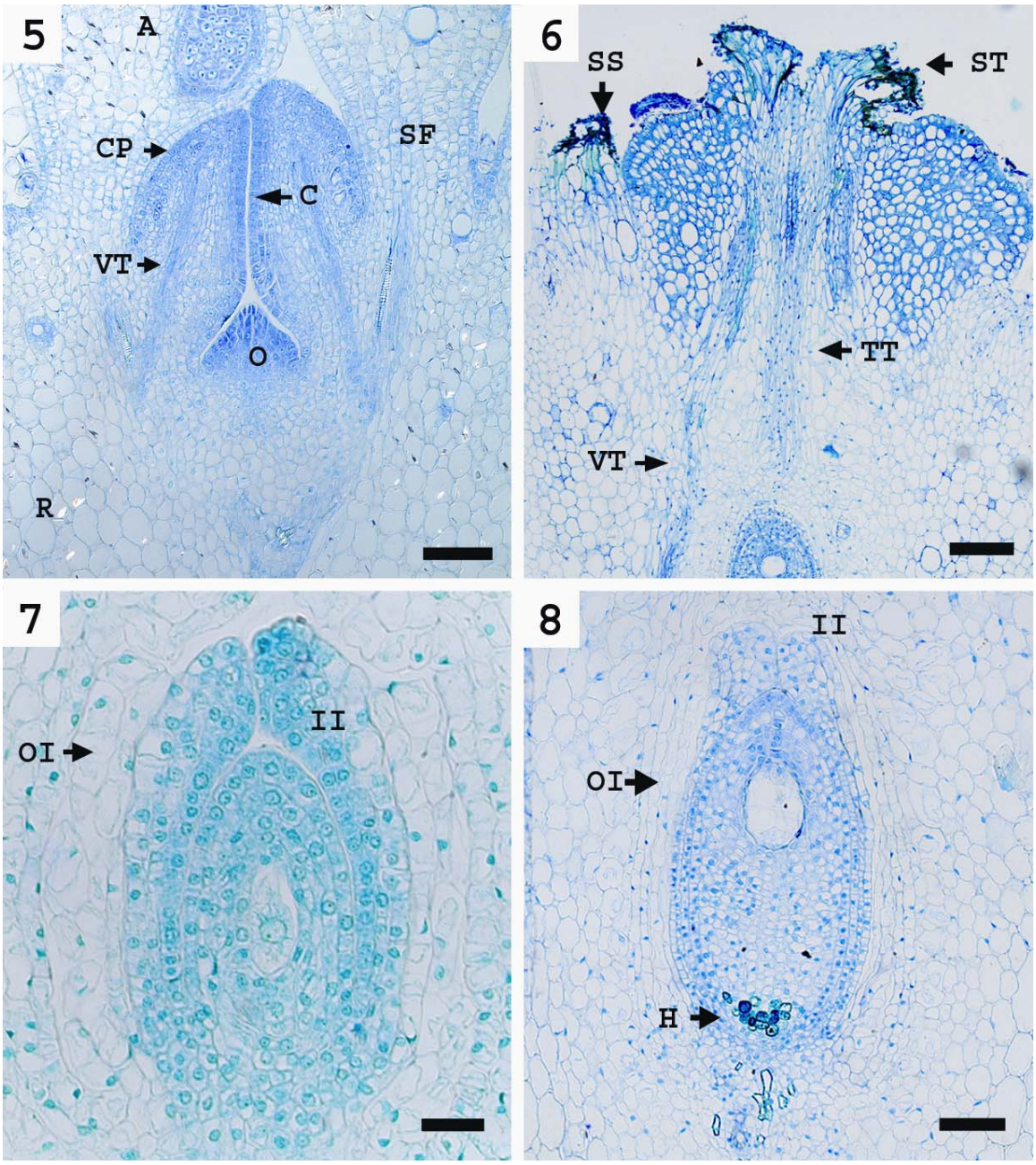
Figure 1-4, continued

Figure 1. Early inflorescence with floral primordial.

