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Floral development and molecular phylogeny support the generic status of *Tasmannia* (Winteraceae)[1]

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The taxonomic status of and evolutionary relationship between *Tasmannia* and *Drimys* (Winteraceae) have been subjects of controversy for many years. In this paper, a molecular phylogenetic analysis of the family with sequences of previously unpublished *Tasmannia* and *Drimys* species confirms earlier conclusions that *Tasmannia* and *Drimys* do not form a monophyletic group, despite the fact that they appear to share distinctive inflorescence and floral morphological attributes. Examination of alternative hypotheses of relationships with likelihood-ratio tests and parametric bootstrapping supports the separation of *Tasmannia* and *Drimys*. A detailed analysis of floral development in *Tasmannia lanceolata* and *T. xerophila* indicates that timing and position of sepal initiation differs between them, but that the position of subsequent organ initiation predictably follows from sepal position. This is in contrast to *Drimys winteri*, where a prolonged delay between sepal and petal initiation leads to the production of many phyllotactic patterns. The prolonged period of calyx tube growth leading to the formation of a calyptra in *Tasmannia* and *Drimys* probably evolved in parallel in the two lineages.

Key words: *Drimys*; floral development; molecular phylogeny; parametric bootstrapping; SH test; *Tasmannia*; Winteraceae.

Tasmannia R.Br. ex DC is an Old World genus of approximately 40 species in the angiosperm family Winteraceae (Smith, 1943a; Vink, 1970). The genus has often been linked with the New World genus *Drimys* J.R. & G. Forst., and has been considered as a section within *Drimys* by some authors (Smith, 1943b; Vink, 1970, 1988). This is because both genera have a monopodial growth habit and a flower with a protective calyptra that is shed just prior to anthesis.

Taxonomic controversy surrounding *Tasmannia* and *Drimys* has been active for over 50 years (Smith, 1943a, b, 1969; Ehrendorfer et al., 1968; Vink, 1970, 1988, 1993; Sampson et al., 1988; Doust, 2000; Endress et al., 2000; Karol et al., 2000). *Drimys*, as delimited by Smith (1943b), was composed of two sections: section *Drimys* in South America and section *Tasmannia* in Australia, New Guinea, Malesia, and the Philippines. However, Smith later changed his opinion and proposed elevating the sections to generic status (Smith, 1969). He was influenced in this decision by the cytological studies of Ehrendorfer et al. (1968), who showed that chromosome numbers in *Tasmannia* are $n = 13$, but in *Drimys* sensu stricto and the other genera they are $n = 43$. Smith (1969) also pointed out differences in anatomy, morphology, and chemical composition as well as the unisexual and dioecious flowers of *Tasmannia*. Vink (1970) disagreed with the elevation of the sections to generic status and refused to recognize the two genera proposed by Smith (1969), citing the monopodial construction of the inflorescence of both *Drimys* and *Tasmannia*, the common presence of a calyptra that encloses the bud until anthesis, and the fact that some flowers on male plants of *Tasmannia* show evidence of bisexuality by occasionally forming fruit.

An initial phylogeny of the family using internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA data indicated that *Tasmannia* and *Drimys* do not form a monophyletic group (Suh et al., 1993). Later molecular analysis using ITS nuclear and *trnL* chloroplast sequence data and a greater number of outgroup taxa showed that *Takhtajania* Baranova & J. F. Leroy was basal in the family and that *Tasmannia* was sister to the group of *Drimys*, *Pseudowintera* Dandy and *Zygogynum* Baill. sensu lato (Karol et al., 2000) (*Zygogynum* s.l. = *Zygogynum* Baill. + *Bubbia* Tiegh. + *Exospermum* Tiegh. [Vink, 1977, 1985]). The clades of both *Drimys* and *Tasmannia* had strong bootstrap support, and there were no molecular data to suggest that the two genera could form a monophyletic group. However, a combined analysis of ITS and morphological data suggested that *Tasmannia* and *Drimys* formed a monophyletic group (Linder and Crisp, 1995). A recent morphological analysis also found the two genera formed a single clade and that *Takhtajania* was part of a clade including *Pseudowintera* and *Zygogynum* s.l. (Endress et al., 2000).

The phylogenetic analyses that group *Tasmannia* and *Drimys* together do so because of the shared characters of a monopodial growth habit and a persistent calyptra. Other genera in the family have a sympodial growth habit and floral calyptras that stop growing and are shed or burst early in the development of the floral bud (Doust, 2000). The shared monopodial growth habit does not extend to shared inflorescence architecture: *Drimys* bears anthotelic racemose florescences in the axils of the inflorescence bracts whereas *Tasmannia* bears either single flowers or multiple flowers in ranks borne on a common mound of tissue (Vink, 1970; Doust, 2000). The shared persistent calyptra is also more complex than at first glance; developmental studies by Tucker and Gifford (1966), Vink

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(1970, 1988), and Sampson et al. (1988) show that *Tasmannia lanceolata* and *Drimys winteri* have lateral sepals, whereas *T. xerophila* and all other *Tasmannia* species that have been observed have only medial sepals (Vink, 1970; Doust, 2000). *Tasmannia lanceolata* also has adaxial and abaxial lobes that have been interpreted as either an inner pair of sepals or as variously lobed regions of the calycine calyptra (Tucker and Gifford, 1966; Vink, 1970; Doust, 2000). *Tasmannia* differs from *Drimys*, and the other genera in the family, because it is the only genus where flowers are unisexual and dioecious (Vink, 1970).

The present study focuses on floral development in *Tasmannia* in relationship to that of *Drimys*, within an expanded phylogeny of the family. Further species of *Drimys* and *Tasmannia* were added to the phylogenetic reconstruction in order to verify that *Drimys* species form a monophyletic group and to ascertain the placement of *Tasmannia lanceolata* within the *Tasmannia* clade. The molecular phylogenies produced were used to test the monophyly of *Tasmannia* and *Drimys* (Vink, 1988), as well as the hypothesis that *Tasmannia* and *Drimys* form one monophyletic clade and that *Takhtajania*, *Pseudowintera*, and *Zygogynum* s.l. form another (Endress et al., 2000). A critical reevaluation of floral development in the light of the present molecular analysis may resolve conflicts between the phylogenies suggested by previously published molecular and morphological data sets and indicate whether *Tasmannia* should be regarded as being part of *Drimys* or recognized as a separate genus.

MATERIALS AND METHODS

Phylogenetic analysis—The molecular data set used for this analysis contained five newly sequenced accessions (see supplementary data accompanying the online version of this article), four of *Tasmannia* and one of *Drimys*. DNA of these taxa was extracted from silica-dried leaf material using the method of Giussani et al. (2001). The ITS genomic region was amplified using the polymerase chain reaction (PCR), using the leu1 and 4R primers (White et al., 1990; Malcomber, 2002). The PCR products of ITS sequences were cloned using the pGEM-T easy vector system (Promega, Madison, Wisconsin, USA) as described in Malcomber (2002). Four clones of each ITS DNA accession were amplified using M13 forward and reverse primers. The PCR products for the *trnL* intron were PCR amplified using primers C and F from Taberlet et al. (1991) and cleaned by gel purification using the QIAquick PCR purification kit (Qiagen, Valencia, California, USA). Cleaned PCR products from both DNA regions were quantified by comparison with DNA of a known concentration (pGEM 10 and 25 ng; Applied Biosystems, Foster City, California, USA) and fluorescence-labelled using the Big Dye (Applied Biosystems) cycle sequencing protocol. Four primers were used for ITS sequencing, the two plasmid primers t7 and sp6, and two ITS specific internal primers, ITS2 (White et al., 1990) and ITS3B (Baum et al., 1994). The *trnL* region was sequenced using primers C, D, E, and F (Taberlet et al., 1991). Both forward and reverse DNA strands were sequenced on an ABI 377 automated sequencer (Applied Biosystems). Contig assembly and editing of sequences used Sequencher, version 3.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Sequences were a minimum of 85% double-stranded. Sequences were aligned by eye with the aligned data set of Winteraceae *trnL* and ITS sequences of Karol et al. (2000), using MacClade 4.0 (Maddison and Maddison, 2000). Accession numbers are listed along with specimen voucher information in the supplementary data accompanying the online version of this article. Selection of outgroup taxa was based on previous analyses by Suh et al. (1993) and Karol et al. (2000).

