MOLECULAR EVIDENCE FOR THE PHYLOGENETIC POSITION OF *TAKHTAJANIA* IN THE WINTERACEAE: INFERENCE FROM NUCLEAR RIBOSOMAL AND CHLOROPLAST GENE SPACER SEQUENCES[1]

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ABSTRACT

The nucleotide sequences of the internal transcribed spacers (ITS 1 and ITS 2) and 5.8S coding region of nuclear ribosomal DNA, as well as the non-coding *trn*L-*trn*F spacer regions of the chloroplast DNA, were determined and analyzed to estimate the phylogenetic position of *Takhtajania perrieri* (Capuron) Baranova & J.-F. Leroy within the Winteraceae. Using representatives of each genus of Canellaceae as outgroups (*Canella, Capsicodendron, Cinnamodendron, Cinnamosma, Pleodendron,* and *Warburgia*), both maximum parsimony and maximum likelihood analyses place *Takhtajania* in a basal position sister to the remainder of the Winteraceae. Although the overall topology within the Winteraceae was mostly congruent between nuclear and chloroplast data sets, the *trn*L-*trn*F data resulted in lower support values in comparison to the ITS data, and failed to resolve basal relationships in the family, yielding alternative equally parsimonious solutions. The combined nuclear/chloroplast data set resulted in a single tree identical to that generated by nuclear data alone, but demonstrated strengthened support for a basal branch leading to *Takhtajania*, as well as for the position of *Tasmannia* as the next branch within the family. Potentially conflicting signals from nuclear and chloroplast data indicate that further taxon sampling or additional sequence data may be required to infer infrafamilial phylogenetic relationships for Canellaceae.

Key words: Canellaceae, combined data, cpDNA, ITS, Likelihood, phylogeny, *Takhtajania*, Winteraceae.

Known until recently from only a single collection made in 1909, *Takhtajania perrieri* has long been an enigma among the basal angiosperm family Winteraceae (Leroy, 1978). Its rediscovery in 1994 (Schatz et al., 1998) has provided the opportunity to reevaluate those morphological features anomalous within the family, as well as to explore the phylogenetic relationship of this isolated, relictual Malagasy endemic. Based upon morphological characters, two alternative hypotheses have been proposed regarding the position of *Takhtajania* within the Winteraceae: (1) *Takhtajania* is a member of a clade also comprising *Pseudowintera, Belliolum, Bubbia, Exospermum,* and *Zygogynum* (the latter 4 genera sometimes combined into a single genus *Zygogynum* s.l.) (Vink, 1988; Endress et al., 2000 this issue); or (2) *Takhtajania* represents the sister taxon to the remainder of the Winteraceae (Leroy, 1978; Vink, 1988). The aim of the present study is to infer the phylogenetic position of *Takhtajania* based upon analyses of molecular sequence data.

A previous molecular phylogenetic study (Suh et al., 1993) utilized the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) to address questions of generic relationships in the Winteraceae. Tissue of *Takhtajania,* however, was unavailable. In addition to presenting a phylogeny

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for the family solely through ingroup analysis, Suh et al. (1993) identified a single gene duplication event in the *Zygogynum* s.l. clade, a possible molecular marker for the inclusion (or exclusion) of *Takhtajania* within the *Belliolum/Bubbia/Exospermum/Zygogynum* assemblage.

If an ITS duplication event either was not identified in *Takhtajania* or occurred independently, then outgroup analysis would become critical for investigating its relationship to the remainder of the family. Both morphological and molecular (*rbc*L) cladistic studies, which examined relationships among basal angiosperms, have identified Canellaceae (represented in these studies by *Canella* alone) as a putative sister taxon to Winteraceae (Donoghue & Doyle, 1989; Qiu et al., 1993; Chase et al., 1993). *Canella* also appeared to be closely related to Winteraceae on the basis of secondary metabolites (Gottlieb et al., 1989). Canellaceae are comprised of six genera found in tropical Africa (*Warburgia*), Madagascar (*Cinnamosma*), and tropical America (*Capsicodendron* and *Cinnamodendron* in South America, *Canella* and *Pleodendron* in the Caribbean) (Cronquist, 1981). Although Suh et al. (1993) had encountered difficulties in the alignment of ITS sequences between *Canella* and Winteraceae, it was decided to reattempt alignment after sequencing additional representatives of Canellaceae.

This paper reports on the implications of new ITS sequence data for *Takhtajania* and the five other genera of Canellaceae; *Canella* ITS sequence had been previously determined by Suh et al. (1992). In addition, sequence data have been generated for two tandem chloroplast encoded (cpDNA) spacer regions, and analyzed both separately and in combination with the ITS sequences. The first spacer is a group I intron and is located between the conserved *trn*L (UAA) 3′ exon and *trn*L (UAA) 5′ exon. The second, an intergenic spacer, is located between the *trn*L (UAA) 5′ exon and *trn*F (GAA) (Taberlet et al., 1991). Restriction fragment length polymorphisms of this molecule have been used to address species-level relationships within Mexican pines (Perez de la Rosa et al., 1995). Sequence data generated from the *trn*L-F region also have been utilized to resolve phylogenetic relationships at both the species level (Mes & Hart, 1994) and the generic level (Compton et al., 1998; McDade & Moody, 1999). Several studies (Gielly et al., 1996; Molvray et al., 1999) have demonstrated the utility of combining the ITS and *trn*L-F spacer sequences for phylogenetic reconstruction. Thus, a complete survey of both ITS and *trn*L-F regions for all genera of both Winteraceae and Canellaceae has been conducted to estimate the phylogenetic position of *Takhtajania*.

Materials and Methods

Species Sampled and Sources of Plant Material

The species included in this study are presented in Table 1, along with voucher information, literature citation, and GenBank accession numbers for both the ITS and *trn*L-F spacer sequences. This list includes at least one representative species from all genera in both the Winteraceae and Canellaceae. In the instances where ITS has been determined previously (all Winteraceae [Suh et al., 1993] except *Takhtajania*, plus *Canella* [Suh et al., 1992]), identical DNA extracts were used to amplify and sequence the *trn*L-F spacers.

DNA Extraction, Amplification, and Sequencing

Standard CTAB methods of DNA isolation (Doyle & Doyle, 1987) were used to obtain total genomic DNA for polymerase chain reaction (PCR) amplification. To generate ITS amplicons, PCR was performed using plant specific primer ITS5 (Suh et al., 1993) and universal primer ITS4 (White et al., 1990) under the conditions described in Suh et al. (1993). To generate the *trn*L-F spacer amplicons, PCR was performed using universal primers "c" and "f" (Taberlet et al., 1991), also under the conditions described in Suh et al. (1993).

Sequence data were generated from PEG (polyethylene glycol) purified double-stranded PCR products (Morgan & Soltis, 1993) with the dye-terminator cycle-sequencing protocol for the ABI 373A Sequencer (Applied Biosystems, Inc.). The entire forward and reverse strand of ITS was determined for each species using the amplification primers (see above) and primers ITS3 (White et al., 1990) and C5.8S (Suh et al., 1993). To determine the sequence for both strands of the *trn*L-F spacers, the amplification primers were used as well as primers "d" and "e" (Taberlet et al., 1991). The resulting chromatograms were edited with Sequencher version 3.1 (Gene Codes, Inc.); regions corresponding to the amplification primers were deleted. The final edited consensus sequences were exported for alignment.

Sequence Alignment

All sequences were aligned manually with the aid of Se-Al version 1.0a1 (Rambaut, 1996) multiple sequence editor.

Table 1. List of specimens used in this study with locality and voucher information, ITS literature citation for previously published sequences, and GenBank accession numbers for both ITS and *trn*L-F.

