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Research article

Links between parallel evolution and systematic complexity in angiosperms—A case study of floral development in *Myrcia s.l.* (Myrtaceae)

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ABSTRACT

The greatest challenge to the modern plant systematist is the interpretation of molecular phylogenies that do not correspond to previous classifications based on morphological data. Characters that on first appearance seem highly diagnostic are brought into focus by phylogeny and frequently shown to have evolved multiple times independently. Parallelism is usually neglected in such systematic studies and the homoplastic distribution of a character in a phylogeny is commonly accredited to convergent evolution. The impact of parallel evolution on angiosperm systematics is examined here using a taxonomically complex and species-rich group of tropical tree genera (*Myrcia*, *Marlierea*, *Calyptranthes*; Myrtaceae) as a case study. These groups are traditionally distinguished by flower characters and have been shown to be polyphyletic by molecular data. Floral ontogeny of distinct lineages is examined using SEM and plotted on a five gene phylogenetic hypothesis to estimate ancestral states and phylogenetic signal for developmental variation. Results show that floral characters responsible for taxonomic confusion are a result of both parallel evolution and convergence. This is contrasted with other diverse and taxonomically complex angiosperm groups and problematic taxonomy appears linked to recent diversification events where the same genetic basis remains latent, demonstrating parallelism to be an important factor in problematic taxonomies. In this study, variations in early stage floral development produce the most labile characters. This is discussed in light of ontogenetic patterns in angiosperms with focus on the evolutionary consequences of homoplastic variation during early vs late floral development. The prevalence of parallelism must be appreciated by taxonomists of complex groups. Future classifications of groups affected by parallelism are likely to require data from detailed, multi-disciplinary studies of key characters to interpret phylogenies correctly.

1. Introduction

Since the 1990s, systematics has been revolutionized as morphological data was sidelined in favor of molecular surveys increasingly used to present evidence of relationships among taxa (e.g. Bruns et al., 1991; Ragan et al., 1994; Baldwin et al., 1995; Shaffer et al., 1997; Soltis et al., 1997; APG, 1998; Goodman et al., 1998). Currently, the advent of phylogenomics has further enhanced this process, rapidly sampling more DNA and more taxa in a quest for better resolved phylogenies (e.g. Delsuc et al., 2005; Burki et al., 2007; McComarck et al., 2012; LPWG, 2013; Nater et al., 2015). This molecular based progress produces topologies that, in conjunction with fossil, geological and ecological data, allow clarification of the environmental history in which taxa evolved (e.g. Hughes and Eastwood, 2006; Simon et al., 2009; Crisp et al., 2011).

The explosion of such phylo-systematic techniques has resulted in the production of increasingly robust, complete and high statistically supported phylogenetic hypotheses. Contrary to expectation, however, systematic and taxonomic confusion has often increased.

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It is not uncommon to find classifications based on morphological characters that are highly incongruent with molecular data. Angiosperms, second only to beetles in species number (Wilson, 1999), have many such examples, particularly at lower taxonomic ranks (e.g. Soltis et al., 1996; Sweeney and Price, 2000; Plunkett et al., 2005; Kim et al., 2007; Goldenberg et al., 2008; Swenson et al., 2008; Lucas et al., 2011; Xue et al., 2011). Systematics of flowering plants is heavily based on reproductive characters (i.e. flower buds, flowers, fruits and seeds) as these present more morphological variation at higher taxonomic rank but are constrained within a species by the need for reproductive success. Nevertheless, since these structures are also susceptible to similar selective pressures in the long term, they often show a high degree of convergence (Tiffney, 1984; Fenster et al., 2004) resulting in common homoplastic patterns.

Recently, the search for morphological homologies that explain and support evolutionary relationships in systematically complex groups has intensified; a process of 'reciprocal illumination' (Hennig, 1966). Plant systematists are now focusing their attention on the development of these reproductive structures, where, in theory, convergences would be clarified by careful analysis of developmental patterns, allowing complex but fundamental characters to finally explain the phylogenetic hypothesis. Such studies, however, are often descriptive (Bernhard, 1999; Buzgo and Endress, 2000; Kocyan and Endress, 2001) or focus on the discovery of single or few synapomorphies (e.g. Endress, 1986; Schönenberger and Endress, 1998; Bernhard and Endress, 1999; Tucker, 2003; Prenner, 2004; Prenner and Rudall, 2007; Vasconcelos et al., 2015). Often ignored are cases where a homoplastic phenotype is found to be the result of similar developmental pathways; i.e. homoplasy at the developmental/structural level (e.g. Bess et al., 2005), suggesting parallel evolution instead of a convergence (*sensu* Patterson, 1982; reviewed by Hawkins, 2002).

Parallel evolution (also referred to as "underlying synapomorphy" by Saether, 1983; or "latent homology" by Cronk, 2002) is the repetition of structural or developmental patterns as a result of the similar genetic basis of closely but not directly related lineages (e.g. nodules in legumes, Doyle, 1994, 1998; zygomorphy in angiosperms, Donoghue et al., 1998; Endress, 1999). This is equivalent to the concept of 'deep homology' and relates to the presence of a genotypic basis that is not always phenotypically expressed in one lineage (see also Endress, 2010). Parallel evolution, or parallelism, has often been discussed in terms of evolutionary patterns that repeat themselves in striking ways in unrelated taxa (e.g. evolution of C4 grasses, Giussani et al., 2011) and has been highlighted in gene-expression studies (e.g. Rodman et al., 1998). Parallelism is, however, rarely taken into consideration in systematic studies and taxonomic decisions. Following radical re-evaluation of relationships between major angiosperm groups in recent years, it is surprising that the implications of parallelism have been almost absent from the systematic debate. The theoretical importance of such evolutionary patterns in plants systematics is discussed by Hawkins (2002) and Scotland (2011), but there is still a lack of comprehensive case studies in flowering plants.

In the new era of systematics, morphology is experiencing a revival (Lee and Palci, 2015; Giribet, 2015), especially at the interface of morphology, development and evolution coined 'MorphoEvoDevo' by Wanninger (2015). To stimulate the discussion of the importance of parallel evolution in the context of systematics, we use a taxonomically complex and species rich tree clade traditionally distinguished by flower characters shown to be polyphyletic by molecular data. We characterize floral development in *Myrcia s.l.* and discuss observed variation in the context of the group's evolution and systematics. The importance of considering parallel evolution as well as convergence when analyzing evolutionary and taxonomic problems in flowering plants is discussed here using floral development in *Myrcia s.l.* as a case study.

2. Methods

2.1. Study group

An example of the conflict between molecular phylogeny and traditional classification can be found in *Myrcia sensu lato* (Lucas et al., 2011), one of the most species-rich Neotropical angiosperm genera (Willmer, 2011). *Myrcia s.l.* includes three genera: *Calyptranthes* Sw., *Marlierea* Cambess and *Myrcia* DC. (*sensu* WCSP, 2016; for detailed nomenclatural chronology see Lucas et al., 2011). These three genera are distinguished by morphological characteristics of the flower, particularly the degree of fusion in the calyx and its behaviour during anthesis (Fig. 1a; Berg, 1856-57; Mc Vaugh, 1968). Both *Calyptranthes* and *Marlierea* are recognized by having an almost or completely closed calyx in the bud, i.e. with indistinct calyx lobes or barely distinct in some *Marlierea.* The calyx opens as a cap-like structure (calyptra) during anthesis in *Calyptranthes* or by tearing regularly or irregularly in *Marlierea*. *Myrcia* on the other hand, is characterized by an open calyx in the bud, i.e. with distinct (usually variable between four or five), free sepals. Although Mc Vaugh (1968) recognized that these generic boundaries were tenuous, this classification based on calyx characters was used until very recently (see also discussion in Staggemeier et al., 2015).