Phylogenetic analyses were performed using both maximum parsimony (MP) and maximum likelihood (ML) algorithms, treating character states as unordered. The best model for each of the maximum likelihood analyses was calculated by Mrmodeltest version 1.1b (Nylander, 2002), using a neighbor-joining tree. Analyses were conducted using PAUP* version 4.0b10 (Swofford, 1999), with heuristic searches, tree-bisection-reconnection (TBR) branch swapping, 100 (MP) or 10 (ML) random addition sequence replicates, and gaps treated as missing data. Full heuristic bootstrap analyses (Felsenstein, 1985) for the parsimony analyses were conducted using 100 (MP) or 1 (ML) random addition sequence replicates and 1000 bootstrapped data sets.

Combinability of the two molecular data partitions was analyzed using ML topologies and the PAUP* implementation of the Shimodaira-Hasegawa (SH) test, with resampling estimated by log-likelihood (RELL) optimization and 1000 bootstrap replicates (Goldman et al., 2000). Two SH tests were performed, one in each data partition (with likelihood parameters being estimated from the data in that partition). Each test compared the likelihood of (a) the best tree topology for that partition (e.g., for the ITS partition, the best ML ITS tree), (b) the best topology for the other partition (e.g., the best ML *trnL* topology from the *trnL* partition), and (c) the best topology for that partition constrained by the topology of the best tree from the other partition (e.g., the best ML ITS tree for the ITS partition, constrained by the *trnL* tree).

A comparison of trees (a) and (b) is a crude test of congruence of tree topologies generated from different data partitions, and can be analyzed either via an exact likelihood ratio test (Huelsenbeck and Bull, 1996) or by a resampling-based approach (SH test; Goldman et al., 2000). However, the results of the comparison of topologies (a) and (b) in the two data partitions via an SH test appeared to be sensitive to differences in the resolution of the trees being compared (see Results), so a comparison of constrained and unconstrained trees calculated from the same data partition (trees [a] and [c]) was also used. This type of comparison has been more commonly used to test how well different topological hypotheses explain the data (Fishbein et al., 2001; Zanis et al., 2002), rather than as an explicit test of combinability, although the logic is similar. Such a comparison, based on the same data partition, circumvents the problem of finding significant differences between trees with similar topologies but very different levels of resolution.

An SH test was also used to examine two main morphological hypotheses of evolutionary relationships in Winteraceae. Topologies encoding (a) *Drimys* and *Tasmannia* as a monophyletic group (Vink, 1988); and (b) *Drimys* and *Tasmannia* as a monophyletic group and *Takhtajania*, *Pseudowintera*, and *Zygogynum* s.l. as a separate monophyletic group (Endress et al., 2000) were used to produce constrained ML molecular phylogenies. These were tested against the unconstrained ML phylogeny.

Parametric bootstrapping was employed to further test the specific hypothesis that *Drimys* and *Tasmannia* should be considered as separate genera (Huelsenbeck et al., 1996; Swofford et al., 1996; SOWH test: Goldman et al., 2000; Fishbein et al., 2001; Buckley, 2002; Zanis et al., 2002). A fully resolved ML tree, calculated under the constraint of *Drimys* and *Tasmannia* being a monophyletic group, was used as the basis for estimating branch lengths under maximum likelihood in PAUP*. The best model for the ML analysis was calculated by Mrmodeltest version 1.1b (Nylander, 2002), using a neighbor-joining tree. Analyses were conducted using PAUP* version 4.0b10 (Swofford, 1999), with heuristic searches, TBR branch swapping, 10 random addition sequence replicates, and gaps treated as missing data. Seq-Gen version 1.2.6 (Rambaut and Grassly, 1997) was then used to simulate 100 data sets using the topology and branch lengths from the constrained tree. Each of these data sets was analyzed in PAUP* without constraint and with the constraint of the tree from which the data sets were simulated. The difference in tree length between constrained and unconstrained trees for each simulated data set was calculated and used to form a null probability distribution. The difference in tree length between trees with *Drimys* and *Tasmannia* as polyphyletic or as a monophyletic group was calculated from the original data set and compared against the null probability distribution.

Developmental characters, defined by comparison of developmental sequences (see below), were optimized on each of the most parsimonious trees using MacClade 4.0 (Maddison and Maddison, 2000). In cases where character states were equivocal at a node, all equally parsimonious reconstructions were examined.

Morphological and developmental analysis—Previous work has shown that there are two main morphological groupings in *Tasmannia* (Smith, 1943a;

TABLE 1. Log likelihood scores for data partition combinability using the Shimodaira-Hasegawa (SH) test (*$P < 0.05$).

Partition	Constraint	Score (−lnL)	Difference (−lnL)	Significance (*P*)
trnl	*trnL* topology	2039.089 86	14.090 56	0.084
	trnL constrained by ITS topology	2024.999 31	0.0001	0.557
	ITS topology	2024.9930	0	(best)
ITS	ITS topology	3502.780 03	0	(best)
	ITS constrained by *trnL* topology	3502.780 03	0	0.819
	trnL topology	3592.312 60	89.532 57	0.001*

Vink, 1970; Sampson et al., 1988). One of these consists only of *Tasmannia lanceolata*, whose first pair of sepals is arranged laterally, while the other group comprises all other species examined and has two sepals arranged medially (adaxially and abaxially). Material at all stages of development of *Tasmannia lanceolata* (Poir.) A.C. Smith and one species from the other group, *T. xerophila* (P. Parm.) Gray were collected for this study from populations at around 800–1000 m a.s.l. in the Great Dividing Range northeast of Melbourne, Australia. Voucher specimens were collected from each plant and are stored in the herbarium at the University of Melbourne (MELU). Fixation, dissection, and scanning electron microscope (SEM) analysis are as detailed in Doust (2000, 2001).

Following the arguments of Hufford (1995) we regard the development of morphological structures as only observable through a succession of instantaneous morphologies (ontogenetic states), such as those provided by SEM micrographs. The description and comparison of ontogenetic states provides an alternative to the view of ontogeny as passing through a prescribed and rigid series of developmental stages. This is necessary because developmental events are often disassociated during morphological diversification of related taxa (heterobathmy; Takhtajan, 1991), so that recognizable and homologous ontogenetic states can potentially arise at spatially or temporally different locations in the developmental process (Hufford, 1995; Doust and Kellogg, 2002b). The molecular phylogeny can be used as a guide to selecting phylogenetically relevant comparisons of ontogenetic states and in inferring direction of character state evolution (Doust and Kellogg, 2002a, b).

RESULTS

Combinability of molecular data sets—The information content was very different between the two data partitions, with the *trnL* partition having 61 out of 989 (6.2%) and the ITS 219 out of 793 (27.6%) informative characters under maximum parsimony. The ITS and *trnL* data sets gave trees for both maximum parsimony and maximum likelihood analyses that were identical in the topological relationships of the genera but differed in the degree of resolution of the terminal taxa. The ITS phylogeny was fully resolved whereas in the *trnL* phylogeny the relationships within each genus were unresolved. The SH test was done in each data partition and was not significant for any comparison in the *trnL* partition, but was significant for the comparison of the unconstrained *trnL* tree topology with ITS topologies in the ITS partition (Table 1). It is likely that this is because of the difference in resolution of the *trnL* and ITS topologies, as the terminal taxa are completely resolved only in the ITS topology. There is no significant difference between topologies when constrained and unconstrained trees are compared in each data partition (Table 1). On the basis of the nonsignificant SH tests between constrained and unconstrained topologies in each data partition, as well as the identical topological relationships of the genera, the data sets were combined, and all analyses were carried out on the combined data set.