Figure 2. Mature inflorescence with mature flowers, receptive stigmas and apical anthers close to open (S).

Figure 3. Close up of flowers in inflorescence, flowers are numbered ,as are associated structures, they are showing stigmatic lobes (ST), bracts (B), apical anthers in maturation (AA) and scars of abscission zones of lateral anthers indicated by arrows for flower 3.

Figure 4. Cross-section of inflorescence and longitudinal section of flower at anthesis (Aniline Blue Staining) showing stigma (ST), oil cells (OC), stylar canal (SC), and postgenital fusion in the lower part of the style (PF) and micropyle (M)



Figures 5-8. Longitudinal sections of flowers and ovules of *Manekia naranjoana* stained with Toluidine Blue. Scale bars Figs. 5 and 8 = 50µm; Fig. 6 = 100µm; Fig.7 = 20µm.

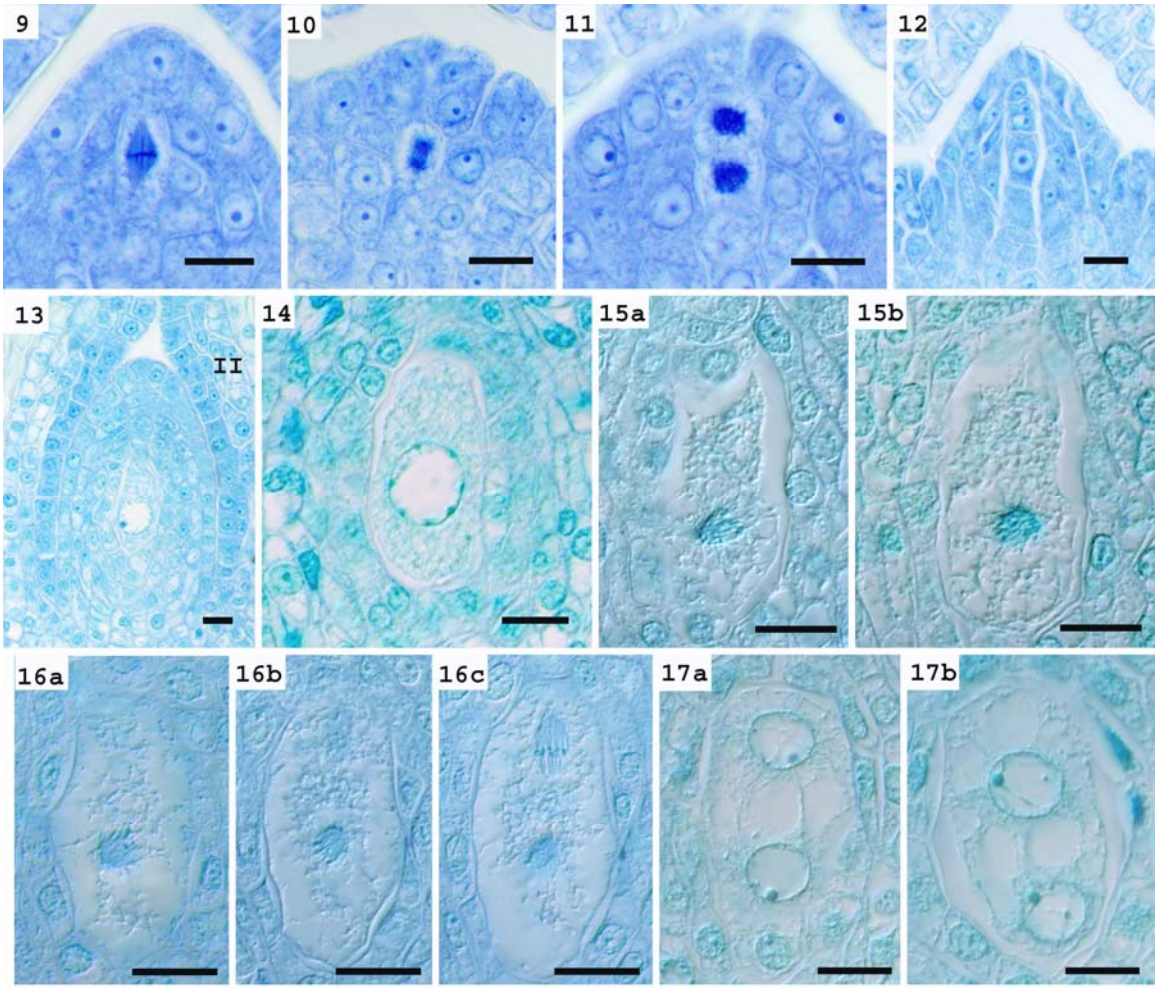
Figure 5 Early flower showing anthers (A), carpels (CP), stylar canal (C), vascular tissues (VT), ovule (O) and rachis (R).