Previous molecular phylogenies of *Myrcia s.l.* (Lucas et al., 2011; Wilson et al., 2016) demonstrate that the nature of the calyx is not representative of natural lineages and found all of the previously recognized genera to be polyphyletic (Fig. 1b). Species originally described as *Calyptranthes* are found in clades *Calyptranthes, Eugeniopsis* and *Aulomyrcia*; species originally described as *Marlierea* are found in clades *Aulomyrcia, Sympodiomyrcia* and *Eugeniopsis*; species originally described as *Myrcia* are found in all clades except *Calyptranthes*. The phylogeny of *Myrcia s.l.* has been regularly revisited (Staggemeier et al., 2015; Wilson et al., 2016; Santos et al., 2016) and nine clades with high statistical support (bootstrap and posterior probability) are currently in preparation for formal publication as sections (Lucas et al., in press; Santos et al., 2016). As most of these nine sections are still not yet formally published, results and discussion of analyses presented here refer to the informal names of the clades shown in Fig. 1b. Species inclusion within informal groups of *Myrcia s.l.* follow the *Myrcia s.l.* scratchpad (*Myrcia s.l. scratchpad*, 2016) database and the suggested taxonomy of Lucas et al. (2011). A tendency of the last ten years has been to transfer published species of *Marlierea* and some *Calyptranthes* to *Myrcia*, and to publish new species that would previously been published as *Marlierea* in *Myrcia* (e.g. *Myrcia rupta* M.L.Kawas. & B.Holst and *Myrcia elevata* M.F. Santos). This is due to anticipated nomenclatural shifts that will be required as the new *Myrcia s.l.* classification is implemented.

While nomenclatural consensus at the rank of genus is stabilizing, the evolution of the floral characters that misled classical taxonomists for nearly two centuries are still poorly understood. Meanwhile, floral characters previously considered of less taxonomic relevance such as anther morphology, pubescence and ovary locularity, have proved to be more systematically consistent (Lucas et al., 2011).

2.2. Myrcia *s.l. flower structure*

Myrcia s.l. flowers are small, radially symmetric, usually ca. 0.5 cm in diameter with calyx and corolla present. The androecium is polystemonous and organized in three whorls, with centripetal development (Werberling, 1989; De Craene and Smets, 1991) and

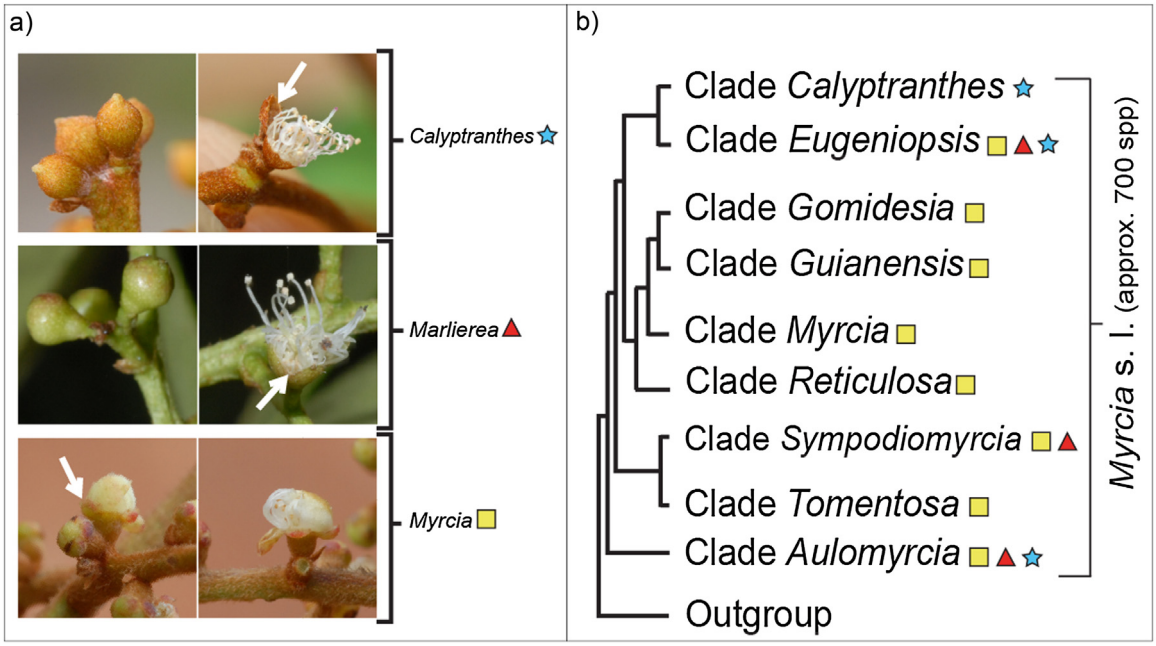

Fig. 1. Simplified traditional classifications of *Myrcia s.l.* based on floral characters (a). Classification of *Myrcia s.l.* after molecular hypothesis (b). Phylogenetic position of species previously placed *Calyptranthes* (star), *Marlierea* (triangle) and *Myrcia* (square). Arrows indicate the calyptra in *Calyptranthes brasiliensis*, the line of rupture of *Marlierea excoriata* and the free calyx lobes of *Myrcia vittoriana*.

inner whorls shorter than outer. The ovary is inferior and usually bi- or tri-locular, with two ovules per locule. Calyx, corolla and stamens are inserted on the rim of the hypanthium, which is often extended above the ovary summit (see scheme in Supplementary material 1). While the number of some floral parts is always constant within a species (e.g. number of locules per ovary), other organ numbers are flexible, even within the same individual. These are, for instance, number of sepals and petals (which usually vary between four or five) and stamen number (usually between 50 and 100, likely related to flower size).

2.3. Sampling

Prior to sampling, a general literature survey (Lucas et al., 2011; Wilson et al., 2016; Santos et al., 2016) was conducted to select species that would represent the highest possible diversity of flower morphology and phylogenetic lineages. A general survey of floral development was carried out to find developmental characters that appear to be fixed in a species. Flowering material representative of this variation was then gathered in Brazil, Jamaica, Costa Rica and the Dominican Republic. Buds were collected in all different stages and, where possible, more than one inflorescence per plant was collected. Inflorescences, buds and flowers were preserved in FAA or 70% alcohol immediately after collection. A few species critical for this study and not recently collected in the field were sampled from herbarium material and were rehydrated in boiling water for 10 min, left to cool overnight and then fixed in 70% alcohol. In total, 97 samples representing 64 species within *Myrcia s.l.* were sampled for comparative ontogenetic analyses. A list of all analysed samples is presented in the Supplementary Material 2.

2.4. Ontogenetic analysis

Floral buds and flowers were dissected in 70% ethanol, dehydrated through an alcohol series to 100% ethanol, and critical-point dried using an Autosamdri-815B critical-point dryer (Tousimis Research, Rockville, Maryland, USA). Dried material was mounted onto specimen stubs, coated with platinum using a Quorum Q-150-T sputter coater (Quorum Technologies, East Grinsted, UK) and examined with a Hitachi cold field emission SEM S-4700-II (Hitachi High Technologies, Tokyo, Japan). Where necessary, different stages of development were viewed from different collections of the same species. Flower developmental stages are described from sepal initiation to anthesis. Images were processed using Adobe Illustrator CC 2015 (version 19.2.0). In total 642 images were taken and analysed.

2.5. Phylogenetic reconstruction

The *Myrcia s.l.* phylogeny was reconstructed using DNA sequences of one nuclear (*ITS*) and four chloroplast (*psbA-trnH*, *trnL-trnF*, *trnQ-rps16*, *ndhF*) regions available from recent molecular studies (Staggemeier et al., 2015; Wilson et al., 2016; Santos et al., 2016). Published regions were sourced from GenBank; unpublished sequences from recently published works (Wilson et al., 2016; Santos et al., 2016) were contributed by the respective authors. Sixty-five species were included, representing all nine clades of *Myrcia s.l.* and four Myrteae taxa were used as outgroups (list in Supplementary material 3). Evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−12337.8258) was used for analysis (Supplementary Material 4). Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Phylogenetic analyses were conducted in MEGA 6 (Tamura et al., 2013). The resulting phylogeny represents c. 10% of the c. 700 species of *Myrcia s.l.*

2.6. Phylogenetic signal analysis

Varying morphological traits associated with different stages of floral development were selected for phylogenetic signal analysis to understand how trait modifications correlate to the phylogeny. Since all characters were analysed in simple binary perspective (presence or absence of a given variation), they were always coded

as discrete, even if apparently continuous (e.g. number of locules per ovary). See Supplementary material 5 and 6 for matrices used in phylogenetic signal estimation.

Phylogenetic signal of variation in developmental characters was analysed using the "fitDiscrete" function in package *geiger* (Harmon et al., 2008) implemented in R (R Development Core Team, 2016). This function uses Markov models of trait evolution (see Geiger package documentation) developed by Pagel (1994) that estimate the potential for a trait to change between species based on a phylogeny scaled for substitutions per site (genetic distance) and uncorrected for molecular clock. The parameter model = Equal Rates (ER) was chosen, since no estimation of evolutionary rate was available, and thus no transformation in the tree (e.g. lambda, kappa) was applicable. The result of this function generates a value of *lnl* (an estimate of log-likelihood) that can be used to rank the phylogenetic signal of discrete traits: the lower the log likelihood, the less well the evolution of a character correlates with the phylogenetic hypothesis. To adjust the phylogeny for this analysis, the function *multi2di,* from package *ape* implemented in R (R Development Core Team, 2016) was used to remove polytomies from the tree. The function "drop.tip" from the same package was used to remove outgroups and species with NA values.