Phylogenetic analyses—Maximum parsimony analyses of the combined data set found three most parsimonious trees of 613 steps each. Differences in the trees are entirely due to rearrangements amongst the outgroup taxa. The best maximum likelihood model calculated for the combined data set was the General Time Reversible with estimated gamma model (Zharkikh, 1994). Maximum likelihood analyses gave two trees with the same likelihood value (to five decimal places). The topologies of the two trees were identical apart from small reciprocal changes in branch lengths. One of the maximum likelihood trees is illustrated in Fig. 1. The relationships suggested

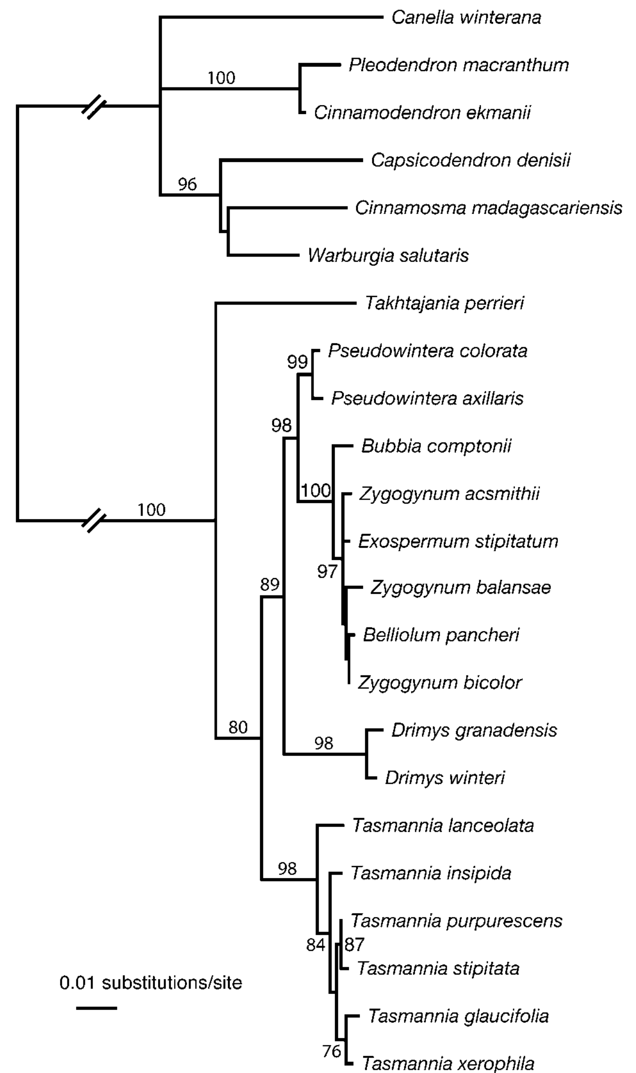

Fig. 1. One of two trees from the maximum likelihood analysis of the *trnL* and ITS data sets (−ln L = 5769.555 07). Topology is identical to that derived from the maximum parsimony analysis. Bootstrap support values from the maximum likelihood analysis are above branches.

TABLE 2. Log likelihood scores for three alternate topologies using the Shimodaira-Hasegawa (SH) test (*$P < 0.05$).

Constraint	Score (−lnL)	Difference (−lnL)	Significance (*P*)
Unconstrained	5769.555 07	0	(best)
(a) *Drimys* + *Tasmannia*	5777.584 91	8.029 84	0.070
(b) *Drimys* + *Tasmannia*, *Takhtajania* + *Pseudowintera* + *Zygogynum* s.l.	5781.296 66	11.741 59	0.036*

by the maximum likelihood trees are the same as for the maximum parsimony trees. Bootstrap values from both maximum parsimony and maximum likelihood analyses were similar and are noted below.

The phylogenetic relationships of the genera are identical to that presented by Karol et al. (2000), with *Takhtajania* sister to the other members of the family. The next clade to diverge is formed by the six species of *Tasmannia*, which are sister to a clade comprising *Drimys*, *Pseudowintera*, and the species of *Zygogynum*, *Exospermum*, and *Bubbia* (*Zygogynum* s.l., Vink, 1985). The clade of *Tasmannia* species has high bootstrap support (M 100%, ML 98%), as does the sister clade of *Drimys*, *Pseudowintera*, and *Zygogynum* s.l. (MP 98%, ML 89%). The two *Drimys* accessions form a well-supported clade (MP 100%, ML 98%), as do those of *Pseudowintera* (MP 99%, ML 99%) and *Zygogynum* s.l. (MP 100%, ML 100%).

Testing alternative hypotheses of relationships—A variety of methods were used to investigate alternative hypotheses of relationships that had been suggested from morphological analyses (Vink, 1988; Endress et al., 2000). In particular, the relationship of *Drimys* and *Tasmannia* in the molecular trees is very different to that found in morphological trees, where the monopodial growth habit and the persistence of the calyptra up until anthesis have been used to unite the two genera.

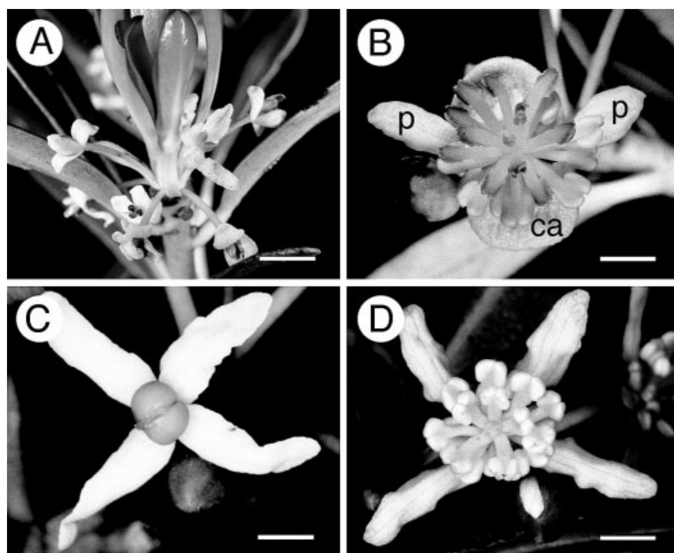

Fig. 2. Inflorescence and flowers of *Tasmannia xerophila* and *T. lanceolata*. (A–B) *Tasmannia xerophila*. (A) Inflorescence bearing pistillate flowers; the apex of the shoot has reverted to producing leaves. (B) Staminate flower showing reflexed calyptra halves and two laterally positioned petals. (C–D) *T. lanceolata*. (C) A pistillate flower showing the regular arrangement of the petals and the single terminal carpel. (D) A staminate flower showing the disymmetrical arrangement of the petals (ca, calyptra; p, petal;). Scale: (A) 15 mm, (B) 4 mm, (C–D) 2.5 mm.

We looked at the trees calculated from the bootstrapped data sets and found that only five out of 11 763 trees in the MP analysis (0.043%) and 0 out of 1173 trees in the ML analysis (0%) had *Drimys* and *Tasmannia* as a monophyletic group. Thus there is little support from the bootstrapped data sets for *Drimys* and *Tasmannia* forming a clade.

To further test the placement of *Drimys* and *Tasmannia*, the SH test was used to examine the hypotheses: (a) that *Drimys* and *Tasmannia* should form a monophyletic group (Vink, 1988); and (b) that *Drimys* and *Tasmannia* form one monophyletic group and *Takhtajania*, *Pseudowintera*, and *Zygogynum* s.l. form a second monophyletic group (Endress et al., 2000). These hypotheses were used as topological constraints to construct constrained ML trees, which were compared to the unconstrained ML trees. Hypothesis (b) was significantly different from the unconstrained tree and therefore can be rejected as an adequate description of the data (Table 2). However, the probability for hypothesis (a) being significantly different was $P = 0.070$, a value that is notable but does not allow us to confidently reject the null hypothesis (Table 2).

We also examined the possible relationships of *Drimys* and *Tasmannia* by parametric bootstrapping and ML analysis (Goldman et al., 2000). The difference in log-likelihood between constrained and unconstrained trees calculated from the original data matrix was $-\ln L = 8.029\,84$, which falls outside the probability distribution calculated from the resampled data sets (largest difference $-\ln L = 7.505\,33$). The difference between the constrained and unconstrained trees is therefore highly significant ($P < 0.01$). However, this result needs to be taken with some care as Buckley (2002) has shown that parametric bootstrapping can produce a high Type I error (probability of false positive results) if the model of sequence evolution is incorrect. However, the results of the three methods of analysis make it unlikely that *Drimys* and *Tasmannia* form a monophyletic group.

Morphology and development—All species of *Tasmannia* have monopodial shoot systems that produce leaves with axillary vegetative buds during the vegetative phase and bracts with axillary floral buds in the reproductive phase. The inflorescence is a raceme terminated by a vegetative bud, and after the production of the flowers, production of leaves recommences (Fig. 2A). In common with most other genera in the family, species of *Tasmannia* have flowers whose free organs show considerable variation in arrangement (Vink, 1970; Doust, 2000). All species are dioecious, with unisexual flowers, although bisexual flowers have rarely been observed (as evidenced by the production of fruit in otherwise staminate flowers; Vink, 1970). The species included in this study usually bear only one flower per floral bract, but other species of *Tasmannia* may have up to 11 flowers per floral bract [various forms of *Tasmannia* (*Drimys*) *piperita* from Papua New Guinea; Vink, 1970].