Species	Voucher	ITS citation	Genbank ITS	Genbank *trn*L-F
Winteraceae				
Belliolum pancheri (Baill.) Vink (= *Zygogynum pancheri* (Baill.) Vink)	New Caledonia, Mt. Koghis, *LBT 205*, NO	Suh et al., 1993	AY004119	AY004135
Bubbia comptonii (Baker f.) Dandy (= *Zygogynum comptonii* (Baker f.) Vink)	New Caledonia, Prokoméo, NW of Canala, *LBT 204*, NO	Suh et al., 1993	AY004123	AY004140
Drimys winteri J.R. Forst. & G. Forst.	South America, Chile, (Berkeley B. G.) 45.307, NO	Suh et al., 1993	AY004126	AY004143
Exospermum stipitatum (Baill.) Tiegh. ex Morot (= *Zygogynum stipitatum* Baill.)	New Caledonia, Mt. Panié, *LBT 202*, NO	Suh et al., 1993	AY004121	AY004138
Pseudowintera axillaris (J.R. Forst. & G. Forst.) Dandy	New Zealand, N. Island, Akatarawa, *LBT 300*, NO	Suh et al., 1993	AY004124	AY004141
Pseudowintera colorata (Raoul) Dandy	New Zealand, N. Island, Akatarawa, *LBT 301*, NO	Suh et al., 1993	AY004125	AY004142
Takhtajania perrieri (Capuron) Baranova & J.-F. Leroy	Madagascar, Anjahanaribe-Sud Special Reserve, *Rakotomalaza et al. 1342*, MO	This paper	AY004129	AY004146
Tasmannia insipida R.Br. ex DC.	Australia, Queensland, Atherton, *LBT 108*, NO	Suh et al., 1993	AY004127	AY004144
Tasmannia lanceolata (Poir.) A.C. Smith	Australia, (Berkeley B. G.) 60.0052, NO	Suh et al., 1993	AY004128	AY004145
Zygogynum acsmithii Vink	New Caledonia, Mts. near Lac Enhuit, *LBT 201*, NO	Suh et al., 1993	AY004122	AY004139
Zygogynum balansae Tiegh. (= *Zygogynum pomiferum* Baill. subsp. *balansae* (Tiegh.) Vink)	New Caledonia, Dzumac Mts., *LBT 203*, NO	Suh et al., 1993	AY004120	AY004137
Zygogynum bicolor Tiegh.	New Caledonia, Plateau de Dogny, *LBT 200*, NO	Suh et al., 1993	AY004118	AY004135
Canellaceae				
Canella winterana (L.) Gaertn.	South America, *LBT 124*, NO	Suh et al., 1992	L03844	AY004152
Capsicodendron dinisii (Schwacke) Occhioni	South America, *Butzke et al. 11521*, US	This paper	AY004132	AY004149
Cinnamodendron ekmanii Sleumer	Dominican Republic, Samaná Penninsula, *García & Veloz 6866*, SD	This paper	AY004133	AY004150
Cinnamosma madagascariensis Danguy	Madagascar, *Lowry 4991*, MO	This paper	AY004131	AY004148
Pleodendron macranthum (Baill.) Tiegh.	Puerto Rico, *Axelrod 10783*, MO	This paper	AY004134	AY004151
Warburgia salutaris (G. Bertol.) Chiov.	South Africa, Mpumalanga, *Goldblatt 11314*, MO	This paper	AY004130	AY004147

Nuclear ITS. The alignment of Winteraceae ITS sequences of Suh et al. (1993) was used as a guide to easily incorporate the *Takhtajania* ITS sequence. The paralogous copies (GenBank AY004111–AY004117) of the ITS region as determined by Buckler et al. (1997) were not included in the alignment.

Alignment of ITS sequences within Canellaceae also was easily determined by eye. Alignment of Canellaceae with Winteraceae sequences into a single data set required additional information to generate a plausible alignment. One region of ITS 1 and another in ITS 2 were unalignable across both families, though these regions were alignable

among families. These regions were treated as non-overlapping insertion/deletion (indel) events in the final data set. Inferred indel regions were included in all phylogenetic analyses with the resulting gaps treated as missing.

Putative secondary structures of ITS 2 were determined for each taxon employing the minimum free-energy program mFOLD (Zuker, 1989) using constraints previously described by Hershkovitz and Zimmer (1996). In their study, the putative secondary structure of *Canella* ITS 2 was determined, and this structure was used as a guide to determine structures for all remaining Canellaceae.

Chloroplast trn*L-F*. The *trn*L-F spacer regions were easily aligned by eye within and between both families, with the resulting indels treated identically to those in the ITS data set.

Combined data set. The aligned ITS and *trn*L-F spacer sequences were then combined into a single data set. Two partitions were defined (ITS and *trn*L-F) and subjected to the data partition-homogeneity test (Farris et al., 1995). A thousand replicates were performed and the resulting P-value was used to determine if using the combined data set for phylogenetic reconstruction would be appropriate.

In summary, three data sets were examined: (1) the ITS data set consisting of the ITS 1, 5.8S, and ITS 2 regions; (2) the *trn*L-F data set consisting of a group I intron, the 5′-*trn*L exon, a non-coding intergenic spacer region, and partial sequence of *trn*F; and (3) a combined data set consisting of both the ITS and *trn*L-F regions.

PHYLOGENETIC ANALYSES

All phylogenetic reconstruction analyses were conducted using version 4.0b1 or 4.0b2 of PAUP* (Swofford, 1998).

Maximum parsimony. For each data set, phylogenetic reconstruction under maximum parsimony (MP) was conducted by utilizing the Branch and Bound search option in PAUP* with TBR branch-swapping, MULPARS, and ACCTRAN options active. Characters were assigned equal weights at all nucleotide positions (Fitch, 1971). Robustness of cladistic linkages was evaluated with 1000 bootstrap replicates (Felsenstein, 1985; Sanderson, 1989). Decay values (Bremer, 1988; Donoghue et al., 1992) for each node were calculated using the TOPOLOGICAL CONSTRAINTS option in PAUP*.

Maximum likelihood. The strategy employed utilized the likelihood-ratio test statistic (Felsenstein, 1981; Goldman, 1993; Yang et al., 1995) to determine the best model of DNA substitution for each of the three data sets described above. The models considered include the general time-reversible model (GTR, equals REV of Yang, 1994a), Hasegawa et al. (1985; denoted HKY85), Kimura (1980; denoted K2P), and Jukes and Cantor (1969; denoted JC69). Equations for calculating likelihoods under these DNA substitution models are given in Swofford et al. (1996). An iterative procedure to first evaluate models and optimize model parameters was used on an initial set of trees generated from the maximum parsimony analysis.

For each model of DNA substitution, models that incorporate rate variation across sites were explored. These included equal rates assumed across all sites, a proportion of sites assumed to be invariable with equal rates assumed across variable sites ("I" [Hasegawa et al., 1985]), all sites assumed to follow a discrete approximation of the gamma distribution ("Γ" [Yang, 1994b]), and some sites assumed to be invariable with gamma distributed rates at variable sites ("I + Γ" [Gu et al., 1995; Waddell & Penny, 1996]).

For the combined data set, a set of models was tested which explored the possibility that the relative rates between the two molecules differed significantly. This class of models assigns sites to classes and then estimates the relative rate for each class separately (Felsenstein, 1991; Goldman & Yang, 1994). Sites were assigned to two classes (ITS and *trn*L-F). This model is denoted as "+ SS."

For each data set, a maximum likelihood heuristic search with TBR branch swapping was then performed using parameters estimated from the tree with the best likelihood score under the best model. If the resulting tree was different in topology from that of the original tree, the resulting tree was used to further optimize the model parameters. With parameters re-optimized, a heuristic search of ten repetitions of random taxon addition and TBR branch swapping was performed using fully defined model parameters. Bootstrap methods (Felsenstein, 1985; Sanderson, 1989) with 1000 replicates were performed to estimate robustness of nodal support.

RESULTS

The combined data set used in this study can be found at TreeBASE (http://www.herbaria.harvard.edu/treebase/).

SEQUENCE ALIGNMENT

Nuclear ITS. ITS sequence alignment proved to be a relatively simple task within both Winteraceae and Canellaceae. With the exception of two regions, one in ITS 1 (bp 1143–1163) and the other in ITS

Table 2. List of the insertion/deletion (indel) events for the *trn*L-F/ITS combined data set. Character name (A–X for *trn*L-F; 1–115 for ITS), position information (character number in the combined data set), indel length (bp), character type (I = parsimony-informative, U = uninformative, H = homoplastic when mapped on Figs. 1 and 2a), and the species where indels were found are noted. Bp = *Belliolum pancheri*, Bc = *Bubbia comptonii*, Dw = *Drimys winteri*, Es = *Exospermum stipitatum*, Pa = *Pseudowintera axillaris*, Pc = *Pseudowintera colorata*, Tp = *Takhtajania perrieri*, Ti = *Tasmannia insipida*, Tl = *Tasmannia lanceolata*, Za = *Zygogynum acsmithii*, Zba = *Zygogynum balansae*, Zbi = *Zygogynum bicolor*, Cw = *Canella winterana*, Cd = *Capsicodendron dinisii*, Ce = *Cinnamodendron ekmanii*, Cm = *Cinnamosma madagascariensis*, Pm = *Pleodendron macranthum*, Ws = *Warburgia salutaris*.