2.7. Ancestral reconstruction of characters

Reconstruction of ancestral characters was performed for the three developmental pathways within *Myrcia s.l.* phylogeny to ensure the characters in question share the same ancestral state, supporting the parallel evolution discussion (according to Scotland (2011), characters obtained by parallel evolution have to share the same ancestral state). Reconstructions were executed using the *ace* function, with the "type = discrete" parameter, from the *ape* package implemented in R (R Development Core Team, 2016). The function "drop.tip" from the same package was again used to remove species with no available information (see Supplementary Material 6).

3. Results

3.1. Myrcia s.l. *floral development survey*

Floral ontogeny in *Myrcia s.l.* can be divided into four stages. Stage 1 concerns the very early development of the flower, which comprises initiation and early development of the outmost whorls in the flower i.e. bracteole, sepals and petals. Stage 2 represents the initiation and early development of androecium and initiation of gynoecium. Stage 3 concerns the development of all floral parts prior to the pre-anthetic stage. Stage 4 represents subsequent growth of the flower when the hypanthium elongates and the bud takes its final shape.

During the general ontogenetic survey, three significantly different floral developmental pathways were observed in different species of *Myrcia s.l.* and these are seen to be the main drivers of bud and flower shape. Differences between these pathways are observable from the first stage of development until anthesis. In all analysed samples, the first organs to develop are the two bracteoles at each side of the floral primordium. Even at this very early stage, distinction between the three developmental pathways are clear; these are most clearly characterised by differential rates of development of the calyx *vs* the hypanthium and gamosepalous (calyx tissue homogenous) *vs* aposepalous (free sepals) calyx development. Below, a description of the three developmental pathways is provided.

3.2. The "aposepalous" developmental pathway

The first pathway (Fig. 2) is here coined the "*aposepalous*" pathway. In this pathway, four or (more commonly) five sepals develop spirally, with the first initiating nearly opposite to the second bracteole and the second opposite to the first and so on (Fig. 2a). The corolla develops as the second whorl, with the first petal initiating between the first and third sepals (Fig. 2b,c) and the next ones following the same spiral sequence, intercalated with the sepals (Fig. 2d,e). In the observed material, the number of petals was always found to be the same as sepals; both whorls develop at a continuous rate and remain free throughout floral development. During the second developmental stage, the androecium is the third whorl of organs to develop. The first stamens initiate below the oldest petal (Fig. 2f) and tissue continues to differentiate under the remaining petals until the first complete ring of stamens is formed. The gynoecium is the last whorl to initiate; it begins as a depression on the floral apex during initiation of the first staminal whorls (Fig. 2f). By expansion of the surrounding tissue an apical pore is formed (Fig. 2g—arrow) as the surrounding tissue swells and extends to form the style (Fig. 2h). This is then followed by Stage 3, the extension of the floral parts during maturation of the bud (Fig. 2i–k). During this stage, a second and third whorl of stamens differentiates below the first following the same order (Fig. 2i). Pre-anthesis (Stage 4), anthers begin to differentiate at the tips of the filaments (Fig. 2l) and the bud takes its final shape. The development of the calyx and corolla continues at an even rate throughout all stages in the "*aposepalous*" pathway. During anthesis the sepals and the relatively showy petals are free and open to reveal the reproductive structures of the flower (Fig. 2m,n).

3.3. The "gamosepalous" developmental pathway

The second developmental pathway is here referred to as the "*gamosepalous*" pathway; the pair of bracteoles initiate on each side of the floral primordium. The first two sepals then also initiate simultaneously (Fig. 3a), followed by two further sepals that produce a decussate pattern relative to the bracteoles (Fig. 3b,c). All calyx lobes are free up to this point but now develop as a gamosepalous structure, completely fused and homogeneous at the base. The tips of the calyx remain free; the calyx is closed at the top of the bud, leaving a discreet mark (Fig. 3d). Petal initiation is simultaneous or nearly simultaneous (Fig. 3e). Unlike the sepals, petals in the "*gamosepalous*" pathway are free throughout floral development (Fig. 3f). The corolla usually does not develop at the same rate as the calyx, remaining poorly developed until anthesis (Fig. 3o). The initiation of the androecium and the gynoecium during Stage 2 and the extension of floral parts to the pre-anthetic stage during Stages 3 and 4 are very similar to the "*aposepalous*" pathway. The first staminal whorl develops under the petals while the stigmatic depression appears, shrinks and extends upwards to form stigma and style (Fig. 3g–m). Staminal whorls develop downwards and the anthers differentiate at the tips of the filaments in Stage 4 (Fig. 3n). This is followed by the pre-anthetic stage (Fig. 3o) and anthesis. In the mature bud, anthesis occurs by tearing of the homogeneous calyx tissue in several ways (see *Parallelism in Myrcia s.l* in Discussion section). Most commonly the weakest point is at the base of the calyx, leading to a cap like structure that dehisces (Fig. 3p,q).

3.4. The "hyper-hypanthium" developmental pathway

The third developmental pathway is here called the "*hyper-hypanthium*" pathway. Similar to the "*gamosepalous*" pathway, four or five sepals initiate either in a decussate or slightly sequential pattern relative to the bracteoles (Fig. 4a,b). The same number of petals then initiate in an intercalated position relative to the sepals