Figure 6 Flower after anthesis showing stigma (ST), abscission zone of stamens (SS), transmitting tract (TT) and vascular tissues (VT).

Figure 5-8, continued

Figure 7 Ovule with megaspore showing outer integument (OI) and inner integument (II).

Figure 8 Ovule at megagametogenesis showing hypostase (H) and outer (OI) and inner (II) integuments.



Figures 9-17. Megasporogenesis of *Manekia naranjoana* (Piperaceae). **Scale bars = 10 µm.**

Figure 9. Metaphase of archesporial cell

Figure 10. Anaphase of archesporial cell.

Figure 11. Telophase of archesporial phase.

Figure 12. Megaspore mother cell and pariental tissue.

Figure 13. Mature megaspore mother cell in a crassinucellar ovule showing the inner integument (II).

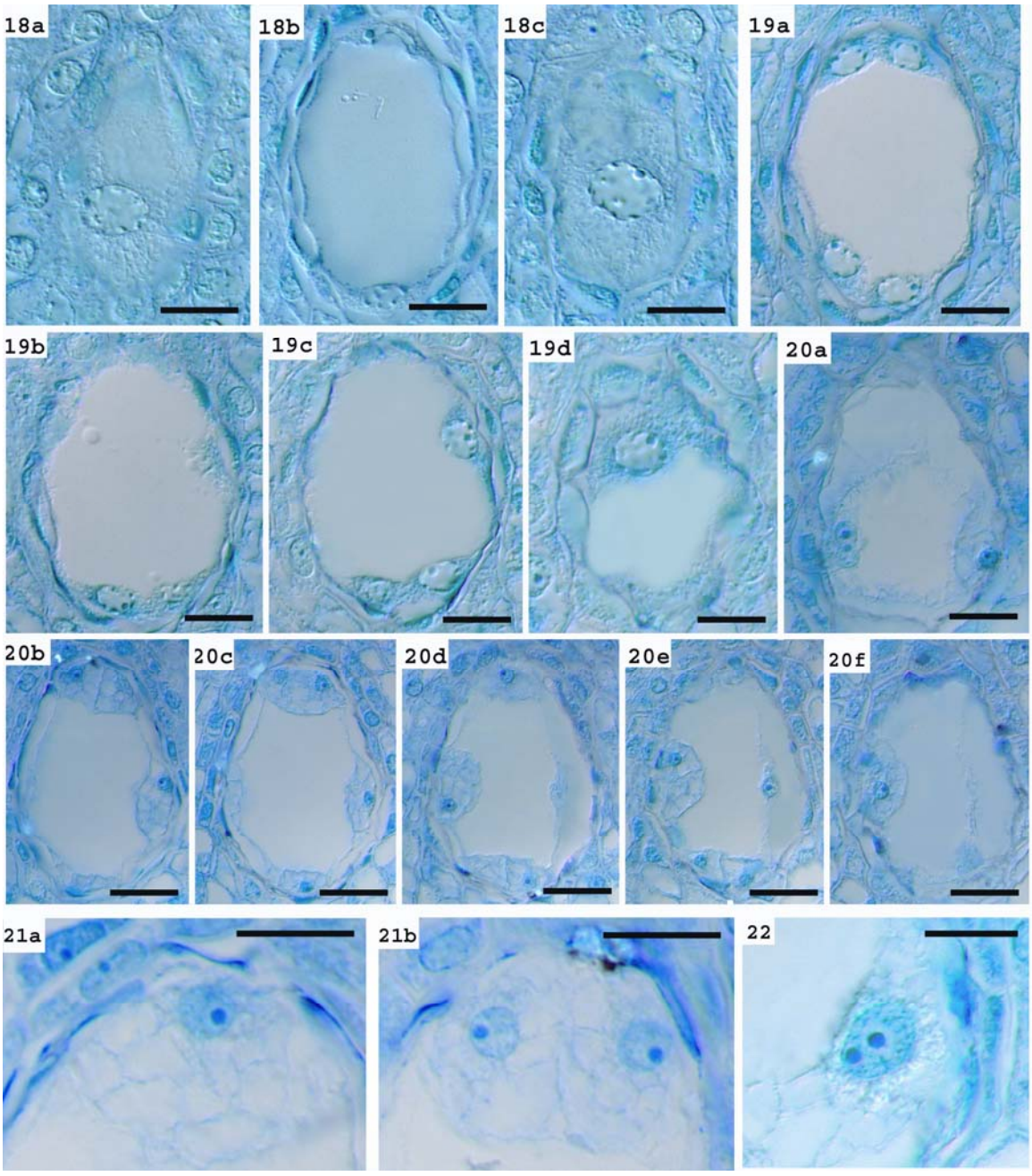
Figure 14 Megaspore mother cell in prophase.