Character	Position in combined data set	Length (bp)	I/U/H	Clade
***trn*L-F**				
A	114	1	I	Winteraceae/Canellaceae
B	179–181	3	I	Winteraceae/Canellaceae
C	207–212	6	U	Tp
D	247–255	9	U	Cw
E	252–256	5	I	Winteraceae/Canellaceae
F	257–264	8	U	Ws
G	282–286	4	U	Cm
H	317	1	U	Cw
I	319–331	13	U	Cw
J	323–331	9	U	Tp
K (a, b, c)	359–364	1	H	a = (Ti, Tl, Tp), b = (Tl, Tp), c = Tl
L	379–384	6	I	Dw, Pc, Pa, Bc, Bp, Es, Za, Zba, Zbi
M	385–388	4	U	Cd
N	414–418	5	I	Winteraceae/Canellaceae
O	586–591	6	H	Run of Adenines (see text)
P	590–694	105	I	Winteraceae/Canellaceae
Q	633	1	U	Dw
R	634	1	U	Tp
S	706	1	I	Winteraceae/Canellaceae
T	710	1	I	Winteraceae/Canellaceae
U	717–718	2	I	Winteraceae/Canellaceae
V	740–750	11	U	Ws
W	772	1	U	Ti
X	897	1	H	Cd, Cm, Ws
ITS-1				
1	993	1	I	Winteraceae/Canellaceae
2	997	1	I	Bc, Bp, Es, Za, Zba, Zbi
3	998–999	2	I	Cd, Ce, Cm, Pm, Ws
4	1000	1	U	Cd
5	1003–1013	11	U	Cd
6	1002	1	I	Pa, Pc
7	1003	1	U	Tp
8	1008	1	I	Winteraceae/Canellaceae
9	1009	1	U	Cw
10	1027–1028	2	I	Winteraceae/Canellaceae
11	1040	1	U	Dw
12	1041	1	I	Winteraceae/Canellaceae
13	1042	1	H	Winteraceae, Cd
14	1045	1	I	Cd, Cm, Ws
15	1048–1049	2	H	Canellaceae, Dw
16	1050–1051	2	I	Winteraceae/Canellaceae
17	1052	1	I	Cd, Cm, Ws
18	1053–1054	2	U	Ws
19	1058	1	H	Canellaceae, Dw
20	1059	1	H	Cd, Dw

Table 2. Continued.

Character	Position in combined data set	Length (bp)	I/U/H	Clade
21	1060–1061	2	U	Dw
22	1068	1	U	Cw
23	1076–1077	2	U	Cw
24	1081–1082	2	I	Tl, Ti
25	1083–1084	2	I	Winteraceae/Canellaceae
26	1090	1	H	Winteraceae, Cm, Pm
27	1091	1	H	Ce, Cw, Pm
28	1094–1106	13	U	Cd
29	1194–1097	4	U	Ws
30	1198–1104	7	H	Ce, Cw, Pm, Ws
31	1105–1106	2	U	Pm
32	1112–1113	2	U	Dw
33	1114	1	I	Winteraceae, Cw
34	1115	1	I	Winteraceae/Canellaceae
35	1116–1118	3	I	Winteraceae, Cw
36	1119–1122	4	I	Winteraceae/Canellaceae
37	1128	1	U	Cw
38	1135	1	I	Winteraceae/Canellaceae
39	1141–1142	2	H	Winteraceae, Cd, Cw
40	1143–1146	4	I	Winteraceae/Canellaceae
41	1147–1163	17	I	Winteraceae/Canellaceae
42	1148–1149	2	U	Cd
43	1158	1	I	Cm, Ws
44	1176	1	U	Cw
45	1185–1188	4	I	Winteraceae/Canellaceae
46	1199	1	U	Dw
47	1206–1225	20	I	Winteraceae/Canellaceae
48	1209–1212	4	I	Ce, Pm
49	1223–1225	3	I	Ce, Pm
50	1234	1	I	Winteraceae/Canellaceae
51	1244	1	U	Cw
52	1245	1	I	Winteraceae/Canellaceae
53	1262–1277	16	I	Winteraceae/Canellaceae
54	1262–1263	2	I	Ce, Pm
55	1272	1	I	Cd, Cm, Ws
56	1273	1	I	Ce, Pm
57	1283–1284	2	H	Ce, Cw, Pm
58	1287	1	H	Cd, Cm, Cw, Ws
59	1292–1293	2	I	Bc, Bp, Es, Za, Zba, Zbi
60	1299	1	H	Cm, Pm
61	1308–1326	19	I	Winteraceae/Canellaceae
62	1311	1	I	Bp, Es, Za, Zba, Zbi
63	1312–1313	2	H	Bc, Pa, Ti, Tl, Tp
64	1314–1316	3	U	Tp
65	1325	1	H	Winteraceae, Ws
66	1326	1	H	Cd, Cm, Cw
67	1331	1	U	Cw
5.8S				
68	1487	1	U	Cw
ITS-2				
69	1498	1	H	Pa, Pc, Bc, Es, Za, Zba, Zbi
70	1504	1	I	Winteraceae/Canellaceae
71	1511–1513	3	I	Winteraceae/Canellaceae
72	1514	1	H	Cd, Ce, Cm, Pm
73	1523	1	H	Cd, Ce, Cw, Pm, Ws

Table 2. Continued.

Character	Position in combined data set	Length (bp)	I/U/H	Clade
74	1524–1525	2	H	Bc, Bp, Cm, Es, Ti, Tl, Za, Zba, Zbi
75	1526	1	I	Bc, Bp, Cm, Es, Za, Zba, Zbi
76	1527	1	I	Winteraceae/Canellaceae
77	1534	1	U	Dw
78	1535–1539	5	I	Winteraceae/Canellaceae
79	1540	1	H	Canellaceae, Bc
80	1543–1544	2	U	Cw
81	1545	1	I	Winteraceae/Canellaceae
82	1553–1554	2	I	Pa, Pc
83	1573–1575	3	I	Winteraceae/Canellaceae
84	1595–1596	2	I	Bp, Zb
85	1603	1	H	Bc, Bp, Ce, Cm, Es, Pm, Ti, Tl, Za, Zba, Zbi
86	1604	1	I	Bc, Bp, Cm, Es, Ti, Tl, Za, Zba, Zbi
87	1612–1618	7	U	Cw
88	1651	1	U	Tp
89	1652–1653	2	I	Winteraceae/Canellaceae
90	1659	1	I	Winteraceae/Canellaceae
91	1662	1	U	Pm
92	1663	1	I	Winteraceae/Canellaceae
93	1672	1	H	Winteraceae, Cm
94	1676	1	I	Cd, Cm, Ws
95	1683–1688	6	U	Ce
96	1690–1691	1	I	Pa, Pc
97	1692–1706	15	I	Winteraceae/Canellaceae
98	1692–1695	4	U	Tl
99	1700–1701	2	U	Tp
100	1707–1722	16	I	Winteraceae/Canellaceae
101	1709	1	U	Ce
102	1713–1716	4	U	Cw
103	1717–1718	2	H	Cw, Pm
104	1719–1720	2	U	Ce
105	1726–1727	2	U	Tl
106	1730	1	I	Winteraceae/Canellaceae
107	1737	1	U	Pc
108	1738	1	U	Pa
109	1736–1748	10	U	Pc
110	1750–1759	11	I	Winteraceae/Canellaceae
111	1757–1758	2	U	Tp
112	1763–1764	2	H	Cw, Cd, Pm
113	1765–1774	10	I	Cd, Cm, Ws
114	1775	1	H	Cd, cm
115	1776–1778	3	U	Cd

2 (bp 1690–1722), the alignment between families was also relatively straightforward. The two problematic regions were partitioned equally between the two families as non-overlapping separate indels (e.g., positions 1143–1153 were coded as gaps in Winteraceae and positions 1154–1163 were coded as gaps in Canellaceae). The effect of this was to reduce the probability of incorrect homology assessment between families while retaining potential phylogenetic information within each family.