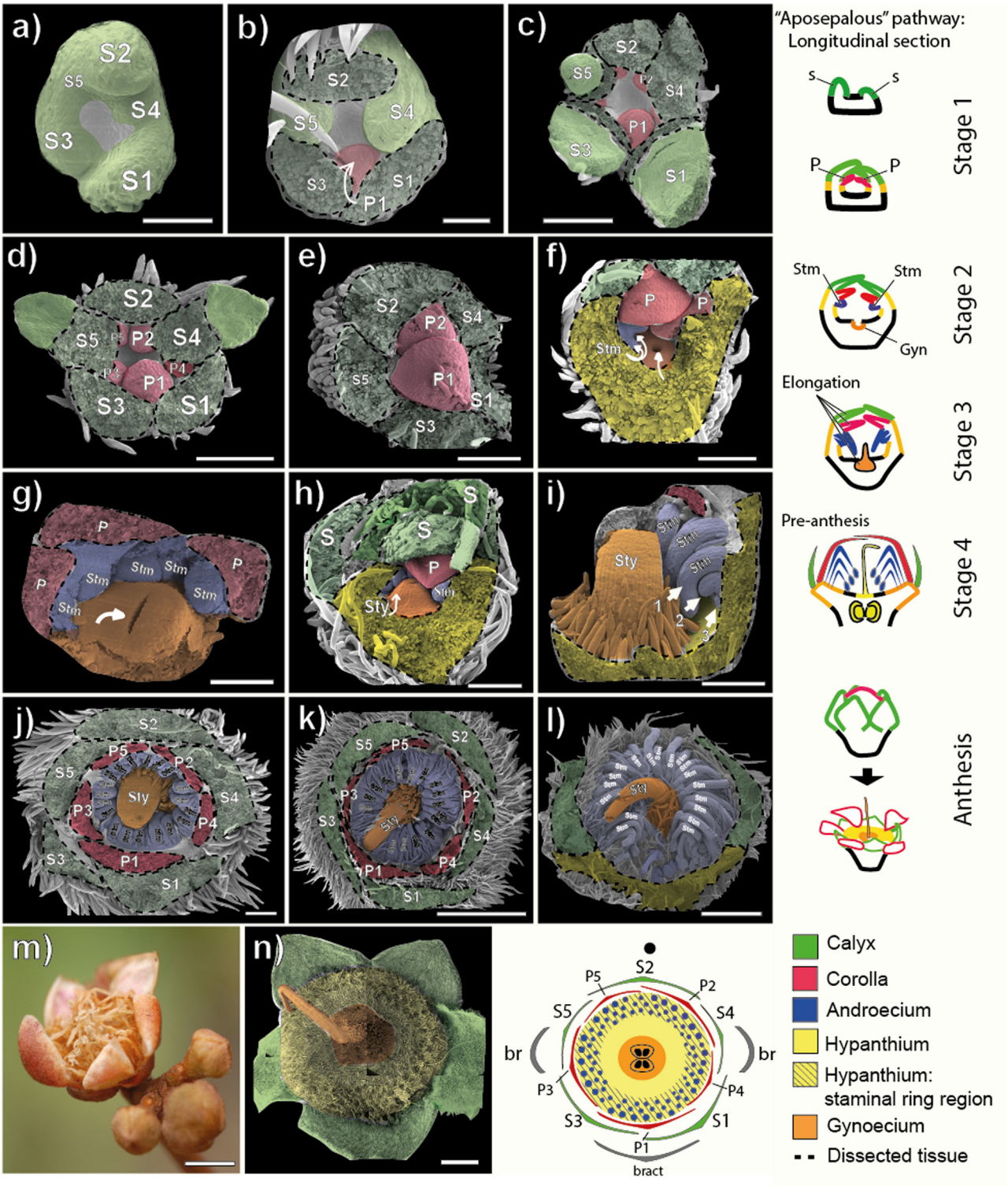

Fig. 2. The "aposepalous" pathway as exemplified by *Myrcia fenzliana* (all images besides "m" and "n") and *Myrcia* sp. T.Vasconcelos 439 ("m" and "n") (both clade *Gomidesia*). Removed structures are represented by a dashed line. The right hand side column summarizes stage by stage organ development; floral diagram is shown at the right bottom corner. a) Spiral sepal initiation. b) First petal initiation between first and third sepals. c,d,e) Spiral petal initiation and early petal development; petals alternating with the sepals. f,g) Initiation of first whorl of stamens and stigmatic depression. h) Upward development of style. i) Longitudinal section highlighting the development of first, second and third staminal whorls. j,k) Extension of floral parts prior to anthesis. l) Longitudinal section of pre-anthetic stage. White circles indicate anthers. m) Anthetic flower, showing free sepal lobes and developed petals. n) Flower after anthesis, stamens and petals removed. Br, bracteole; S, sepal; P, petal; Sty, style; Stm, stamen; Scale bars = 50 μm (a, b, g), 100 μm (c, d, e, f), 150 μm (h, i, j), 500 μm (j, k, l), 1 mm (n), 3 mm (m).

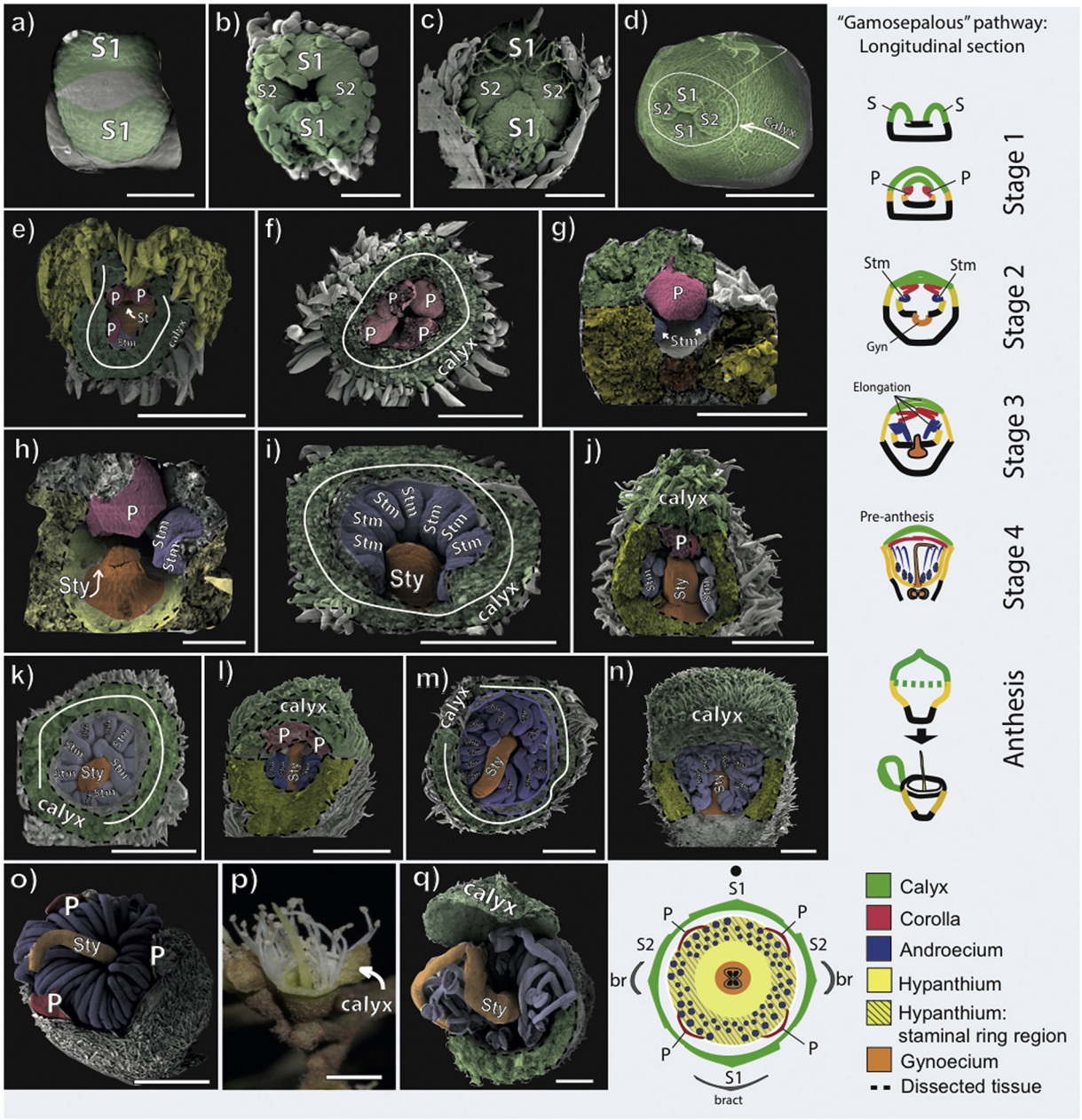

Fig. 3. The "gamosepalous" pathway, as exemplified by *Calyptranthes pallens* (Clade *Calyptranthes*, all images besides "a" and "d") and *Calyptranthes multiflora* (Clade *Aulomyrcia*, "a" and "d"). Removed structures are represented by a dashed line. The right hand side column summarizes stage by stage organ development; floral diagrams are shown at the right bottom corner. a) Initiation of the two first sepals in median position. b,c) Initiation and early development of the four decussate sepals. d) Calyx with the free distal sepal lobes and fused base. e) Simultaneous or near simultaneous petal initiation. Depression in the young gynoecium becomes visible. f) Early development of the four petals. g) Longitudinal section showing initiation of first stamens under a petal. h) Early development of stamens and style. i-n) Sequence of floral part elongation prior to anthesis. o) Pre-anthetic phase, calyx removed. p,q) Anthesis highlighting the cap-like structure of the calyx. Br, bracteole; S, sepal; P, petal; Sty, style; Stm, stamen. Scale bars = 50 μm (a, b, c) 100 μm (d, e, f, g, h), 300 μm (h, i, j, k, l, n), 1 mm (o, q), 3 mm (p).

(Fig. 4c,d). Sepals and petals remain free and their rate of growth becomes imperceptible. In contrast, hypanthium growth accelerates, extending massively and giving the appearance that in stage 2 stamen are formed on the inside of the bud apex, growing upside down (Fig. 4e). Gynoecium initiation and early development is similar to the other pathways (Fig. 4d,e). Most of what appears to be the outside of the bud at this point is actually extended hypanthium with very reduced calyx lobes remaining at the apex (Fig. 4f). During Stage 3, the hypanthium continues its extreme extension, "carrying" the staminal whorls upwards (Fig. 4g–j). At Stage 4 anthers differentiate at the tips of the filaments (Fig. 4k–m). The pre-anthetic bud from the outside resembles the pre-anthetic bud from the "gamosepalous" pathway. However, most of what is seen from the outside represents the long hypanthium extension, with very reduced calyx lobes on the top of the bud (Fig. 4n). At anthesis the reduced calyx lobes of the mature bud move apart; the subsequent opening is too small to reveal the floral display and the pressure inside the bud increases. The hyper-extended hypanthium then tears along fissures below the sepals to expose the stamens (Fig. 4o,p). This anthesis behaviour produces a display where the showiest parts of the flower are the hypanthia slices that hold the stamens. Calyx and corolla don't seem to play an important role as attractive to pollinators (see red circle in Supplementary material 1c).