Figure 9-17, continued

Figure 15a-b. First meiotic division, anaphase I of the megaspore mother cell, serial and adjacent sections.

Figure 16a-c. Second meiotic division, serial and adjacent sections. Figs. 16a-b. Chalazal cell in anaphase II. Fig. 16c. Micropylar cell in metaphase II.

Figure 17a-b. Tetraspore, serial but not adjacent sections.



Figures 18-22. Megagametogenesis of *Manekia naranjoana* (Piperaceae). **Scale bars = 10 µm.**

Figure 18-22

Figure 18a-c. First mitotic division of linear tetraspore in prophase, serial but not adjacent sections. Fig. 18a. central nucleus in prophase. Fig. 18b. Chalazal and micropylar nuclei, the chalazal one at prophase. Fig 18c. Central nucleus at prophase.

Figure 19a-d. Eight nucleate stage in prophase (serial and adjacent sections). Fig. 19a. Four nuclei in prophase in this section, two at the micropylar end and two at the chalazal one. Fig. 19b. One nucleus in prophase at the chalazal end. Fig. 19c. Two nuclei in prophase one at the micropylar end and one at the chalazal one. Fig. 19d. One nuclei in prophase at the micropylar end.

Figure 20.a-f. Sixteen nuclei female gametophyte. Fig. 20a. Central cell nucleate. Fig. 20b. Section with two nuclei, one at the micropylar end and one at the chalazal one. Fig. 20c. Four nuclei, two at the micropylar end and two at the chalazal. Fig. 20d. Two nuclei at the chalazal end. Fig. 20e. Four nuclei at the chalazal end. Fig. 20f. One nucleus at the chalazal end.

Figure 21 a-b. Egg apparatus. Fig. 21a. Egg. Fig 21b. Synergids.

Figure 22. Detail of a central cell nucleus, formed by fusio of two polar nuclei in the chalazal region.

Figures 23-28. Post-pollination events in *Manekia naranjoana*. Scale bars in Figs. 25, 26, 27 = 10 µm; Fig. 28 = 15 µm ; Fig. 25 = 10 µm; Fig.24 = 20µm; Fig.23 = 40µm.

Figure 23 Pollen tube (PT) growing in the transmitting tube.