The organs that are initiated first in the developing flower

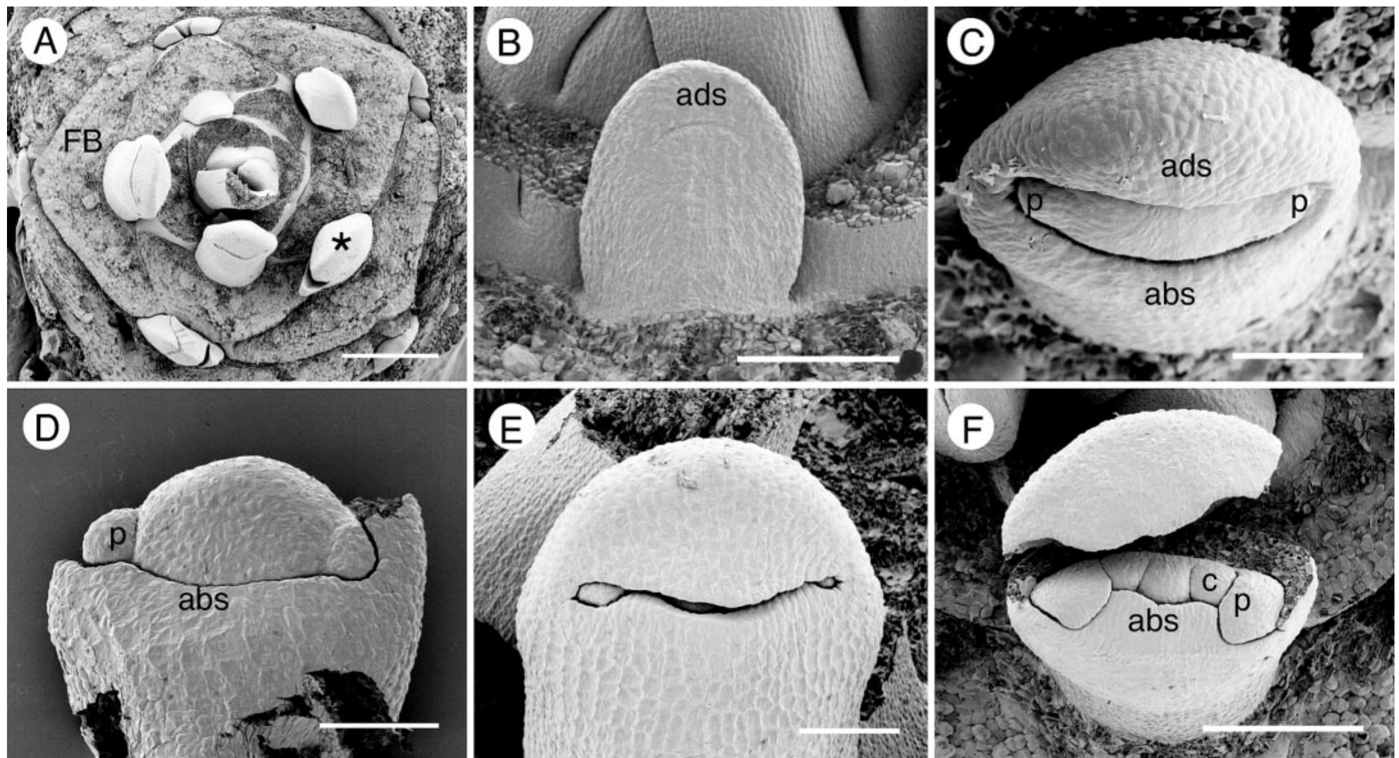

Fig. 3. Development of sepals in *Tasmannia xerophila*. (A) Young inflorescence showing normal buds near the center of the inflorescence and anomalous buds with bracteoles towards the edges of the inflorescence. Floral bracts have been removed. (B) Lateral view of a bud with the adaxial sepal initiated; floral bract removed. (C) Apical view showing overarching adaxial sepal and line of developing abaxial sepal. Petal primordia have been initiated. (D) Lateral view showing abaxial sepal and petals. (E) Lateral view showing the floral apex covered by the growth of the adaxial sepal. (F) Apical view with adaxial sepal partially removed, showing the limited growth of the abaxial sepal and the developing petals and carpels (abs, abaxial sepal; ads, adaxial sepal; c, carpel; FB, floral bract; * bud with basal bracteole). Scale: (A) 500 μm, (B–E) 100 μm, (F) 200 μm.

are here described as sepals while the inner series of petaloid organs are described as petals, as the two organ types are developmentally and morphologically distinct from each other (Smith, 1943b; Tucker and Gifford, 1966). An alternative interpretation would be to interpret the first and second pair of organs in *T. lanceolata* and the first pair of organs in *T. xerophila* as prophylls, and the inner petaloid organs as tepals. In other members of the Winteraceae the inner series of organs are more tepaloid, as they can form a gradation from thick outer to thin inner petaloid structures. However, in *Drimys* and *Tasmannia*, all of the inner series of organs are alike and petaloid. It is not possible to definitely differentiate between sepals and petals vs. prophylls and tepals for flowers in Winteraceae, but the particular interpretation adopted does not affect the comparisons of floral development detailed below. It may be that patterns of gene expression will be able to differentiate between prophylls and sepals, but in the absence of such evidence we have labelled the first initiated organs as sepals rather than prophylls and the inner series of organs as petals.

Tasmannia xerophila—During development the floral buds are protected by a number of tightly enclosing straw-colored floral bracts, with the most basal bracts being the largest. These bracts abscise just before anthesis. In more distal positions on the inflorescence all of the flowers are solitary but towards the base of the inflorescence some of the axillary floral buds produce a bracteole or two on their pedicels (Fig. 3A). In the very lowermost positions these bracteoles may themselves have axillary buds, at least some of which are floral buds. Staminate flowers produce stamens and a nonfunctional carpel; pistillate flowers produce a number of lateral carpels. The processes of sepal and petal initiation are similar in both staminate and pistillate flowers.

The first discernable event in the development of the flower is the appearance of the floral primordium in the axil of the subtending bract. After its appearance the floral primordium elongates, and sepals are initiated when the meristem is 200–300 μm high, and has a projected surface area between 2.0×10^4 and 3.6×10^4 μm^2 (surface area when measured in apical view). The adaxial sepal is initiated first, in an approximately apical position (Fig. 3B), and the abaxial sepal is initiated soon after in a position lower down the abaxial side of the floral bud (Fig. 3C, D). The floral apex at this time is not at the topographical top of the flower but rather more towards the abaxial side. The placement of the sepals is parallel to the subtending bract. The adaxial sepal grows more quickly than the abaxial one, further emphasizing the abaxial position of the floral apex.

Soon after the initiation of the petals the sepals enclose the floral apex, the adaxial sepal overlapping the abaxial sepal (Fig. 3E). The extent of this overlap can be seen when the adaxial sepal has been removed (Fig. 3F). The sepal tips are then borne aloft by the action of an intercalary meristem, which forms the tube of the calyptra. Growth of the calycine calyptra keeps pace with the development and growth of the developing bud. At anthesis the calyptra splits into two halves,