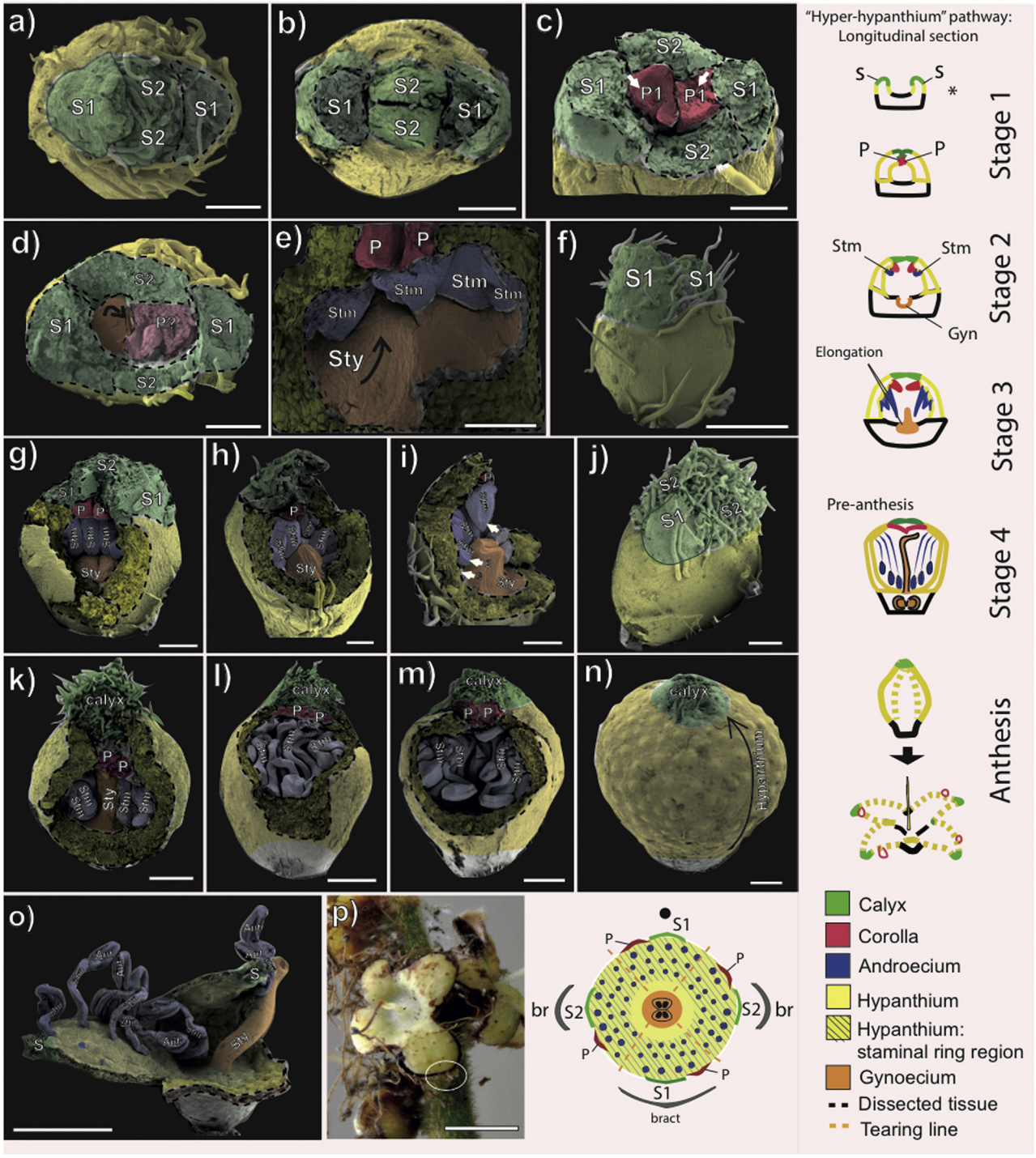

Fig. 4. The "hyper-hypanthium" pathway, as exemplified by *Marlierea umbraticola* (clade *Aulomyrcia*). Removed structures are represented by a dashed line. The right hand side column summarizes stage by stage organ development; floral diagram is shown at the right bottom corner. * the earliest initiation of sepals was not observed. a,b) Early development of the four decussate sepals. Apical view. c) Simultaneous or nearly simultaneous initiation of petals alternating with sepals. Lateral view. d) Same bud as in 'a'-'c'; sepals and petals were partially or completely removed to show depression on the young gynoecium (highlighted by black arrow). Apical view. e) Initiation of stamens below petals and on top of style. Longitudinal section. f) External view of bud in Stage 2. g-i) Longitudinal sections showing early extension of floral parts. j) External view of bud in Stage 3. k-m) Longitudinal sections showing floral parts continuous elongation prior to anthesis. n) External view of pre-anthetic bud in Stage 4. o) Anthesis, showing deep tearing lines up to the top layer of the ovary. Note the stamen insertion on the hypanthium and the tiny sepals on the tip of the hypanthium p) Old buds with the position of sepals encircled. Br, bracteole; S, sepal; P, petal; Sty, style; Stm, stamen; St, stigma. Scale bars = 50 μm (e), 100 μm (a, b, c, d, g, h, i), 200 μm (f, k, l, m, n), 1 mm (o), 4 mm (p).

3.5. *Specific stage character variation*

In addition to the three distinct pathways, further variation in floral characters was observed. These variations are independent of the developmental pathway and occur during developmental stages 2–4. In all material analysed of clades *Myrcia*, *Gomidesia* and *Reticulosa*, single-celled hairs were observed growing at the base of the staminal ring, usually appearing during early development of the first stamens or the initiation of the second staminal ring in Stage 2 (Fig. 5a). The staminal rings are glabrous in all other clades.

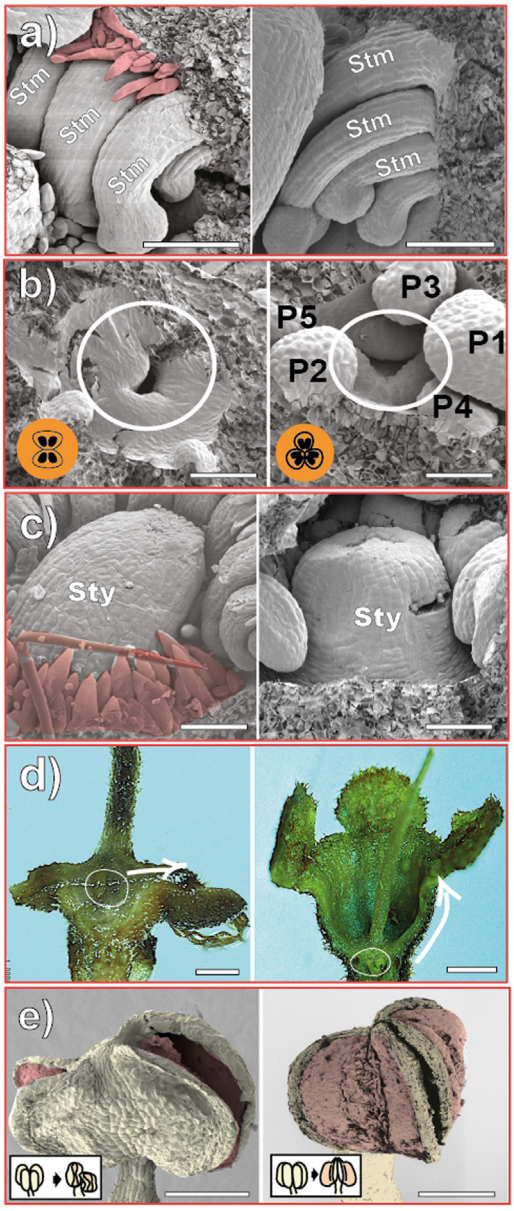

Fig. 5. Stage specific variation in *Myrcia s.l.* floral organ development a) Hairy or glabrous base of staminal ring (initiates in Stage 2). b) Definition of bi or tri ovary locularity (Stage 2). c) Hairy or glabrous base of style (initiates in Stage 3). d) Flat or vertically extended hypanthium in longitudinal section (Stage 4). Ovary position is circled. e) Uneven or even growth of anther connective leading to different anther openings (Stage 4) (inside of thecae highlighted in red). Stm, stamen; Sty, style. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

During the same developmental stage, variation in the shape of the stigmatic depression was observed. This corresponds to the number of locules in the ovary, with a triangular form observed in species with three locules and an "H-shaped" form in species with two locules (Fig. 5b). During Stage 3, the base of the style was observed to be glabrous in most samples, however, in all material studied of clades *Myrcia*, *Gomidesia* and *Reticulosa* epithelial cells develop into single-celled hairs during style elongation (Fig. 5c).