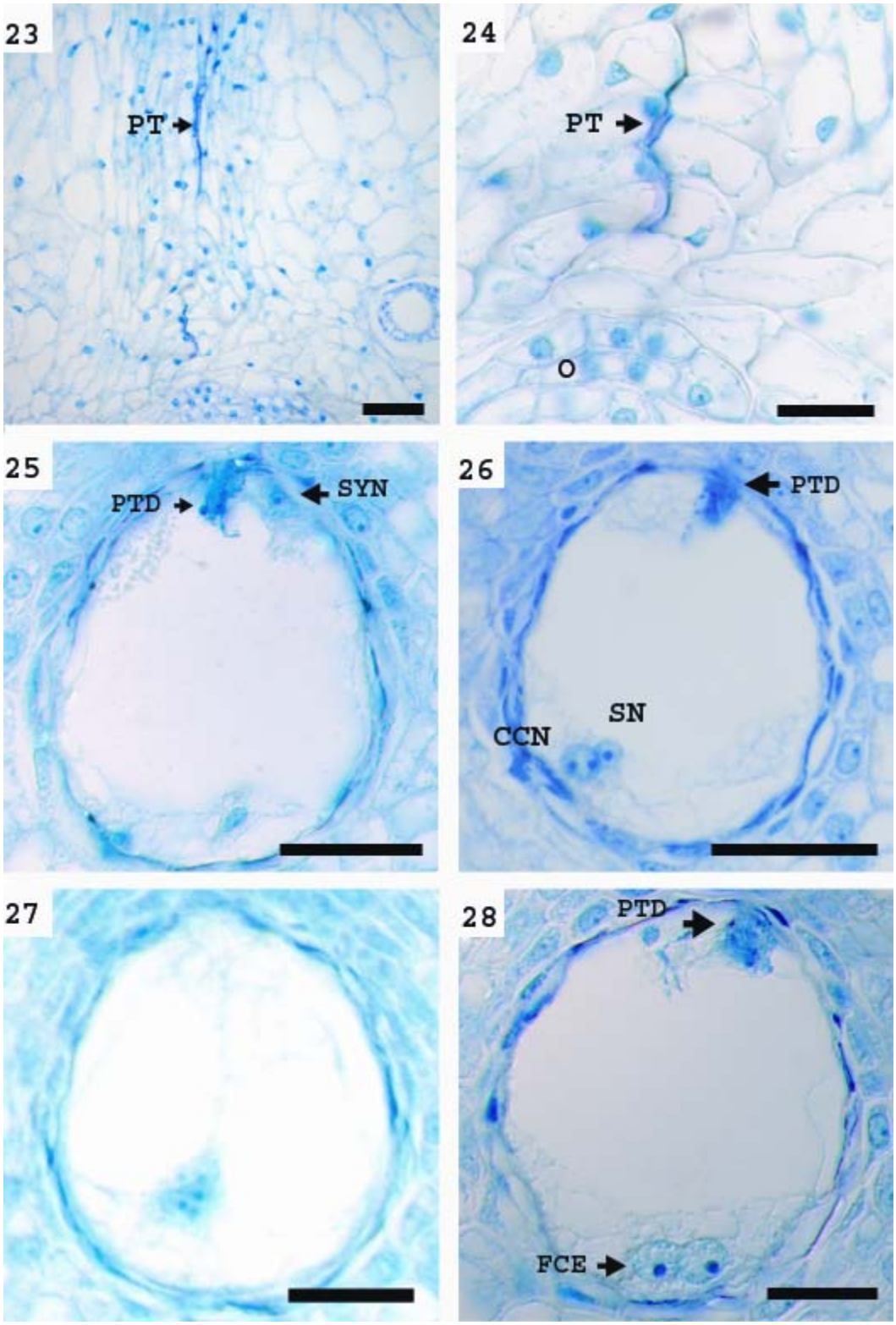
Figure 24. Pollen tube reaching the ovule (O).

Figure 25 Pollen tube contents being discharged in the micropylar end of the female gametophyte, pollen tube discharged (PTD), synergid (SYN).

Figure 26. Central cell nuclei (CCN) fusing with a sperm nuclei (SN) in a female gametophyte,.