Putative secondary structures for ITS 2 were determined for each sequence, and these structures were generally consistent with the final alignment (data not shown). An apparent contradiction between secondary structure and DNA alignment was found in the v5 region (see Hershkovitz & Zimmer

[1996] for details) and corresponds to bp 1704–1734. Although this region consistently formed a bulge in secondary structure analyses across all individuals, the base composition and sequence length clearly differed between families (11–15 bp with 46–60% G/C in Winteraceae and 7–16 bp with 75–85% G/C in Canellaceae). This result further supports treatment of this region as non-overlapping indel events. No known secondary structure exists for ITS 1; consequently, structural evidence supporting the alignment of ITS 1 could not be generated.

A total of 115 indels were identified in the ITS alignment, of which 53 (46%) were parsimony-informative, 38 (33%) were autapomorphic, and the remaining 24 (21%) were homoplastic. Table 2 presents these indels along with position information (character number in the data set), indel length, and the species where indels were found.

Chloroplast trn*L-F*. The *trn*L-F spacer regions were easily aligned by eye within and between families. Overall, a total of 24 indels (Table 2: A–X) were identified in the alignment. Of them, 9 (37.5%) were parsimony-informative, 12 (50.0%) were autapomorphic, and the remaining 3 (12.5%) were homoplastic. Especially noteworthy was indel P (Table 2) with 105 bases present in the Winteraceae, including *Takhtajania*, but this indel was not present in the Canellaceae.

Combined data set. Two partitions of the combined alignment were defined, (1) *trn*L-F (bp 1–987) and (2) ITS (bp 988–1778). These partitions were tested for congruence with the data partition-homogeneity test (1000 replicates) and yielded a P value of 0.367. This result fails to reject the null hypothesis of congruence between the two partitions. Therefore, all phylogenetic analyses were performed on both the individual and combined data sets.

PATTERNS OF SEQUENCE EVOLUTION

The aligned combined data set was 1778 characters long. Positions 1 to 987 represented the *trn*L-F chloroplast sequence region; positions 988 to 1778 represented the ITS nuclear ribosomal sequence region including the 5.8S coding region. Unlike the situation for certain genera of Winteraceae (*Belliolum, Bubbia, Exospermum*, and *Zygogynum*), no polymorphisms were detected in the ITS sequence for *Takhtajania* or any member of the Canellaceae.

The ITS 1 sequences in Winteraceae ranged from 238 bp in *Takhtajania* to 252 bp in *Drimys*. In Canellaceae, ITS 1 sequences ranged from 244 bp in *Capsicodendron* to 274 bp in *Pleodendron*. The 5.8S coding region was 164 bp in all individuals of both families, although it was only 163 bp long in *Canella*. ITS 2 sequences in Winteraceae ranged from 215 bp in *Pseudowintera colorata* to 228 bp in *Belliolum*, with *Takhtajania* having a 222 bp sequence. Canellaceae ITS 2 sequences ranged from 187 bp in *Cinnamodendron* to 211 bp in *Canella*.

The Winteraceae chloroplast group I intron sequences ranged from 490 bp in *Tasmannia insipida* to 508 bp in *Takhtajania perrieri*. In Canellaceae, these sequences ranged from 477 bp in *Canella winterana* to 495 bp in *Cinnamosma madagascariensis*. The 5′ *trn*L exon sequences were 50 bp in all individuals of both families. The intergenic spacer sequences in Winteraceae ranged from 360 bp in *Drimys* to 368 bp in both *Tasmannia insipida* and *Takhtajania perrieri*. All Canellaceae sequences were markedly shorter, ranging from 247 bp in *Warburgia salutaris* to 259 bp in *Canella winterana, Cinnamodendron ekmanii*, and *Pleodendron macranthum*. The 40 bp partial *trn*F exon sequences were identical in length in all individuals of both families.

PHYLOGENETIC ANALYSES

Maximum parsimony

Nuclear ITS. Using the Branch and Bound search procedure in PAUP*, with equal weighting of positions and Canellaceae selected as the outgroup, a single most parsimonious tree of 489 steps (366 steps excluding uninformative characters) was obtained with the ITS data set. The consistency index (Kluge & Farris, 1969) was 0.8221 (0.7623 excluding uninformative characters) and retention index (Farris, 1989) was 0.8793. Overall, 317 variable nucleotide positions were counted; of these, 206 (or 26% of the 791 total characters) provided parsimony-informative changes.

To assess robustness of these results, the bootstrap procedure (1000 replicates) and decay analyses were run in PAUP* (TOPOLOGICAL CONSTRAINTS were used to determine decay values for each node). These results are shown in Figure 1.

A polytomy including *Belliolum, Exospermum*, and *Zygogynum* (*Zygogynum* s.l.) was supported as monophyletic and sister to *Bubbia* by a 97% bootstrap value and decay index of + 4. The *Bubbia* + *Zygogynum* s.l. clade was monophyletic and sister to *Pseudowintera* (represented by two species, monophyletic in 99% of the bootstrap iterations [decay = + 5]), supported by a 100% bootstrap value (decay = + 10). *Drimys* was sister to this