In species that follow the "*aposepalous*" or "*gamosepalous*" pathways, the final shape of the hypanthium in the bud varies depending on developmental differences during late bud development (Stage 4). In flowers of the *Myrcia*- and *Gomidesia*-clades, vertical extension of the hypanthium is limited, growth then stops and hypanthial tissue expands horizontally, leaving the mature bud and opened flower without a tube or hypanthial cup as in all other clades (Fig. 5d). In the *Myrcia*- and *Gomidesia*- clades the inner surface of the short hypanthium wall is covered in hairs, while it is glabrous in all other clades of *Myrcia s.l.* In all sections, anther development is similar until Stage 4, when differential growth of the connective dislocates anther thecae in the *Gomidesia* clade (Fig. 5e), forming a structure that resembles a pore during anthesis. In all other clades, anthers open via longitudinal slits, forming an angle of nearly 180° between the thecae (Fig. 5e).

3.6. Phylogenetic signal of developmental variation

Results presented here describe three developmental pathways that initiate in the first stage of development and other trait variation that appears in later stages of *Myrcia s.l.* floral development. Key differences were observed during the described developmental stages and can be summarised as follows: Stage 1–Pathway determination; Stage 2—Growth of hairs at the base of the staminal ring during androecium development (observed in all analysed samples of clades *Myrcia*, *Gomidesia* and *Reticulosa*), and determination of locule number (trilocular in samples of clades *Guianensis* and *Reticulosa*, bilocular in the other sections); Stage 3—Growth of hairs at the base of the style (observed in all analysed samples of clades *Myrcia*, *Gomidesia* and *Reticulosa*), Stage 4—fixation of hypanthium shape (flat and pubescent in all samples of clades *Myrcia* and *Gomidesia*, extended and glabrous in the rest), unequal growth of the anther connective (only in clade *Gomidesia*). Even though other small variations of floral development could be observed (e.g. four to five sepals), these were not considered here due to inconsistency at the intraspecific level. The non-independence of traits such as hairs at the base of stamen and style was considered as they are always found in the same species. They were not merged however, as the hairs appear at distinct development stages.

Correlation of key developmental characters with available phylogenetic hypotheses are shown (Fig. 6) estimated by means of log likelihood from the correlation of each character with the phylogeny. Results indicate that during floral development in *Myrcia s.l.*, the earlier the development of a structure varies, the lower the phylogenetic signal of this variation is (Fig. 6). Consequently, early developing characters are more homoplastic and less congruent with the phylogeny. The most striking example of this is the developmental pathway determination in Stage 1. These show a clearly homoplastic pattern when correlated with the phylogeny and return the lowest log likelihood values (e.g. "*aposepalous*" pathway: −18.73 "*gamosepalous*" pathway: −16.58) in comparison to variation in later developed characters. The "*hyper-hypanthium*" pathway is exceptional in this case, scoring a slightly higher value of log likelihood (-13.38) as it occurs exclusively within the clade *Aulomyrcia*. The homoplastic pattern of similar developmental pathways increases evidences for parallelism in *Myrcia s.l.*

In contrast, characters that undergo later stage developmental variation return higher values of log likelihood and seem to be more phylogenetically congruent. For example, hairy staminal ring and style base, always observed to occur in the same species, return a moderately high (−13.07) log likelihoods; the same is true for the characters of hypanthium elongation and pubescence (−11.55). Uneven growth of the connective, a variation that occur in the last stages of development, is exclusive found Gomidesia and thus score the highest phylogenetic signal (−5.73). Locule number, although consistent in the lineage where it is found, scores the lowest phylogenetic signal (−13.78) among late developed characters.

3.7. Ancestral reconstruction of developmental pathways

Ancestral reconstruction of the three developmental pathways indicate that the "*aposepalous*" pathway is the ancestral state for

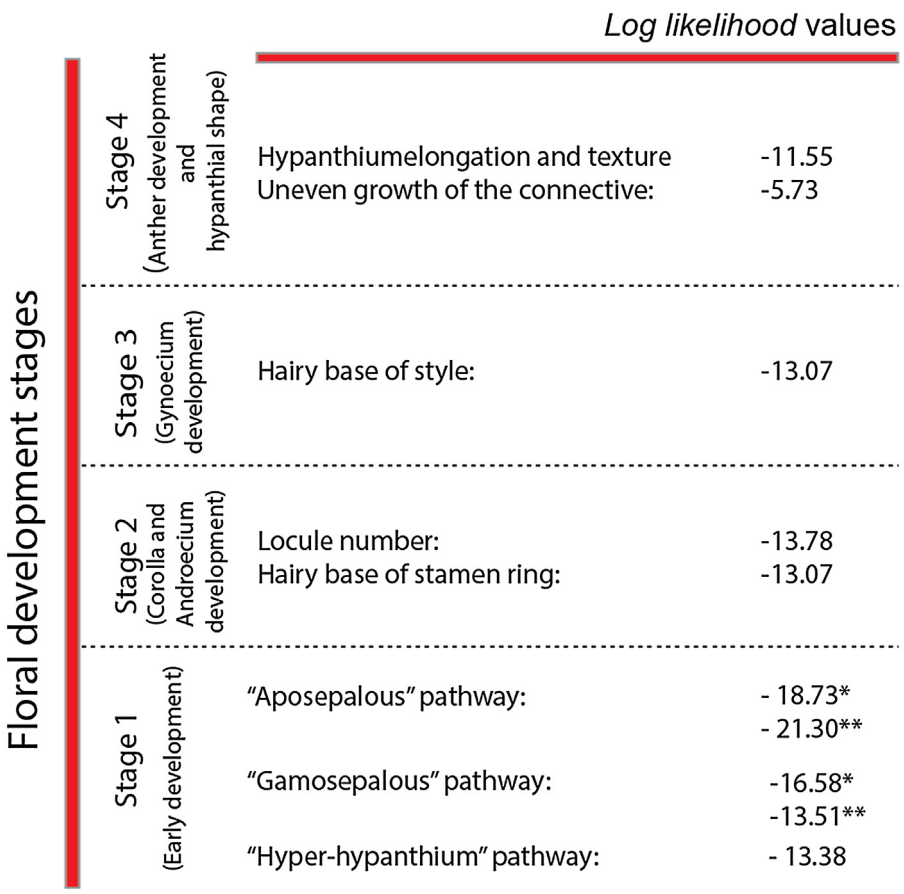

Fig. 6. Comparison between phylogenetic signal based on *log likelihood* of character variation in distinct developmental stages. * *Marlierea glazioviana* is considered "*Gamosepalous*"; ** *Marlierea glazioviana* is considered "*Aposepalous*".

Myrcia s.l. (Fig. 7). The "*hyper-hypanthium*" pathway appears to have evolved twice independently inside clade *Aulomyrcia*. The "*gamosepalous*" pathway evolved independently four times in four different clades. Reversal from "*hyper hypanthium*" or "*gamosepalous*" pathway to "*aposepalous*" was not observed. Results show that a given developmental pathway always arises from the same ancestral state regardless of its phylogenetic position. *Marlierea glazioviana* was observed to present both pathways in the same individual. The results show that similar developmental pathways appear independently but can still present the same ancestral state. This is another evidence of parallelism in the evolutionary history of *Myrcia s.l.*

4. Discussion

4.1. *Parallelism in* Myrcia s.l.

Developmental variations in the calyx and hypanthium, which before were considered systematically consistent, can be categorized into three distinct developmental pathways that are shown to be examples of parallel evolution (Fig. 8a–c). These pathways are polyphyletic and thus score low phylogenetic signal, reinforcing their ineffectiveness for classification of *Myrcia s.l.* The "*aposepalous*" pathway (Fig. 8a) has evolved just once (the ancestral state). However it has very low phylogenetic signal because it occurs in most lineages and therefore it is not a useful systematic character either. The "*gamosepalous*" pathway (Fig. 8b) is also of low systematic value because it has evolved independently at least four times and it scores a low phylogenetic signal. The "*hyper-hypanthium*" pathway (Fig. 8c) has more systematic relevance because it is found in a single lineage and scores the highest phylogenetic signal among the developmental pathways. It has, however, evolved at least twice independently within clade *Aulomyrcia* and still returns a value of phylogenetic signal lower than all later developed traits, with the exception of ovary locularity. Variation in floral characters acquired later in the development present a stronger correlation with the phylogeny and the combination of these characters can be more reliably used to accurately classify species.