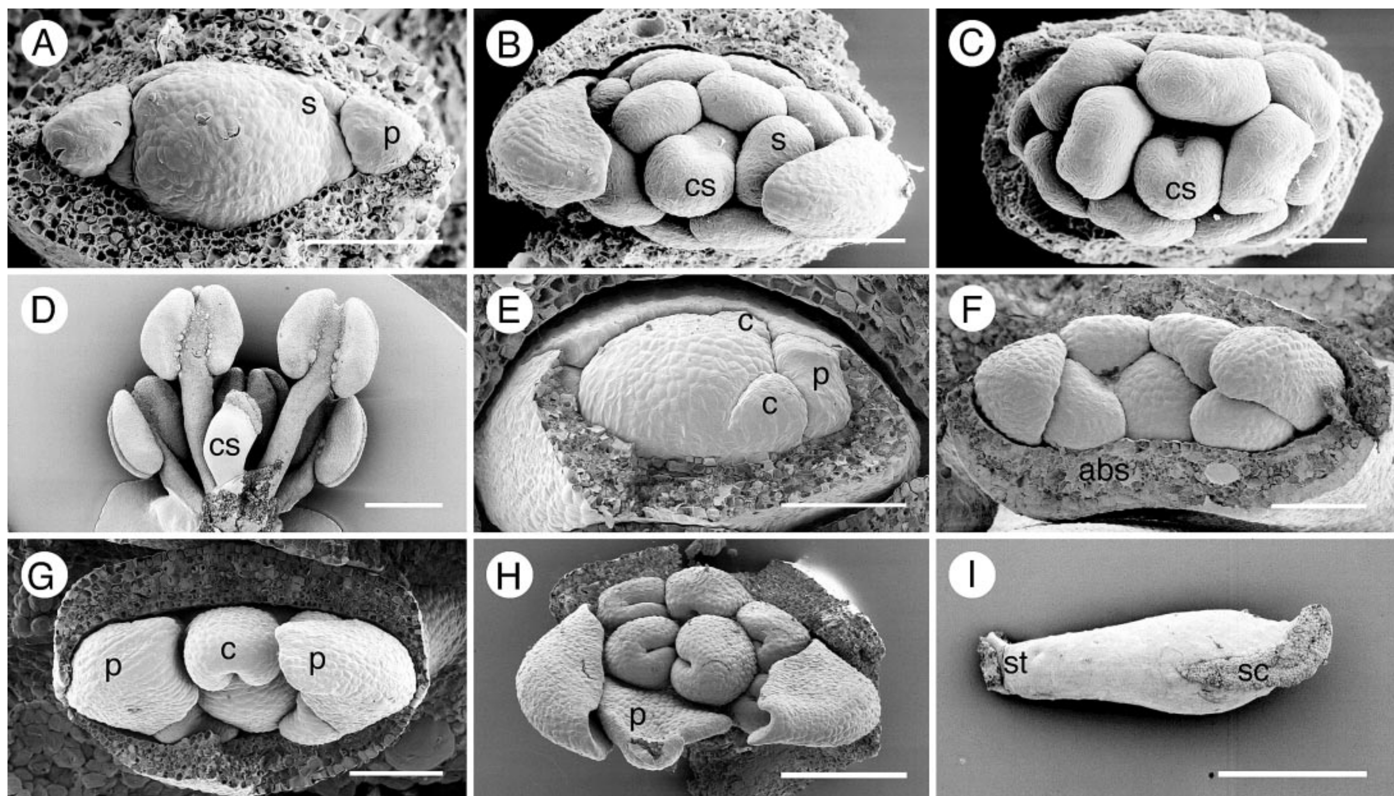

Fig. 4. Development of petals, stamens, and carpels in *Tasmannia xerophila*. (A) Apical view of lateral petal and stamen primordia. Stamen primordia are more developed towards the lateral poles of the elliptic floral apex. (B) Apical view of stamen development showing stamens initiated all around the meristem; central organ is sterile carpel. (C) Apical view of male flower showing stamens starting to differentiate into filaments and anthers and sterile carpel beginning to become grooved. Stamens are arranged in more or less regular tetramerous whorls. (D) Lateral view of a dissected staminate flower showing the small sterile carpel surrounded by stamens; longest stamens are around the carpel and the shortest at the base of the flower. (E) Lateral view of early carpel development showing a petal flanked by two carpel primordia. (F) Early carpel development showing lateral petals and four carpels. Note the greater space available on the abaxial as opposed to the adaxial side of the flower. (G) Apical view of carpel development showing carpels at different heights on the floral apex. (H) Carpel development with the margins of the carpels growing together as the cleft deepens. There are two lateral and one abaxial petal, with the abaxial petal occupying the space where a carpel would usually develop. (I) A mature carpel of *T. xerophila* showing the locular region with the stigmatic crest and the basal stipe (cs, sterile carpel; s, stamen; sc, stigmatic crest; st, stipe). Scale: (A–C) 100 μm, (D) 1 mm, (E–G) 100 μm, (H) 200 μm, (I) 1 mm.

the splits appearing between the adaxial and abaxial sepal positions. The two halves of the calyptra then reflex to a horizontal position (Fig. 2B).

Petals are initiated soon after the sepals, before the floral apex is covered by the growth of the calyptra. Two petals are initiated, one at each pole of the elliptic floral apex, lateral to the placement of the sepals and to the subtending floral bract (Figs. 3C, D, 4A). The floral apex is at this stage approximately 50 μm high and has a projected surface area of approximately 2.0×10^4 μm^2. Further petals may be initiated, appearing in either an abaxial or adaxial position (Fig. 4H).

In staminate flowers, stamens are initiated soon after the petals and after the growth of the calycine calyptra has effectively covered the floral apex. They appear near the poles of the elliptic floral apex on either side of each petal primordium (Fig. 4A). Further stamen primordia are initiated towards the poles of the elliptic floral apex before initiating on the abaxial and adaxial sides of the floral apex (Fig. 4B). Stamens may appear to be initiated in alternating whorls or in an irregular spiral (Fig. 4B, C). The last organ to be initiated in the staminate flower is the nonfunctional carpel, which resembles the fertile carpel of the pistillate flower except that ovules are absent and the stigmatic crest is not as well developed (Fig. 4C, D).

In pistillate flowers there are generally no stamens or staminodes, and carpel initiation directly follows on from the initiation of the petals. There are between three and 10 carpels laterally initiated on the floral apex; the first four are initiated on either side of the petal primordia, towards the poles of the elliptic apex (Fig. 4E, F) and further carpels are initiated to fill in the remaining space on the meristem. The placement of the carpels can vary in both height and transverse position on the apex (Fig. 4G). In some flowers, primordia are differentiated into petals in some of the positions where carpels would otherwise differentiate (Fig. 4H). The carpel develops as an open structure until fusion of the edges occurs (Fig. 4I). Later in development a stipe starts to grow beneath the region of the locule, elevating it above the receptacle.

Tasmannia lanceolata—During development, the floral buds are tightly enclosed by red floral bracts; basal bracts are shorter than more distal ones. Floral buds are usually single in the axil of each bract. As in *T. xerophila*, staminate flowers produce stamens and a nonfunctional carpel; however, pistillate flowers generally produce only a single terminal carpel. The processes of sepal and petal initiation are similar in both staminate and pistillate flowers.