Figure 27. Central cell nuclei (CCN) with four nuclei participating in the fusion.

Figure 28. First mitosis of the endosperm, first cells of the endosperm (FCE).


23
PT
24
PT
O
25
PTD
SYN
26
PTD
SN
CCN
27
28
PTD
FCE

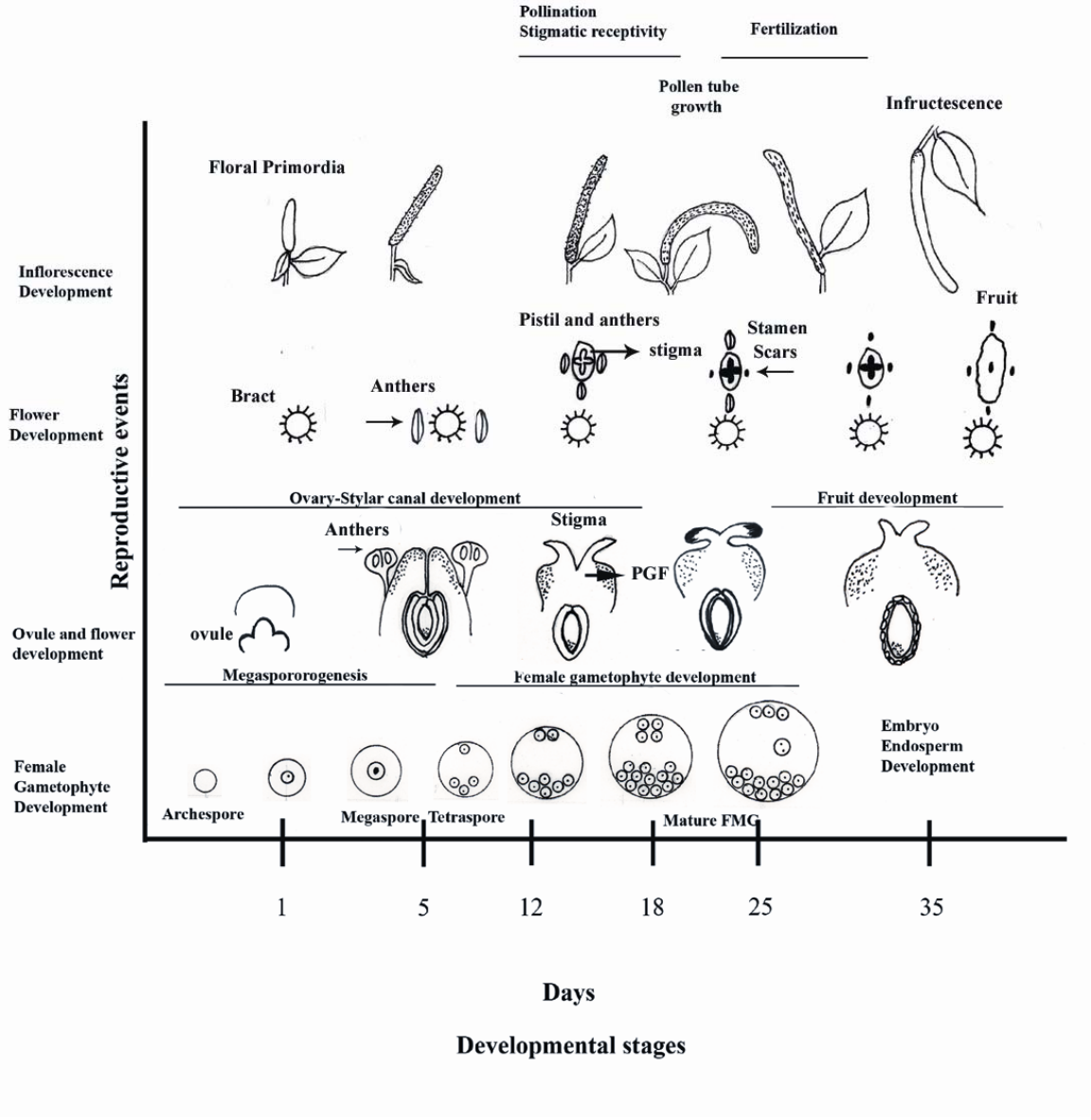


Figure 29. The timeline of reproductive events in *Manekia naranjoana* including inflorescence, flower and female gametophyte development. (PGF) postgenital fusion