Systematic problems that arise from parallelisms in *Myrcia s.l.* might be further aggravated by errors of interpretation resulting from the three developmental pathways. These mistakes in interpretation are the root of the observed convergent bud types and behaviours at anthesis. Closed Myrtaceae buds have been associated with protection from dehydration and predation (Werberling, 1989) and prior to anthesis, buds resulting from the "*gamosepalous*" and "*hyper-hypanthium*" pathways can be indistinguishable. This has resulted in arbitrary assignments of species to *Marlierea* and *Calyptranthes* (Fig. 8d,e). The lack of material of *Myrcia s.l.* in adequate phenological phases (i.e. anthetic flowers), in conjunction with very small flowers from which it is difficult to determine hypanthium length, added to the taxonomic confusion.

The nature of opening of the bud is also a taxonomically problematic, convergent character as all three developmental pathways have potential to tear at anthesis (Fig. 8f–k). This has given rise to nomenclatural confusion involving the genera *Marlierea* and *Calyptranthes*. Despite the developmental pathway, most species with buds tearing at anthesis were described in *Marlierea* (Fig. 8f–i). In species of clade *Eugeniopsis*, however, tearing at anthesis can result in a greater portion of calyx tissue remaining on one side of the hypanthium (Fig. 8j) producing a structure reminiscent of, or

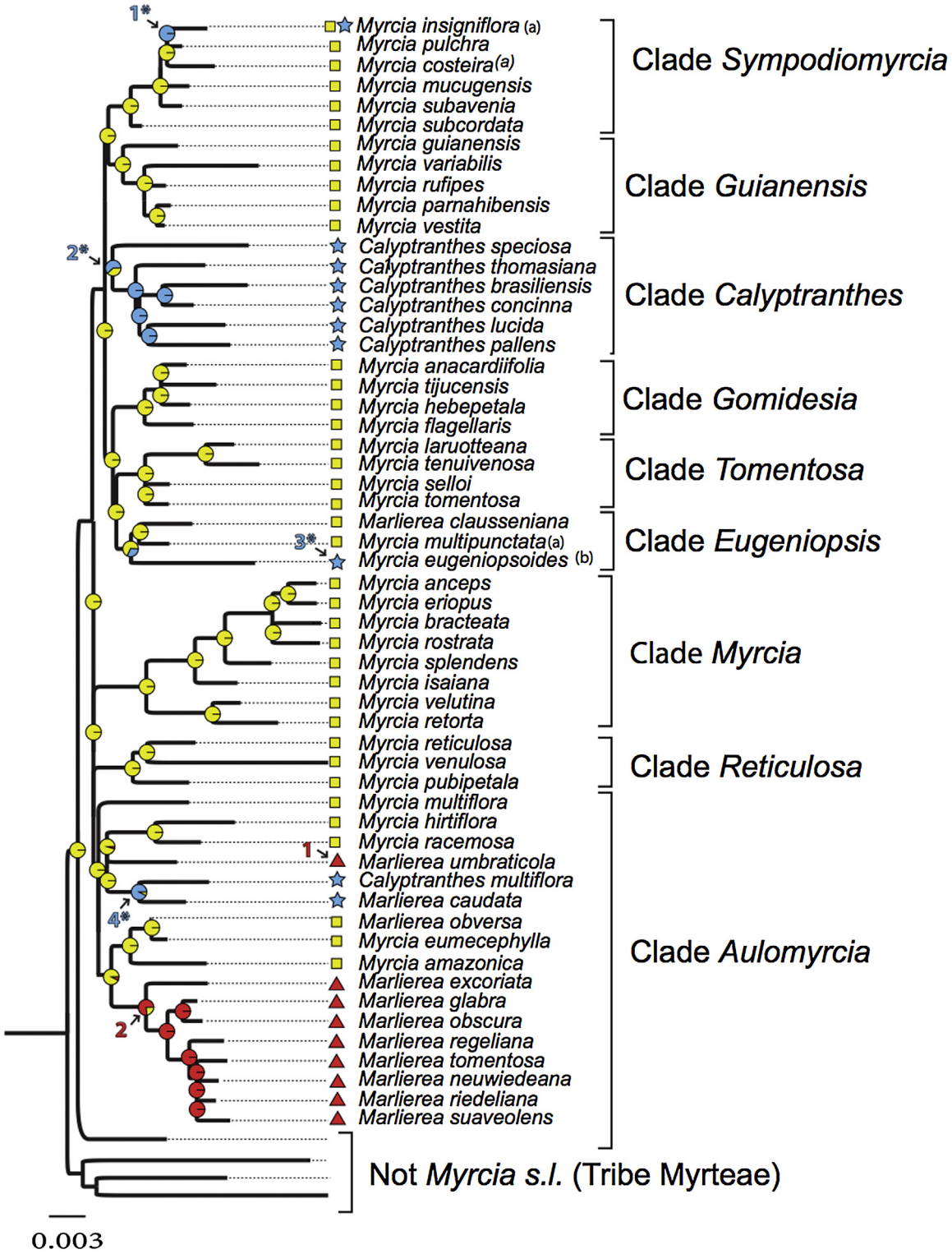

Fig. 7. Distribution of the three *Myrcia s.l.* developmental pathways. Yellow squares—*"aposepalous"* pathway; Red triangles—*"hyper-hypanthium"* pathway; Blue stars—*"gamosepalous"* pathway. Shifts from ancestral *"aposepalous"* to *"gamosepalous"* pathways are marked * (1–4); shifts from ancestral *"aposepalous"* to *"hyper-hypanthium"* are labelled (1,2). (a) Names first described in *Marlierea*. (b) Names first described in *Calyptranthes*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

convergent with, a calyptra (Fig. 8k). A systematic example is *Myrcia eugeniopsoides* that opens as described and was first described as *Calyptranthes* (1962; as *C. eugeniopsoides* D. Legrand & Kaussel) then transferred to *Marlierea* (1975; as *M. eugeniopsoides* D. Legrand & Kaussel (Legrand)) and finally placed in *Myrcia* (2014; as part of a current trend transferring all names to *Myrcia*; see Mazine et al., 2014). Species that follow the "*aposepalous*" pathway without tearing at anthesis (Fig. 8l,m) were always described in *Myrcia* regardless in which clade they are found.

This study shows that both parallelism and convergence are responsible for the two-century long problems with an accurate *Myrcia s.l.* taxonomy which has resulted from extreme emphasis