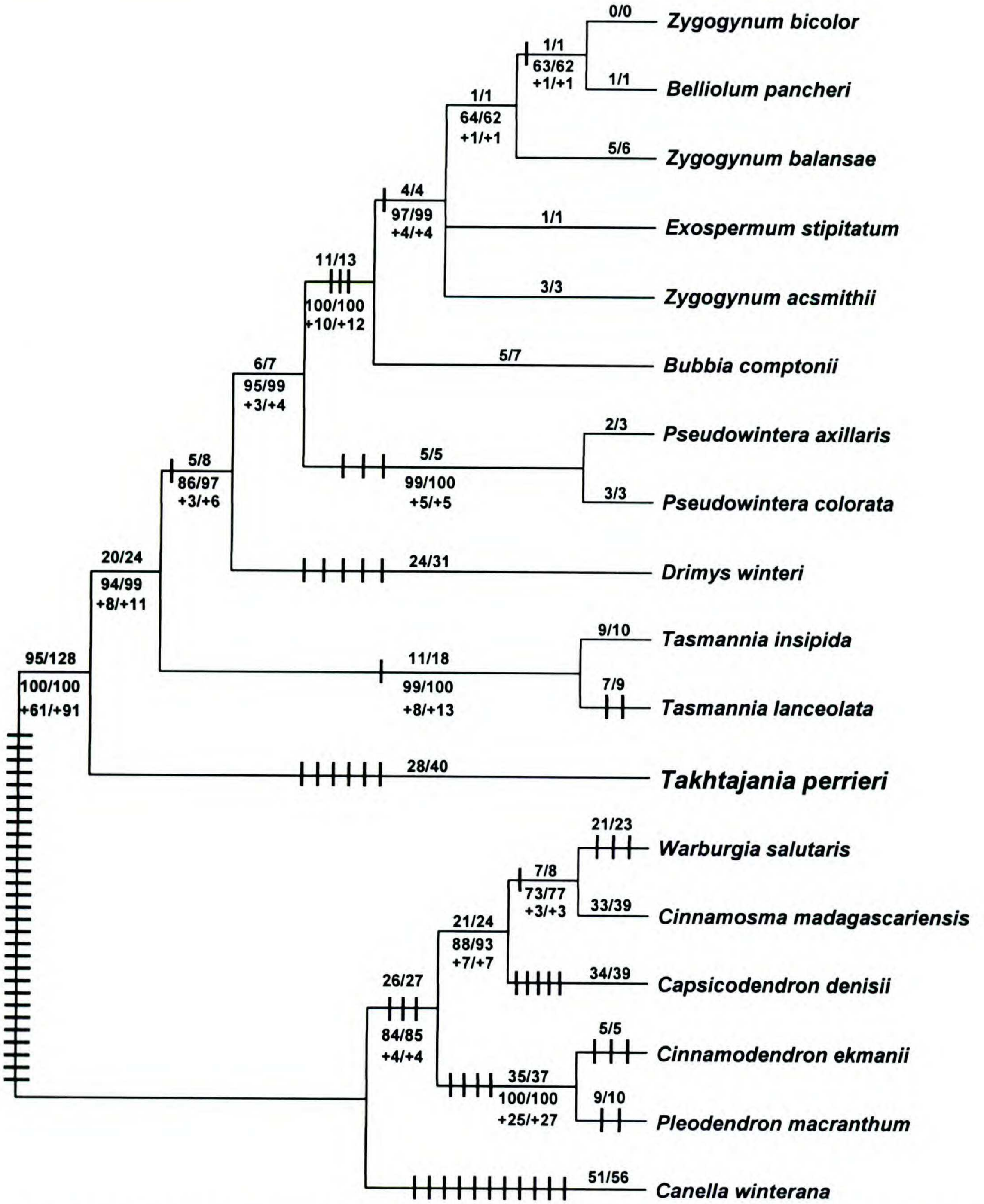

Figure 1. Single most parsimonious tree generated both from the ITS data set alone and the combined ITS/*trn*L-F data set. Tree length for ITS tree = 489 steps (366 steps excluding uninformative characters), consistency index = 0.8221 (0.7623 excluding uninformative characters), and retention index = 0.8793. Length of combined tree = 593 steps (434 steps excluding uninformative characters), consistency index = 0.8381 (0.7788 excluding uninformative characters), and retention index = 0.8994. Numbers to the left of the slash represent those generated from ITS data alone, while numbers to the right of the slash represent those generated from the combined data set. Branch lengths shown above branches, bootstrap values (1000 replicates) and decay values shown below branches, respectively. Dark bars represent non-homoplastic insertion/deletion events inferred from the ITS alignment (see Table 2).

assemblage in 95% of the bootstrap iterations (decay = + 3). *Tasmannia* (represented by two species) was monophyletic with 99% bootstrap support (decay = + 8) and sister to the *Drimys* + *Pseudowintera* + *Bubbia* + *Zygogynum* s.l. clade in 86% of the bootstrap iterations (decay = + 3). *Takhtajania* was found sister to the remainder of the Winteraceae with a bootstrap value of 94% (decay = + 8).

In the Canellaceae, *Cinnamosma* and *Warburgia* formed a clade sister to *Capsicodendron* in 73% of the bootstrap replicates, with a decay index of + 3. *Cinnamodendron* and *Pleodendron* formed a well-supported clade (100% of the bootstrap replicates, decay = + 25) sister to the *Capsicodendron* + *Cinnamosma* + *Warburgia* clade in 88% of the replicates (decay = + 7). Finally, *Canella* was found sister to the remainder of the family with 84% bootstrap support (decay = + 4).

Chloroplast trn*L-F*. Using the Branch and Bound search procedure in PAUP*, with equal weighting of positions and Canellaceae selected as the outgroup, two most parsimonious trees of 103 steps (67 steps excluding uninformative characters) were obtained with the *trn*L-F data set. The consistency index was 0.9223 (0.8806 excluding uninformative characters), and the retention index was 0.9657. Overall, 90 variable nucleotide positions were counted; of these, 55 (or 5.6% of the 987 total characters) provided parsimony-informative changes. These two trees are presented in Figure 2 along with bootstrap values and decay indices.

With the exception of alternative equally parsimonious basal relationships, the topology generated by the *trn*L-F sequence data for Winteraceae was not in conflict with the tree generated by the ITS data, albeit there were both lower resolution and lower bootstrap support values. The polytomy of *Belliolum*, *Exospermum*, and *Zygogynum* (*Zygogynum* s.l.) expanded to include *Bubbia* (78% bootstrap and decay = + 2). The two species of *Pseudowintera* were unresolved with respect to each other and to the *Bubbia* + *Zygogynum* s.l. polytomy, and together these were only weakly supported as monophyletic and sister to *Drimys* (69% bootstrap and decay = + 1). The two species of *Tasmannia* were strongly supported as sister taxa in 98% of the bootstrap iterations (decay = + 5). Finally, the *trn*L-F sequence data could not resolve basal relationships within the family; both *Takhtajania* (Fig. 2a) and *Tasmannia* (Fig. 2b) as sister to the remaining Winteraceae were equally parsimonious.

Within Canellaceae, except for a strong sister relationship between *Cinnamodendron* and *Pleodendron* (88% bootstrap and decay = + 2), the topology generated by *trn*L-F data resulted in much lower levels of bootstrap support, and, moreover, suggested a fundamental split between Old World and New World lineages. Thus, rather than being sister to the remaining Canellaceae as suggested by the ITS-based phylogeny, *Canella* was nested within a weakly supported clade of New World genera. To force such a division of the family into Old World and New World clades requires an additional 11 steps in the ITS-based phylogeny.

Combined data set. Using the Branch and Bound search procedure in PAUP*, with equal weighting of positions and all Canellaceae genera selected as outgroups, a single most parsimonious tree of 593 steps (434 steps excluding uninformative characters) was obtained with the ITS/*trn*L-F combined data set. The consistency index was 0.8381 (0.7788 excluding uninformative characters) and the retention index was 0.8994. The topology derived from this combined data set was identical to that generated from the ITS data alone. The branch lengths, bootstrap values, and decay indices are presented in Figure 1.

Overall, with the addition of the *trn*L-F data, bootstrap values either remained similar or increased in comparison with the ITS-based phylogeny. Within the Winteraceae, the most notable increases resulted in stronger support for basal relationships in the family. The position of *Takhtajania* as basal and sister to the rest of the family was further strengthened (ITS = 94% bootstrap and + 8 decay index vs. combined data = 99% bootstrap and + 11 decay index), as was the position of *Tasmannia* as the next branch within the family. The combined data set provided especially strong support for the inclusion of *Drimys* in the clade consisting of all other members of the family and sister to *Tasmannia* (ITS = 86% bootstrap and + 3 decay index vs. combined data = 97% bootstrap and + 6 decay index). Within the Canellaceae, bootstrap support also remained similar or increased only slightly with the addition of the *trn*L-F data (Fig. 1).

Maximum likelihood

Nuclear ITS. For the ITS data set, the best model of DNA substitution was the general time-reversible model with a proportion of sites assumed to be invariable and equal rates assumed across variable sites (GTR + I). With this model, parameters were fully optimized using the tree generated from the parsimony search (Fig. 1). With the model parameters defined, a heuristic search was con-