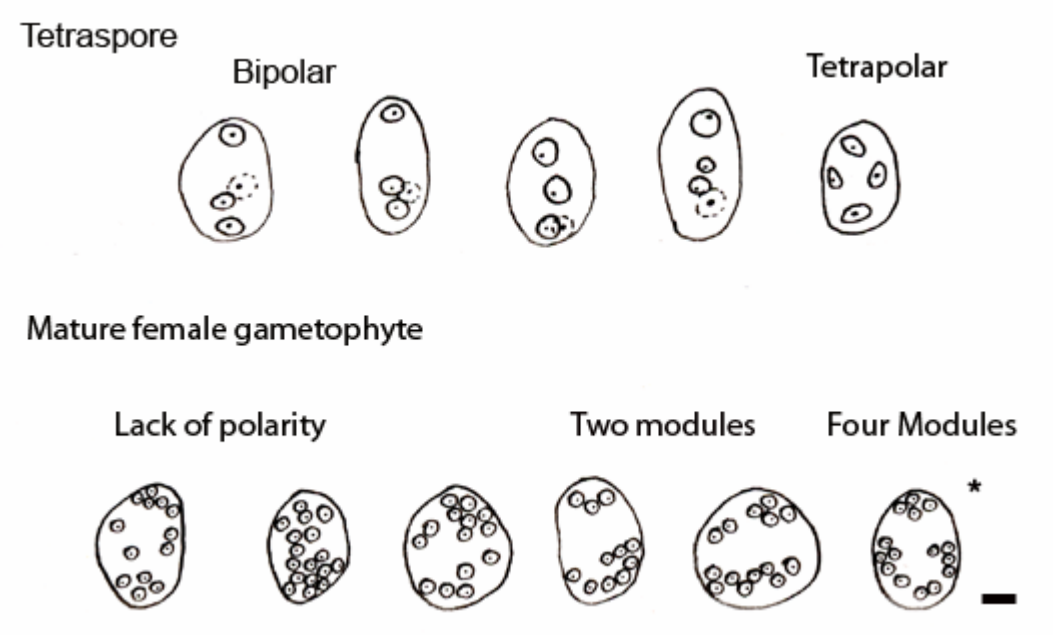


Figure 30. Variation in polarity of the tetraspore and the mature female gametophyte found in *Manekia naranjoana*. Tetraspore with strong early bipolar organization gives rise to a female gametophyte with two modules, while tetraspores that lack strong, early bipolar organization gives rise to a female gametophyte with four modules. * From figure 20 a-f