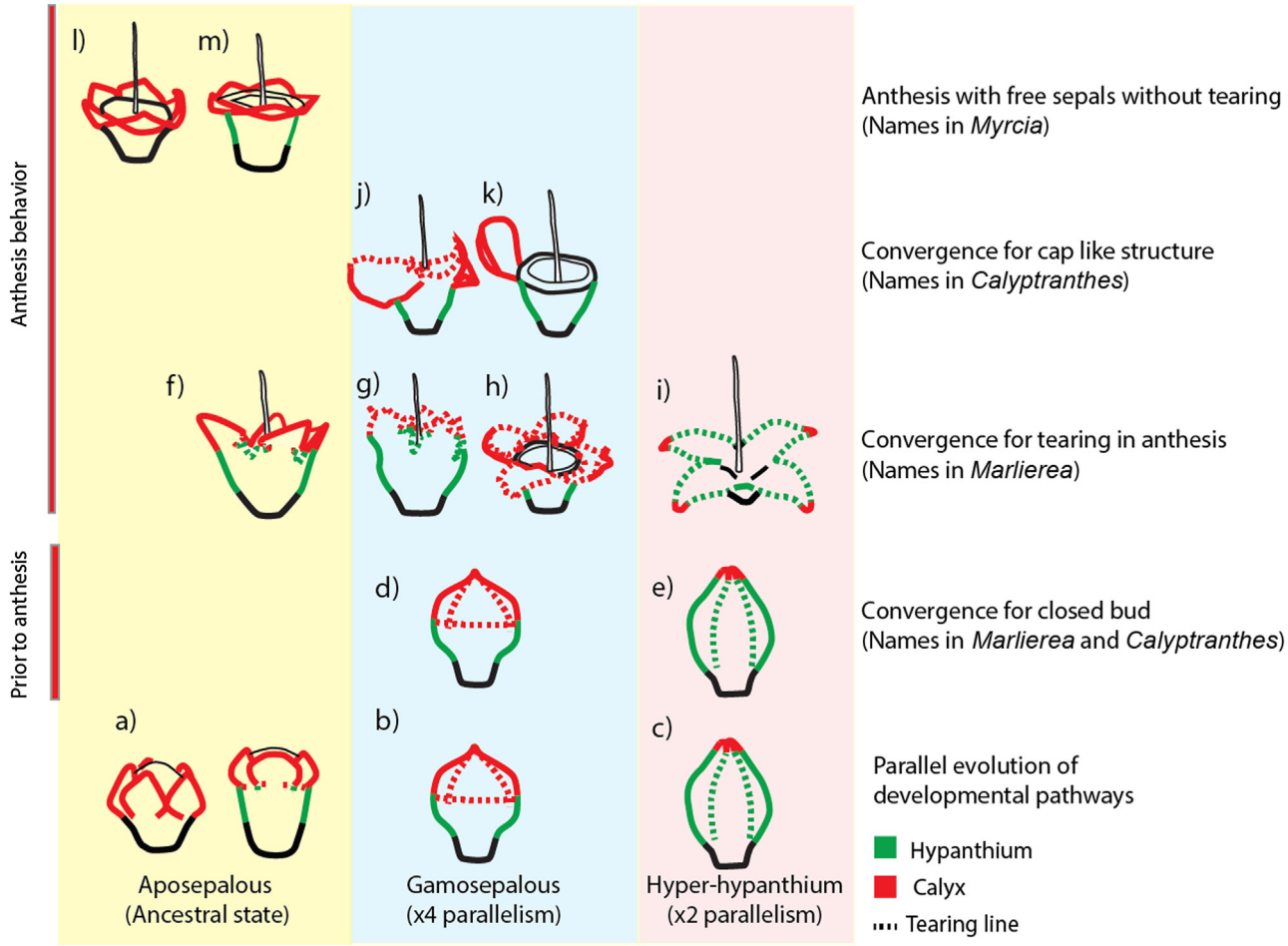

Fig. 8. Historical taxonomic problems in *Myrcia s.l.* as a result of parallel (a–c) and convergent (d–m) evolution. Right hand column names show genera in which species are most commonly housed.

on characters of the calyx, hypanthium and the nature of anthesis (see Lucas et al., 2011). In conclusion for the study group, characters related to the hypanthium and the mode of calyx dehiscence cannot be used alone to define a group of species at any systematic level. Characters with stronger phylogenetic signal, are the best for this purpose (e.g. presented here: hairs on the style base or staminal disk, locule number). The best approach to classification in such a group uses molecular data in combination with multiple characters from a broad range of parts of the plant. The floral characters investigated here can only imperfectly indicate broad species relationships and may only be used reliably for classification at the species level, and even then they must be used with care.

4.2. *Parallelism in systematics of flowering plants*

The persistent lack of consideration of parallelism in systematics is likely due to confusion between convergence and parallelism in the literature (Gould, 2002; Diogo, 2005). Saether (1983) described "shared common internal constraint of homologous genes or developmental pathways" as underlying synapomophy; this is here considered parallelism. Today homoplasy and homology are used as antagonistic terms. However homoplasy was first defined as a sub-category of homology (Lankester, 1870) as it is characteristic of a single lineage; today this is referred to as parallelism.

Parallelism is likely to be more common than previously considered and in some groups of angiosperms it is a significant source of systematic strife as shown in the present study for *Myrcia s.l.* Patterns of homoplasy that can be attributed to parallelism are frequently found in other angiosperms groups (e.g. inflorescences in *Panicum*, Bess et al., 2005; stamens in *Miconia*, Goldenberg et al., 2008). These incidents are not always clearly cited as parallelism and are often considered to "result from a shared developmental program (. . .) that is flexibly turned on and off during evolution" (Hearn, 2006, for growth form in *Adenia*). In other words, even if expression of these different developmental pathways remains latent for long periods of evolutionary history, they can occasionally be re-expressed and fixed following a specific selective pressure or genetic drift.

Nevertheless, such latent developmental pathways may be silenced through genetic mutation over time that would represent the end of potential for parallelism (Wagner, 1998; Hawkins, 2002). This may explain why systematic problems related to phenotypic polyphyly appear more prevalent in recently diversified groups where latent molecular signals for expression of similar structures can still be triggered, with abundant examples throughout flowering plants (e.g. *Adenia*—26mya, Hearn, 2006; *Disa*—c. 18mya, Bytebier et al., 2007, 2010; *Miconia*—c. 10mya, Goldenberg et al., 2008; Berger et al., 2016; *Panicum* c. 15mya, Bouchenak-Khelladi et al., 2010; Giussani et al., 2011; *Mimosa*—c. 15mya, Simon et al., 2009, 2011) including the case study presented here (*Myrcia s.l.*, 22mya; Thornhill et al., 2015); whereas older groups are more likely to have matching morphology and phylogeny (e.g. *Piper* and *Peperomia*—Late cretaceous, Jaramillo and Manos, 2001; Quijano-Abril et al., 2006; Smith et al., 2008; Jaramillo et al., 2008).

Time-scale is therefore important when dealing with parallelisms in a systematic context. Estimates of a time limit for the

re-expression of silenced genetic mechanisms that could lead to parallelism are ca. 6 million years (Marshall et al., 1994, using empirical data of genes with different degrees of mutational rate). This can however, be much longer for traits that affect distantly related clades of a large group such as flowering plants. In the example of *Myrcia s.l.* the period between the occurrence of a calyptra in *Calyptranthes multiflora* (clade *Aulomyrcia*) and species within clade *Calyptranthes* is estimated at approximately 20 ma (Santos, 2014). When considering a single character for the whole Myrtaceae family it is possible to observe that a calyptra reoccurs in more distant lineages along similar developmental pathways (i.e. *Calyptranthes* and *Eucalyptus*, Weberling 1989; this study) even though their last common ancestor was ca. 65 ma (Thornhill et al., 2015).

4.3. The impact of parallelism on flower evolution

This study also provides insight into the evolution of floral development. Although recent floral evolution studies have mainly focused on classic evo-devo approaches (such as the ABC model, Erbar, 2005), macro-evolutionary and systematic aspects of flower evolution have also become more common. In such studies, stable early floral development within a lineage and homoplastic late floral development are considered the norm. Tucker (1992, 1997, 2003) found this arrangement in Fabaceae, another mega-diverse group of eudicot angiosperms. Re-expression of early developing characters in independent lineages of *Myrcia s.l.,* perhaps as a result of parallel evolution, makes floral development proceed in the exact reverse. In this case, characters that differentiate in later stages have higher phylogenetic signal than in earlier stages (Fig. 6). In contrast to the studies of Tucker, this pattern was also recently found in the Fabaceae tribe Cassinae (Marazzi and Endress, 2008); it is possible that these contrasting findings may be linked to extremely variable Fabaceae floral morphology. Such early stage changes might be important components of late flower display and have consequences for pollination. In *Myrcia s.l.* for example, different floral development pathways might bring discreet changes in post anthetic display, where the calyptra or undeveloped petals of some pathways might play a role in flower presentation to pollinators.

These labile structural changes in early floral development that lead to morphological variation in the mature flower and that are responsible for flawed systematic interpretations may also play an important role in angiosperm evolution. The labile nature of these changes adds weight to suggestion that major changes in floral morphology evolve fast (Vasconcelos and Proença, 2015) rather than gradually, resulting in evolutionary jumps (Eldredge and Gould, 1972) and thereby increasing short-term adaptability and fitness of a lineage. Such flexibility is likely to have contributed to angiosperm success.

4.4. Conclusions, final considerations and future perspectives

This study shows in-depth ontogenetic and anatomical research of apparently similar structures to be important in the detection of parallelism. Parallel evolution, as well as convergence, misleads taxonomists and evolutionists when searching for characters to define natural lineages (=morphological synapomorphies). Different developmental pathways can be labile and repeat themselves in non-related lineages of recently diversified groups, probably due to underlying homology in the genetic expression of these characters. It is therefore clear that morphological synapomorphy is particularly difficult to define in the presence of parallel evolution. The question then is: how to interpret homology when structural variation remains latent for long periods of evolutionary history, appearing just occasionally. This question is challenging, especially in the phylogenomic era where systematists have the benefit of robust hypotheses of species relationships that are often incongruent with morphology. Modern plant systematists must be comfortable to define and classify complex groups using combinations of characters rather than searching for or relying on a single homology. Furthermore, a better understanding of the development may clarify homoplasies as potential parallelisms instead of only convergences, additionally sharpening the focus on trait evolution in plants. Future studies are required that will investigate how genetic mechanisms are silenced and then re-expressed over time and the role of hybridization and introgression, known to be integral drivers of plant diversification (e.g. Gargiulo et al., 2015), in the context of maintaining such parallelisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ppees.2016.11.001.

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