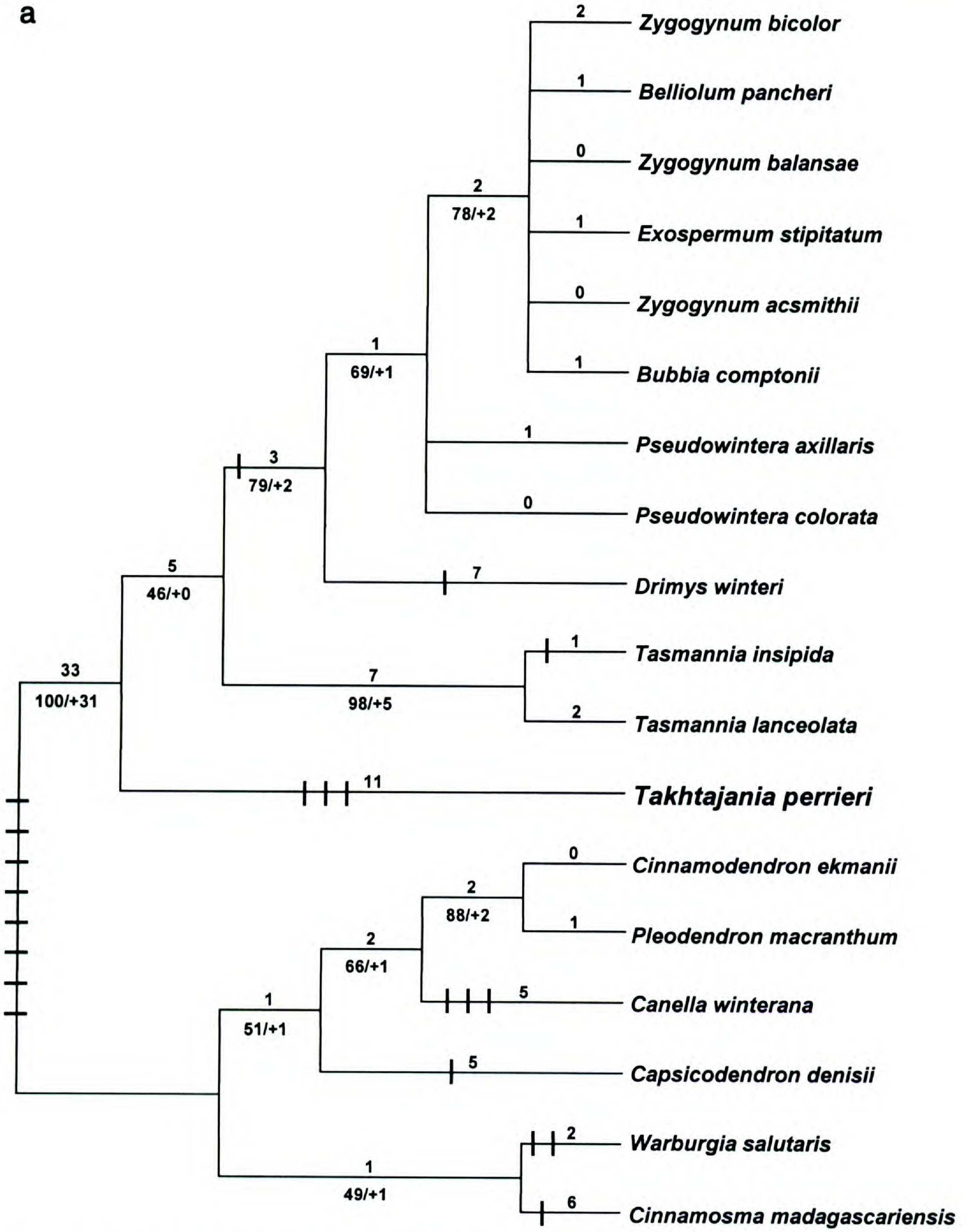

Figure 2a, b. Two most parsimonious trees generated using *trn*L-F data. Tree length = 103 steps (67 steps excluding uninformative characters), consistency index = 0.9223 (0.8806 excluding uninformative characters), and retention index = 0.9657. Branch lengths shown above branches, bootstrap values (1000 replicates) and decay values shown below branches, respectively. Dark bars (Fig. 2a) represent non-homoplastic insertion/deletion events inferred from the *trn*L-F alignment (see Table 2).

b

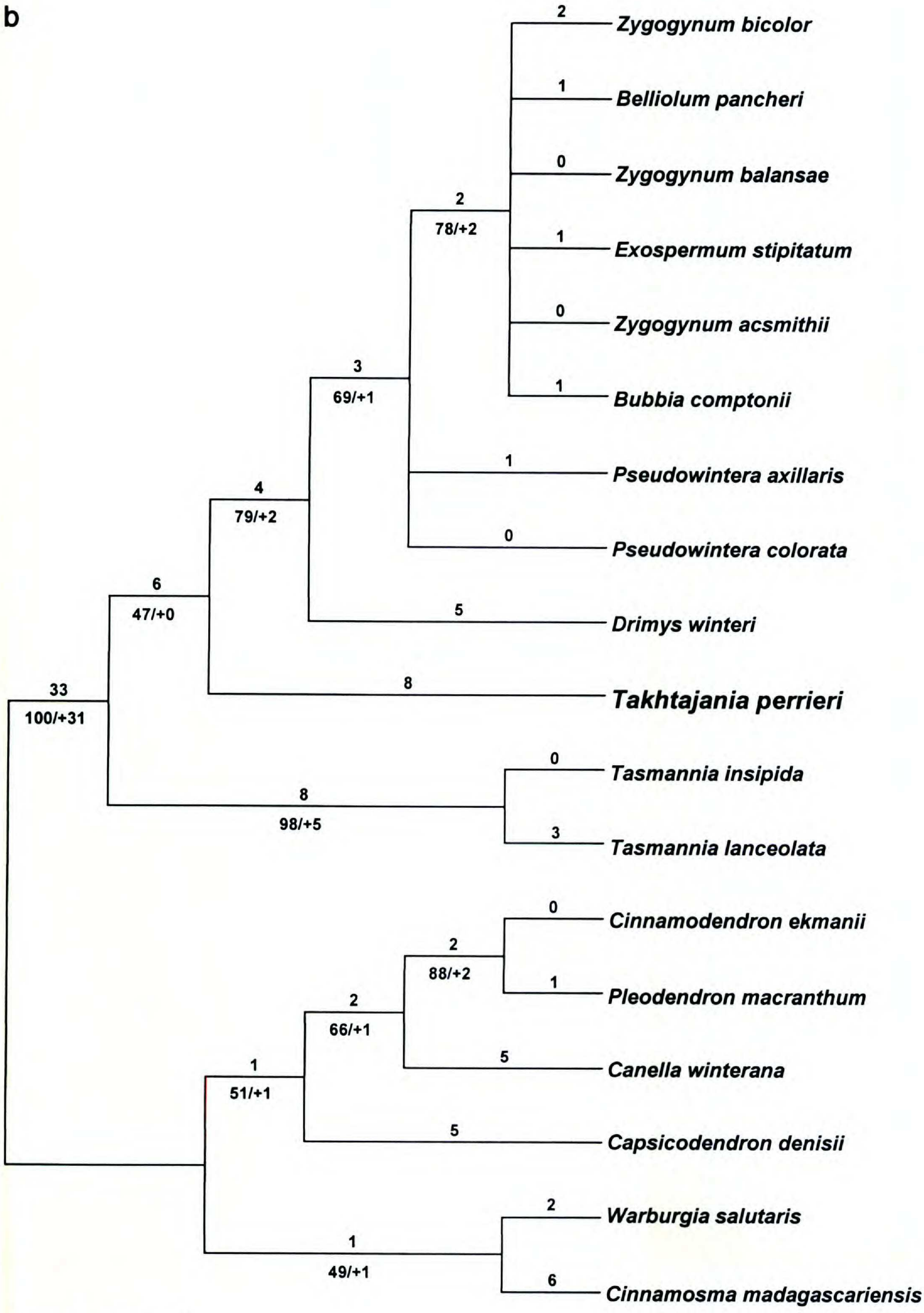

Figure 2. Continued.