Scale bar= 10 µm

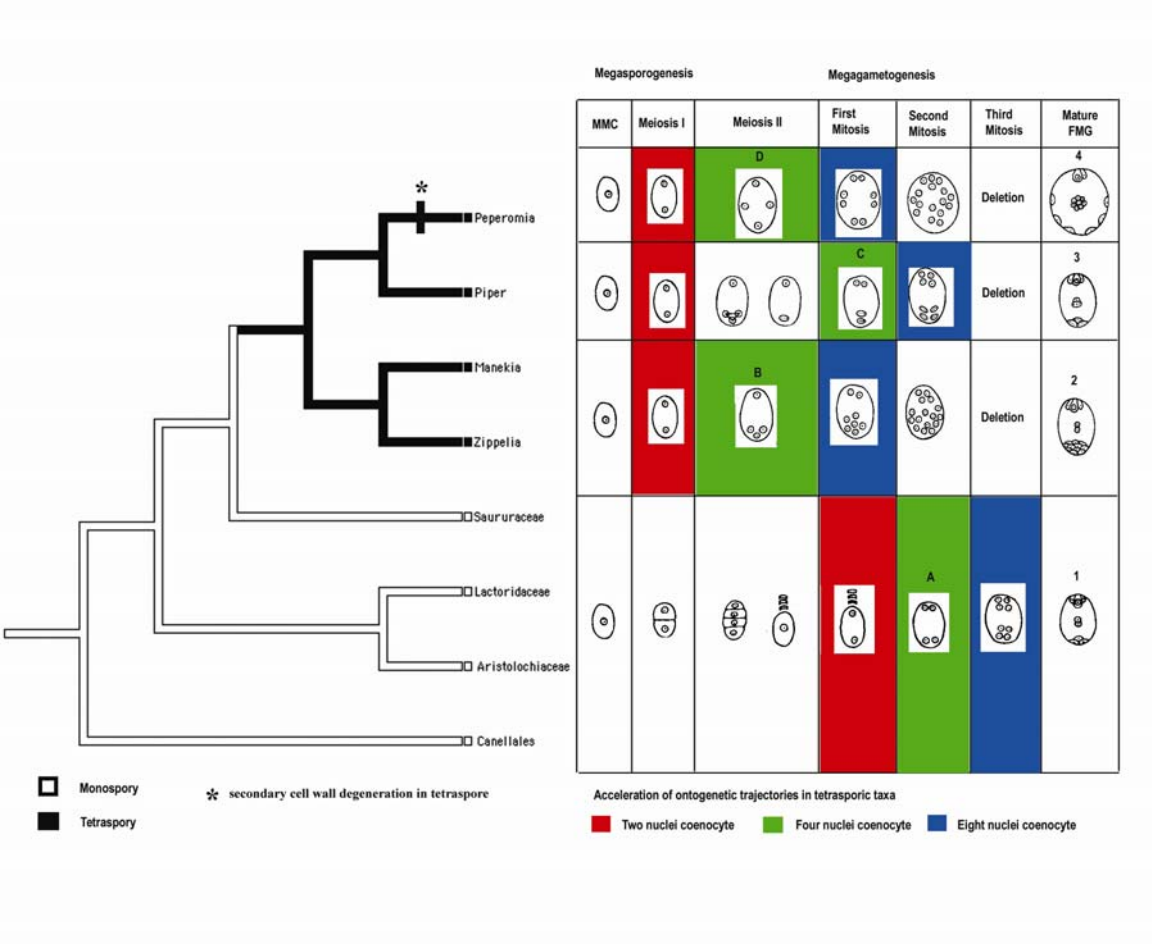


Figure 31. Simple parsimony reconstruction of female gametophyte ontogenetic sequences. A: two modules, bipolar organization, one haploid nucleus initiates each module. B: two modules, bipolar organization, one haploid nucleus initiates the micropylar module and three haploid nuclei initiates the chalazal module. C: two modules, bipolar, one haploid nucleus initiate the micropylar module and one triploid nucleus initiates the chalazal module. D. Four modules, tetrapolar, one haploid nucleus initiates a module in each pole. 1: both modules with the same number of nuclei, same ploidy, and equal genetic contribution to the central cell nuclei. 2: three times more nuclei in the chalazal vs. the micropylar module, but nuclei with same ploidy and equal genetic contribution to the CCN. 3: both modules with the same number of nuclei, but ploidy of each chalazal nucleus is three times higher that the ploidy in the micropylar nuclei, and unequal contribution to the CCN. 4: Four modules with the same number of nuclei each, same ploidy, and equal contribution of nuclei to the CCN.

VITA

Tatiana Arias was born in Medellín, Colombia in May 17 of 1979. She first went to school to get a Bachelor in Biology at La Universidad de Antioquia in Medellin, Colombia. Then she traveled to The United States to complete a Master degree at The University of Tennessee, Knoxville. She wrote a thesis about the female gametophyte development in *Manekia naranjoana* and its implications for the origin of the sixteen nuclei female gametophyte in Piperales. She graduated as masters in Science from the University of Tennessee in spring 2007.