The first two sepals initiated are at the poles of the elliptic

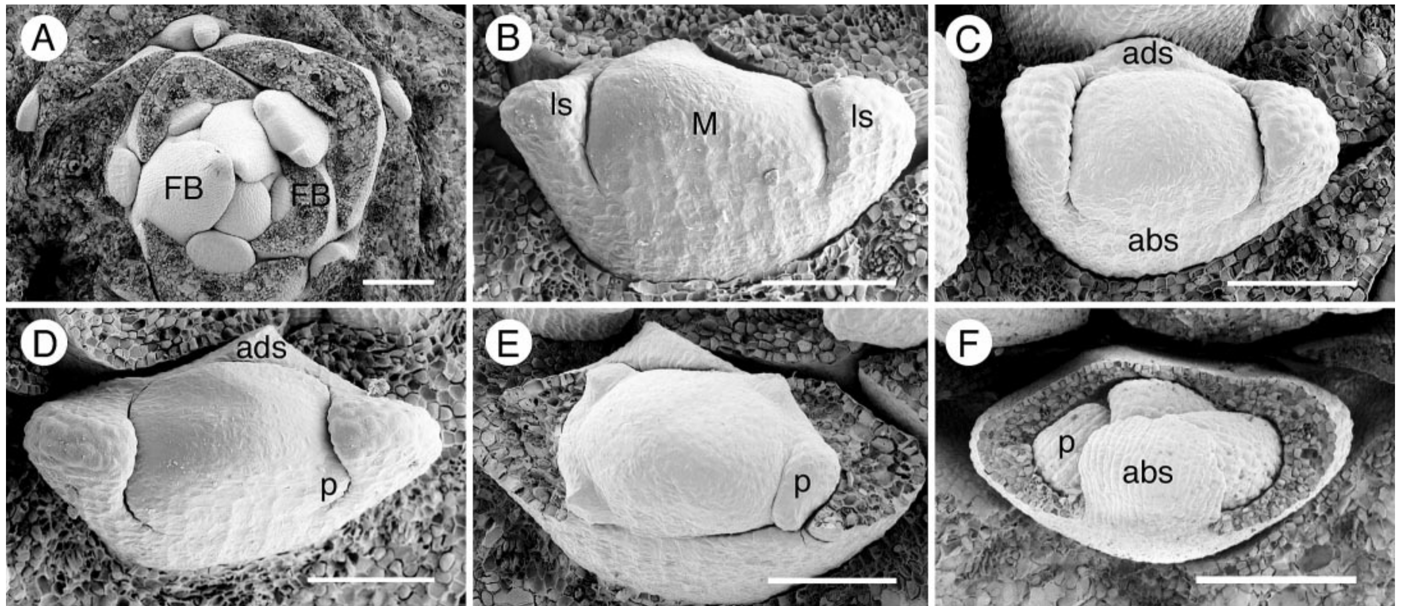

Fig. 5. Sepal and petal development in *Tasmannia lanceolata*. (A) Young inflorescence showing the developing flowers and the subtending floral bracts (some bracts have been removed). (B) Early sepal initiation, showing the production of two sepals lateral to the floral apex meristem. (C) Sepal initiation, showing initiation of two lateral sepals and an adaxial sepal. The abaxial sepal is very shallow with no prominent extension outside the line of the floral apex at this stage. (D) Petal initiation in the angles between the four sepals, adaxial sepal indicated. (E) Petal initiation with lateral sepals removed. Note the difference in size of the petals, suggesting that they may be initiated sequentially rather than synchronously. (F) Late development, showing the growth of the abaxial sepal. Lateral and adaxial sepals have been removed (ls, lateral sepal; M, floral apex meristem). Scale: (A) 200 μm, (B–E) 100 μm, (F) 200 μm.

floral apex and, unlike *T. xerophila*, are oriented laterally to the subtending bract (Fig. 5A, B). The floral primordium is at this stage 100–130 μm high and has a projected surface area between 1.1×10^4 and 2.0×10^4 μm^2. Two more sepals are initiated adaxially and abaxially soon after the first two sepals (Fig. 5C). The adaxial sepal is the more prominent of this second pair of sepals and has a more or less triangular shape; the abaxial sepal is often no more than a line of tissue with no free portion, although it occasionally is more pronounced (Fig. 5F). Both lateral and medial sepals are the result of unequal growth of the calyx rim. The continued growth of the calyx occurs via an intercalary meristem, as in *T. xerophila*, so that a calycine calyptra is formed, bearing the sepal tips at its apex. Some further growth of the two medial sepal tips may also occur (Fig. 5F). In most flowers, the presence of the adaxial and abaxial sepal tips continues to separate the lateral sepal tips throughout the growth of the bud. At anthesis, the calyx calyptra splits in two and the halves reflex. The dehiscence line of the calyptra is between the adaxial and abaxial sepals and bisects the lateral sepals.

Four petal primordia are initiated in a whorl alternating with the four sepals but positioned rather more towards the lateral poles of the elliptic floral apex (Fig. 5D, E). These are initiated before the calyptra has enclosed the floral apex. The floral primordium is at this stage 50–80 μm high and has a projected surface area between 1.0×10^4 and 2.1×10^4 μm^2. More than four petals may eventually be initiated, and up to eight have been counted in staminate flowers. These further petals are initiated first on the adaxial and abaxial sides of the flower and then laterally, in line with the lateral sepals.

Stamens are generally found only in the staminate flowers, although two examples were found of a single stamen being initiated in a pistillate flower. Stamen initiation commences before the calyptra has enclosed the floral apex (Fig. 6A), and stamen primordia appear first towards the lateral poles of the more or less elliptic floral apex although they are very soon initiated on the abaxial and adaxial sides of the apex as well (Fig. 6B). The stamens can be positioned in whorls or spirals (Fig. 6C, D).

The last organ to be initiated in the staminate flower is the nonfunctional carpel, which resembles the fertile carpel of the pistillate flower except that ovules are absent and the stigmatic crest is not as well developed (Figs. 2D, 6D).

In pistillate flowers the floral apex continues to grow after petal formation and may become more circular in cross-section (Fig. 6E). The initiation of the single terminal carpel uses up the entirety of the floral apex. During differentiation of the carpel, two parallel ridges appear, oriented in a roughly medial plane, creating a furrow or cleft at the apex of the carpel (Fig. 6F). The ridges continue growth, but the dorsal ends of the ridges together with the dorsal bridge between them grows at a faster rate than the ventral ends of the ridges (Fig. 6G). For most of its development the carpel grows as an open U-shaped structure, which finally fuses by the intertwining of the papillae at the margins. However, there is a short section towards the base of the carpels where the carpel grows as a completely closed ring. This ring of tissue can be seen as a zone between where the carpel starts to swell from the top of the floral meristem and where the lower edge of the cleft begins (Fig. 6H, I). At maturity the carpel has a stigmatic crest running almost to the base of the carpel on the ventral side (Figs. 2C, 6H), and the distinction between the stipe and the cross-zone is obscured (Fig. 7I).

Patterns in floral organ arrangement—In both *Tasmannia xerophila* and *T. lanceolata* there is a basic early pattern of organ initiation consisting of firstly two floral organs, then another two at right angles to the first, and then four organs