a

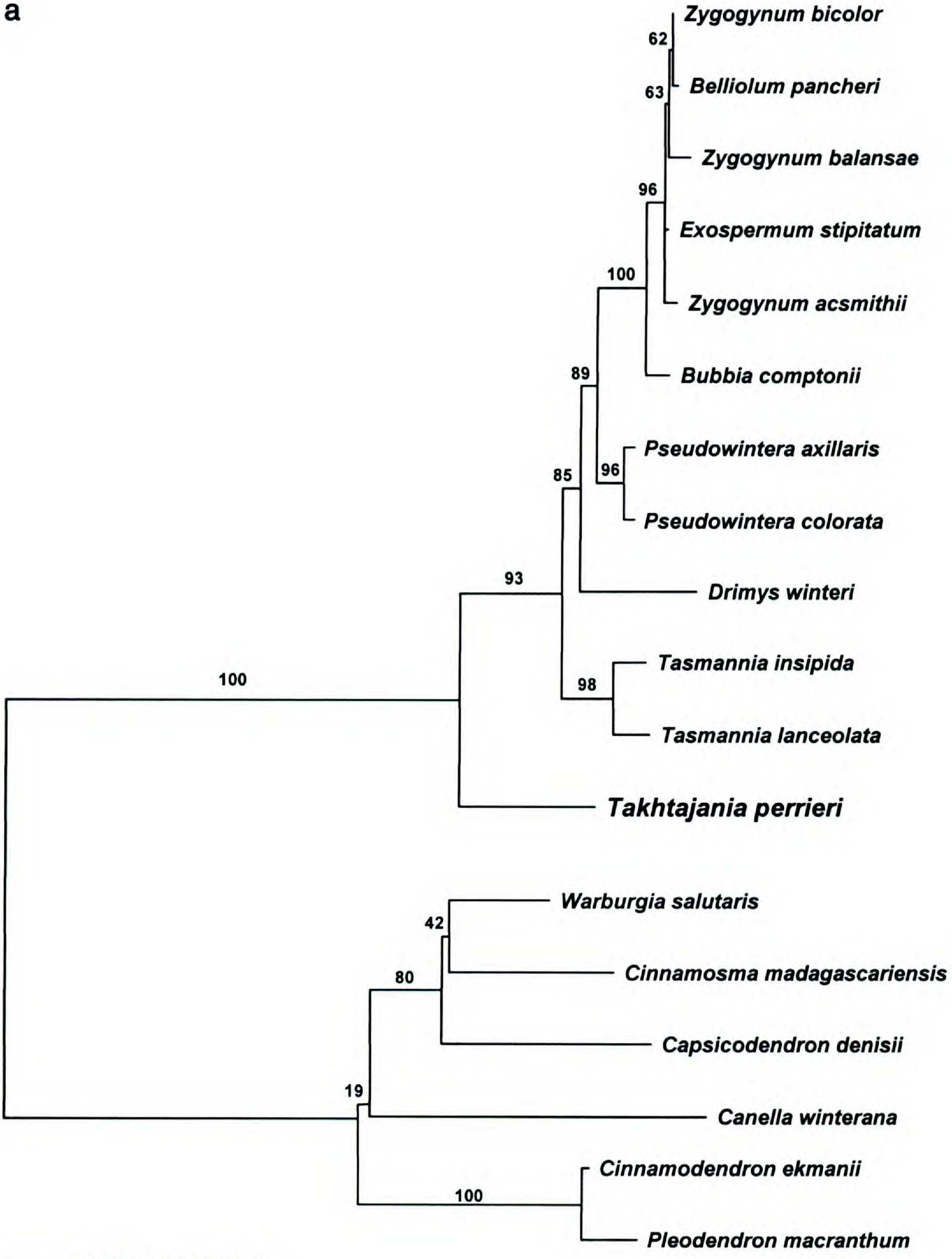

Figure 3a. One of four most likely phylograms generated with ITS data (−ln = 3371.16) with bootstrap values (1000 replicates) above branches.

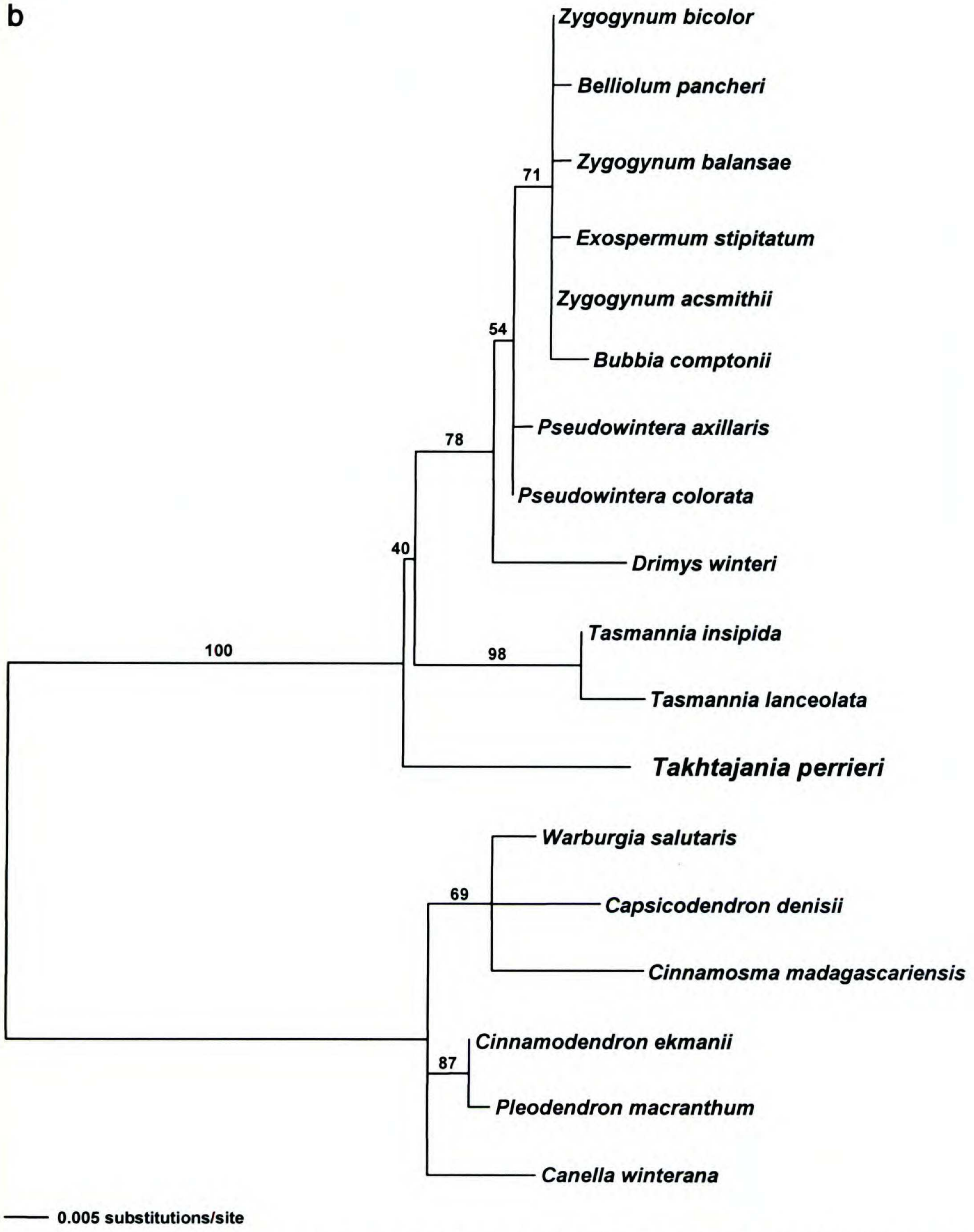

Figure 3b. Single most likely phylogram generated with *trn*L-F data (−ln = 1996.3473) with bootstrap values (1000 replicates) above branches. Figure 3c is on page 428.

ducted that resulted in three trees, one of which was identical in topology to the most parsimonious tree (Fig. 1). These three trees were used to further optimize model parameters. The resulting parameter values did not differ from those generated by using the single maximum parsimony tree. Thus, ten heuristic searches (with random taxon addition, multrees, and steepest descent active) were conducted using the parameters defined in the initial search. These ten random taxon addition searches generated a total of four equally likely trees (−ln = 3371.16), one of which is presented in Figure 3a.