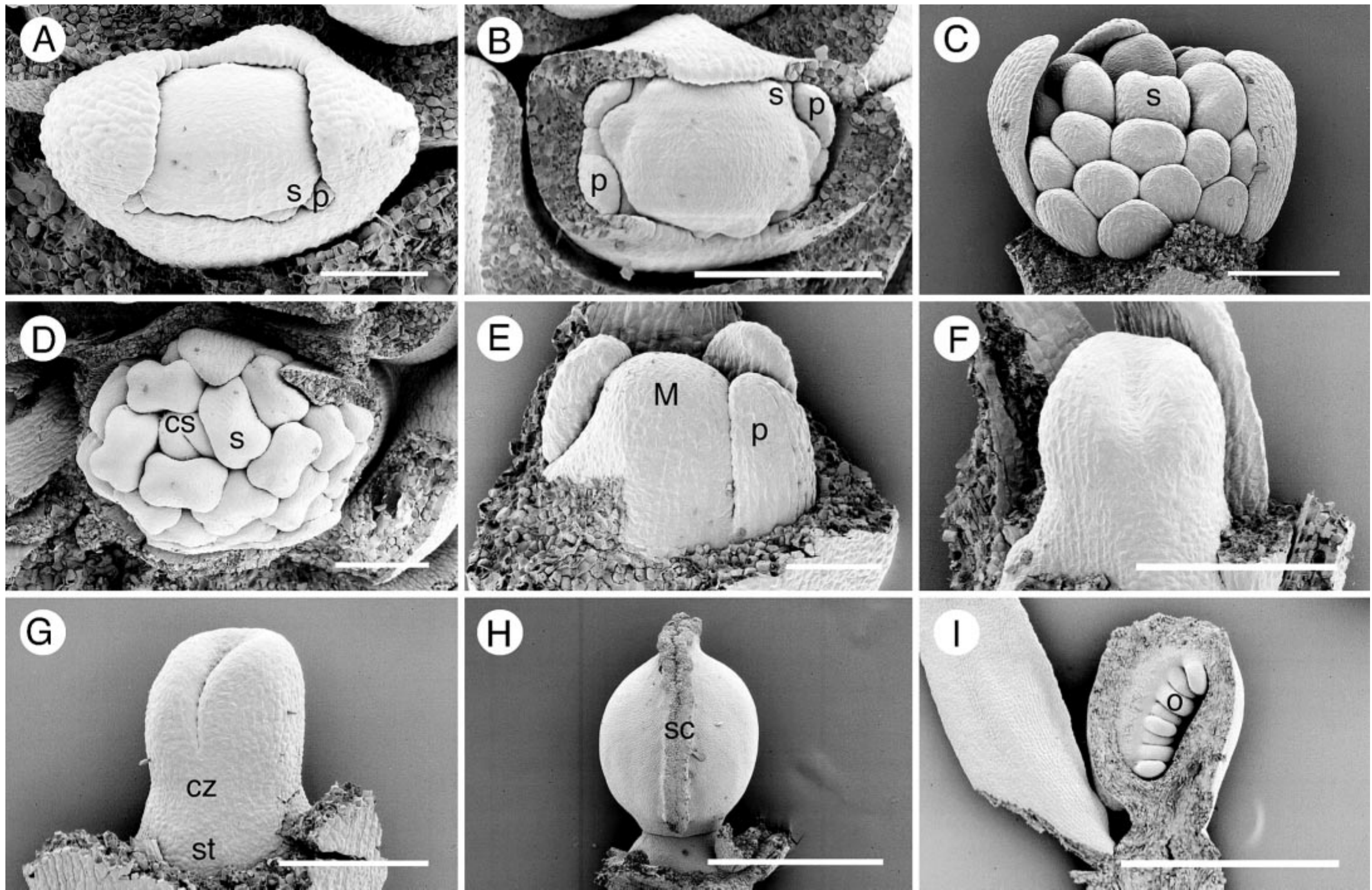

Fig. 6. Stamen and carpel development in *Tasmannia lanceolata*. (A) Early stamen initiation, with stamens being initiated before the growth of the calycine calyptra covers the floral apex. (B) Early stamen initiation, with the lateral sepals removed, showing that stamens initiate first towards the lateral poles of the ellipse before initiating on the adaxial and abaxial sides of the apex. (C) Late stamen development; side view, showing tiers of stamens in an approximately whorled arrangement. (D) Late stamen development, showing an irregular spiral arrangement of the stamens and the grooved sterile carpel at the center of the flower. (E) The pistillate flower just before carpel initiation showing the enlarged floral apex and the developing petals. (F) Early carpel development showing the formation of a groove on the apical and ventral face of the carpel. (G) Mid-carpel development, lateral view, showing the enlargement of the groove and the elongation of the carpel. Note the cross-zone, the area in the waisted region of the carpel which is below the level of the margins of the carpel but above the thickening of the floral apex/stipe. The cross-zone is a region that has a continuous circular meristem that produces a short tube at the base of the carpel. (H) Late carpel development showing the fusion of the margins of the carpel via the interdigitation of the papillae of the stigmatic crest. There is a small zone of continuous tissue (cross-zone) beneath the stigmatic crest and above the stipe. (I) Longitudinal section of the carpel showing eight ovules on a placenta that runs the full length of the carpel. (cz, cross-zone; o, ovule). Scale: (A) 100 μm, (B–D) 200 μm, (E) 100 μm, (F–G) 200 μm, (H–I) 1 mm.

alternating with the first four organs (Fig. 7). However, differences between the two species involve both the position of the first initiated organs, the whorls to which those organs belong, and the identity of the organs into which the primordia differentiate. The two sepals of *T. xerophila*, and all other *Tasmannia* species excepting *T. lanceolata*, are initiated abaxially and adaxially. These two primordia are followed by two petals initiated laterally. A whorl of four stamens or carpels follows the whorl of two sepals and of two petals (Fig. 7). By contrast, in *T. lanceolata* the first two sepals are initiated laterally, followed closely by two more abaxially and adaxially (Fig. 7). The two pairs of sepals are initiated sequentially but appear to be in the same organ whorl. A further whorl of four petals follows the two pairs of sepals. In both species there is a 2 + 2 + 4 timing of organ initiation, but position and organ identity varies. Primordium position is not necessarily closely tied to organ identity as further petals may initiate in both species from primordia that would otherwise have been stamens or carpels. Male and female flowers of each species show similar patterns of organ position and identity until the initiation of stamens or carpel(s). At these later developmental stages organ identity changes but position is essentially conserved, except that the generally larger size of the carpels compared to stamens reduces the number that are initiated.

Evolution of floral development—Figure 7 illustrates the developmental sequence of the two *Tasmannia* species and of terminal and lateral flowers of *Drimys winteri*. Developmental time is relative, being confounded with size, so that increased size may be due to longer or faster periods of growth. In this diagram size is used as a proxy for time and the lengths of the lines connecting the various defined developmental stages signify the amount of time/size change that occurs between stages. Wavy lines show where time/size has increased relative to other taxa. The approximate height of the floral primordium at sepal initiation and the half-ellipsoid surface area (area = basal perimeter length multiplied by height) of the floral meristem at petal initiation stages are indicated. There are a num-

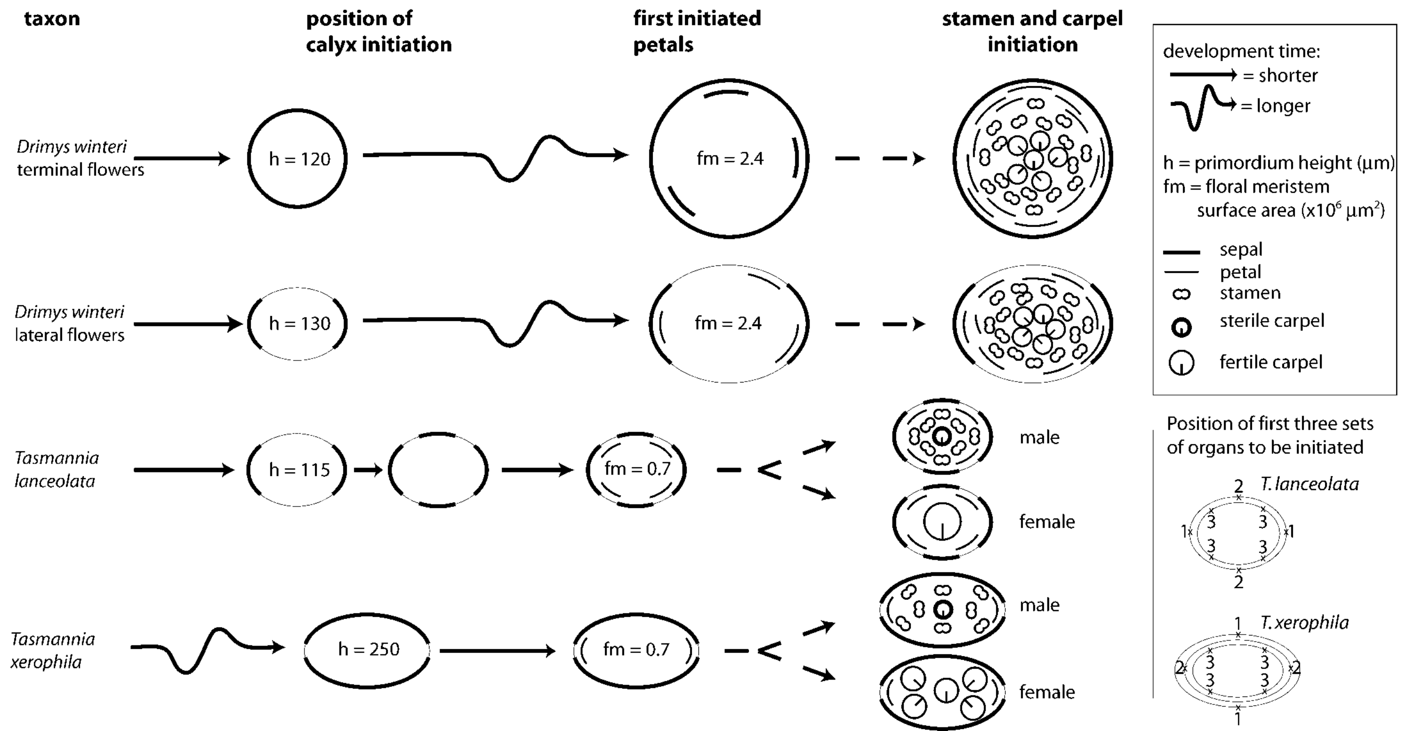

Fig. 7. Comparison of developmental sequences for terminal and lateral flowers of *Drimys winteri* and flowers of *Tasmannia lanceolata* and *T. xerophila*. Numbers inside diagrams at sepal and petal stages are height (h, in μm) of the floral primordium (sepal stage) and hemi-elliptical area (fm, in μm^2) of the floral meristem (petal stage). Wavy lines connecting stages imply greater time to get to that stage, with a concomitantly larger meristem relative to the other taxa at that stage. Stages between petal initiation and the fully developed flower have been omitted (indicated by dashed lines), as these are similar in the three species. Not all of the organs that are typically present in the staminate and bisexual flowers have been shown due to lack of space. In the bottom right hand corner of the figure are diagrams of the positions of the first three groups of organs initiated in flowers of *Tasmannia xerophila* and *T. lanceolata*, with an x marking the position of each organ.

ber of points throughout the developmental trajectory of these species where differences arise. An early difference is that *T. xerophila* achieves a larger floral primordium size and is twice the height of the other taxa before sepals are initiated and that the sepals of *T. xerophila* are initiated medially rather than laterally. The increased floral primordium height in *T. xerophila* may have facilitated the novel substitution of medial for lateral sepals. Another difference at sepal initiation is that the terminal flowers of *D. winteri* inflorescences initiate a ring meristem whereas lateral flowers, that have elliptic floral meristems, initiate two lateral sepals (Doust, 2000, 2001). This suggests that formation of lateral sepals may be linked to shape of the meristem. The lateral sepals in *T. lanceolata* are very similar to those in the lateral flowers of *D. winteri*, and an occasional lobe which resembles a medial sepal can also be found in *D. winteri*. Thus *T. lanceolata* and *D. winteri* are developmentally similar in these early ontogenetic stages.

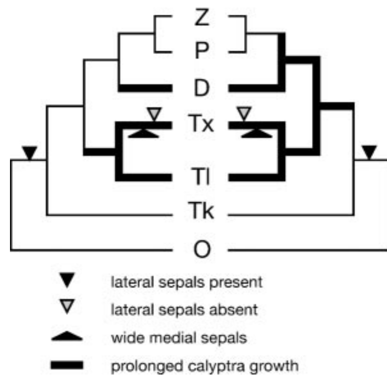

Fig. 8. Optimization of calyx and calyptra characters onto one of the three most parsimonious trees from the MP analysis. Two optimizations for calyptra growth are shown; the thick black line indicates prolonged growth of the calyptra (see Results). Initials indicate the major clades in Winteraceae: Z = *Zygogynum* s.l., P = *Pseudowintera*, D = *Drimys*, Tx = *Tasmannia* species other than *Tasmannnia lanceolata*, Tl = *T. lanceolata*, Tk = *Takhtajania*, O = outgroup.

Many of the developmental changes mentioned above are autapomorphies, yet the extended growth of the calyptra can be optimized onto the phylogeny either as a gain each in the lineages leading to *Tasmannia* and *Drimys* or a gain on the branch between *Takhtajania* and *Tasmannia*, followed by a loss on the branch between *Drimys* and *Pseudowintera* (Fig. 8).

DISCUSSION

Phylogenetic relationships—The combined ITS + *trnL* phylogeny shows that there is good support for *Tasmannia* and *Drimys* being separate taxa and does not support their inclusion into a single genus. These results are consistent with the two other molecular phylogenies that have used fewer representatives of *Tasmannia* and *Drimys* (Suh et al., 1993 [ITS]; Karol et al., 2000 [ITS + *trnL*]). Additional taxa of *Tasmannia* and *Drimys* do not change the basic phylogenetic relationships that had been described by those studies.

These molecular phylogenies conflict with hypotheses of relationships between *Drimys* and *Tasmannia* based on morphological data (Vink, 1993; Endress et al., 2000). The SH test indicated the most constrained hypothesis, that of a tree with *Drimys* + *Tasmannia* as one monophyletic group and *Takhtajania* + *Pseudowintera* + *Zygogynum* s.l. as another, is significantly less likely than the unconstrained molecular tree. The less constrained hypothesis, that *Drimys* + *Tasmannia* form a monophyletic group, was notably less likely than the unconstrained tree on the SH test ($P = 0.070$), but not significant at the $P < 0.05$ level. However, less than one-tenth of one-percent of MP trees and no ML trees from the pseudoreplicated bootstrap data sets had *Drimys* and *Tasmannia* grouped together. As well, the results of the parametric bootstrap test showed that topologies where the two genera were constrained to be monophyletic were significantly less likely than topologies where the two genera were separate. These methods offer different insights into the topologies suggested by the data, but support the topologies that were consistently produced in the phylogenetic analysis, and the finding that *Tasmannia* and *Drimys* should be regarded as separate genera rather than a monophyletic group. It is possible that the marginal significance for the SH test for this hypothesis may reflect a lack of power due to too few informative characters, a deficiency that could be resolved by adding more molecular data.

Development—The patterns of development show that there is a mixture of similarities and differences between *Tasmannia lanceolata*, *T. xerophila*, and *Drimys winteri* (Fig. 7). In fact, the only consistent similarity between the two genera is the extended growth of the calyptra, which has two equally parsimonious optimizations onto the molecular topology (Fig. 8). Consistent developmental differences between the two genera include the delay in petal initiation in *Drimys winteri* and the divergence of development into unisexual male and female flowers in *Tasmannia*. However, there are also marked differences between the two species of *Tasmannia*, both in the size of the floral primordium when sepals initiate, the position where sepals first initiate, the number of sepals initiated, and the number and placement of carpels (several and lateral in *T. xerophila*, single and apparently terminal in *T. lanceolata*). Sepal initiation in *T. lanceolata* is more similar to the lateral flowers of *Drimys winteri*, yet this pattern is found throughout the family and does not provide evidence for monophyly for the two species (Doust, 2000).

Tucker and Gifford (1966) state that two lateral sepals are initiated in *T. lanceolata*, and that the margins of the two sepals eventually become appressed and fused edge to edge, so that the lower portions grow as a cylinder. No evidence was found in this study for sepal fusion, and the growth of the calyptra as a collar, bearing the free sepals aloft, appears to better describe the development of the calyptra. Vink (1970) considered that only two lateral sepals are initiated but that it was a matter of opinion whether the adaxial and abaxial lobules were interpreted as a second pair of sepals or a result of the unequal growth of the ridges connecting the lateral sepals. However, as the lateral sepals in all genera are themselves the result of unequal growth this does not preclude the medial sepals likewise being so. The developmental analysis shows that adaxial and abaxial structures are more appropriately interpreted as sepals because the placement of the first whorl of petals alternates with the positions of both sepal pairs.

The shared position of the medial sepals in *T. lanceolata* and *T. xerophila* might be considered as evidence for their homology, although their appearance and manner of initiation are quite different. In *T. lanceolata* the medial sepals are the second set of primordia initiated and are relatively narrow and at approximately equal heights on the floral apex, whereas in *T. xerophila* they are the first set of primordia initiated and are wide and at different heights on the floral meristem. Thus it seems unlikely that the two forms of medial sepals are homologous. More sampling of the morphological diversity within *Tasmannia* is needed to shed light on the possible homology of the two forms of medial sepals.

In both species of *Tasmannia* the position of initiation of further petals, stamens, and carpels depends on the position of those already present. In a number of cases, more petals are differentiated than is the norm and are found where carpels or stamens would normally have been differentiated. This indicates that organ identity may be somewhat flexible in the flower but that organ position is less so. This is also evidenced by the conserved position yet changed organ identity of stamens and carpels in male and female flowers of *Tasmannia xerophila*. In later flower development in both species the initially regular whorled patterning evident in early flower development may become more irregular. This is most noticeable in male flowers, because more and smaller organs (stamens) are initiated in male as opposed to female flowers. An explanation of the irregular arrangement of floral organs in *Tasmannia* was first attempted by Vink (1970), who attributed the lack of regularity to the influence of an uneven base to the flower. Vink also noted that sepals and petals in *Tasmannia* were more regularly arranged and considered it likely that sepal and petal arrangement were under separate genetic control from that of stamens and/or carpels. However, there is no need to posit two sets of control factors for organ arrangement, because the smaller size of the stamen and carpel primordia makes it easier for disturbances in arrangement to emerge in that part of the flower. It is likely that irregularities in organ arrangement are due to irregularities in floral meristem shape, as shown for *Drimys winteri* (Doust, 2001).

The differences in sepal initiation and floral organ arrangement between *Drimys* and most species of *Tasmannia* do not support grouping of the two into a single genus. The initiation of lateral sepals in *T. lanceolata* is similar to *D. winteri*, yet later patterns of floral organ initiation are more similar to other species of *Tasmannia*. The production of a calyptra tube by an intercalary meristem occurs throughout the family, and it is only its continued growth that provides a morphological link between *Tasmannia* and *Drimys*. The developmental evidence presented above makes it likely that the continued growth of the calyptra has been separately derived in each of the two lineages. Developmental analysis reveals little evidence for uniting *Drimys* and *Tasmannia*, but rather supports the molecular phylogenetic findings that they are better regarded as separate monophyletic genera.

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