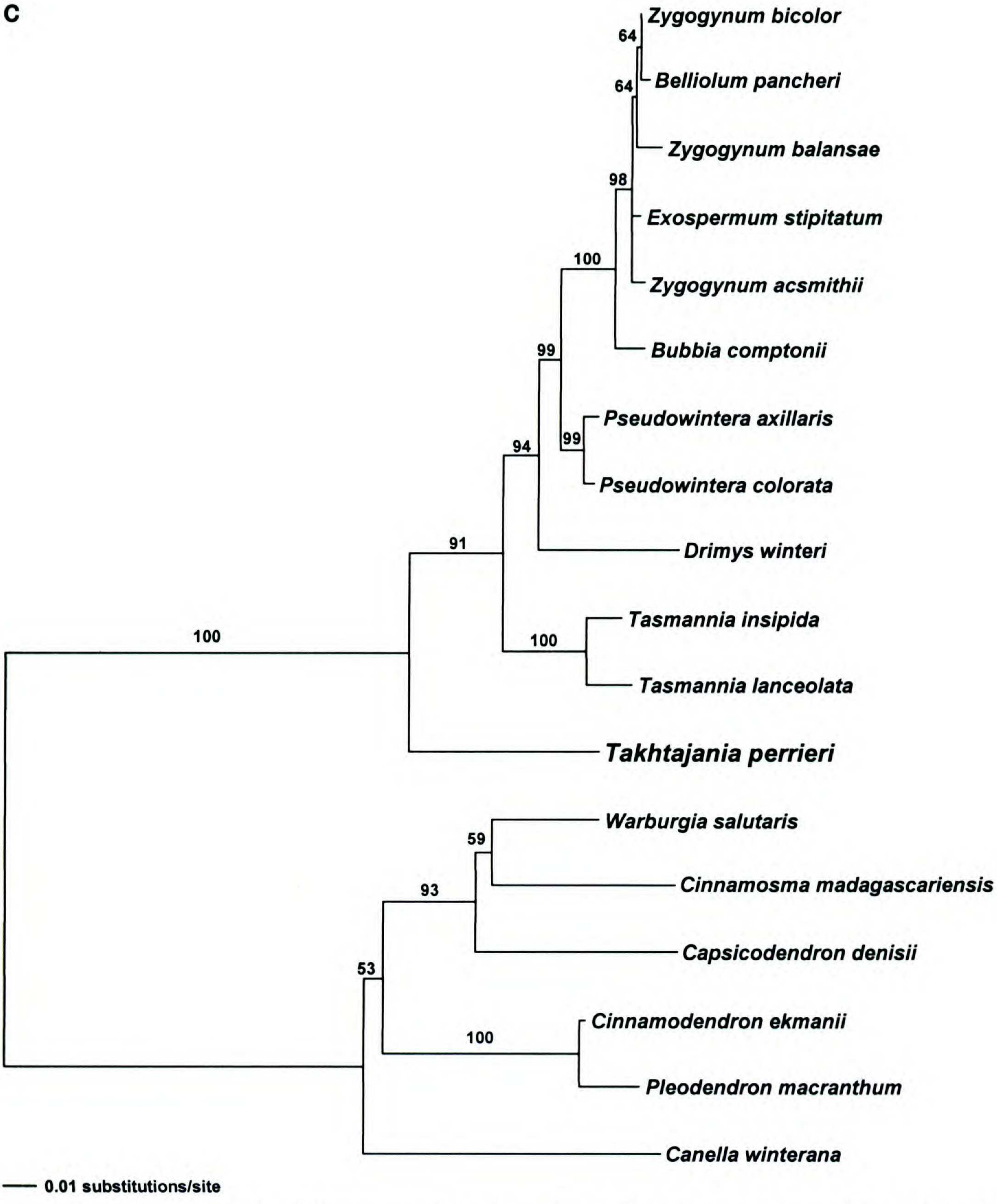

Figure 3c. Single most likely phylogram generated with combined ITS/*trn*L-F data set (ln = −5508.26165) with bootstrap values (1000 replicates) above branches.

In all four trees the relationships within Winteraceae were identical to those generated by the MP search, with *Takhtajania* sister to the remainder of the Winteraceae. The four trees differed in the position of *Canella* in the Canellaceae. Either *Canella* was sister to the *Warburgia* + *Cinnamosma* + *Capsicodendron* clade, or sister to the *Cinnamodendron* + *Pleodendron* clade, or sister to the remainder of the family, or, finally, in an unresolved polytomy with the three above-mentioned clades. Although *Canella* is included within a monophyletic Canellaceae in 100% of the bootstrap iterations, bootstrap values do not support a resolved phylogenetic position of *Canella* within the family.

Chloroplast trn*L-F*. For the *trn*L-F data set, the best model of DNA substitution was the HKY85

model with a proportion of sites assumed to be invariable and equal rates assumed across variable sites (HKY85 + I). With this model, parameters were fully optimized using the two trees generated from the parsimony search (Fig. 2a, b). With the model parameters defined, a heuristic search was conducted that resulted in a single tree identical in topology to one of the four most likely trees generated from the maximum likelihood ITS search (Fig. 3a). Using this tree, parameters were re-estimated and the resulting optimized values were used for ten heuristic searches (random taxon addition, multrees, and steepest descent active). The result of these searches generated a single most likely tree (−ln = 1996.3473), which is presented in Figure 3b along with bootstrap values for each node.

The single tree from the maximum likelihood search was identical to one of the two trees generated by the maximum parsimony analysis using *trn*L-F with one exception. The placement of *Canella* sister to *Pleodendron* and *Cinnamodendron* was not supported. Rather, *Capsicodendron*, *Cinnamosma*, and *Warburgia* were supported as an unresolved trichotomy in 69% of the bootstrap iterations. *Cinnamodendron* and *Pleodendron* were supported as sister taxa in 87% of the bootstrap iterations, and finally *Canella* was found in an unresolved trichotomy with the two above-mentioned clades in a monophyletic Canellaceae in 100% of the bootstrap replications. Within the Winteraceae an unresolved polytomy including *Belliolum*, *Bubbia*, *Exospermum*, and *Zygogynum* was found to be sister to the two species of *Pseudowintera* in 71% of the bootstrap replicates. In 78% of the bootstrap iterations, *Drimys* joined the above in a monophyletic clade, but was only weakly supported by a 54% bootstrap value as sister to them. There was less than 50% bootstrap support for the basal position of *Takhtajania* sister to the remainder of Winteraceae.

Combined data set. For the combined data set, the best model of DNA substitution was the general time-reversible model. Among site rate variation was accommodated for by dividing the data into two partitions (ITS and *trn*L-F), thus allowing among site rate variation to be estimated for each molecule separately (GTR + SS). With this model, parameters were optimized using the tree generated from the maximum parsimony search (Fig. 1). With the model parameters defined, a heuristic search was conducted that resulted in a single most likely tree identical in topology to the most parsimonious tree using the same data set (Fig. 1). This tree was used to further optimize model parameters. The resulting parameter values did not differ, and thus ten heuristic searches (random taxon addition, multrees, and steepest descent active) were conducted using the parameters defined in the initial search. These ten random taxon addition searches generated a single most likely tree (ln = −5508.26165). The bootstrap values are presented in Figure 3c.

In general, with the exception of support for *Canella* as basal within Canellaceae, the bootstrap values were consistent with those generated by the parsimony analyses of the combined data set. In the parsimony analysis, *Canella* was sister to the remainder of the Canellaceae in 85% of bootstrap iterations, whereas the likelihood analyses recovered this relationship in only 53% of the bootstrap replicates.

Discussion

In the initial molecular phylogenetic study of Winteraceae, Suh et al. (1993) found it difficult to align the ITS region with certainty for several exemplar outgroup species. The present study increased generic sampling in Canellaceae from a single exemplar (*Canella*) to a representative of all genera, which greatly facilitated homology assessment between the two families. In addition, the more slowly evolving *trn*L-F chloroplast regions, for which no difficulty in alignment was encountered, were investigated. With Canellaceae as the outgroup, the rooting of Winteraceae is no longer ambiguous. Phylogenetic analyses of ITS and combined ITS/*trn*L-F DNA sequences provided a well-resolved molecular phylogeny for the Winteraceae that placed *Takhtajania* sister to the remaining genera in the family. Analysis of *trn*L-F sequence data alone failed to resolve the basal topology within the family, with either *Takhtajania* or *Tasmannia* equally parsimonious as the basal branch. Nevertheless, the addition of *trn*L-F sequences to a combined ITS/*trn*L-F analysis resulted in increased support values for the same topology derived from ITS sequences alone. Maximum likelihood analyses of each of the three data sets mirrored those obtained through maximum parsimony.

ITS and *trn*L-F sequences provided generally weaker support for phylogenetic relationships within Canellaceae. Although parsimony analysis of ITS data revealed moderate support for a basal position of *Canella* sister to the remaining members of the family, parsimony analysis of *trn*L-F data weakly suggested a fundamental New World/Old World split in Canellaceae. Parsimony analysis of the combined molecular data set resulted in a topology identical to that generated by ITS data alone, with only slight increases in bootstrap support and iden-

tical decay values. Maximum likelihood analyses of each of the three data sets further demonstrated the poor resolution these molecules provide for estimating basal relationships in Canellaceae. Nevertheless, ITS, combined ITS/*trn*L-F, and all maximum likelihood analyses strongly suggest that the southeastern Brazilian endemic *Capsicodendron* forms a clade with the African/Malagasy genera *Warburgia* and *Cinnamosma*.

The alignment of ITS and *trn*L-F sequences for the 12 species of Winteraceae and 6 species of Canellaceae revealed numerous probable insertion-deletion events. When mapped on the trees, these indels furnished additional support for certain clades. A total of 37 indels (29 in ITS [Fig. 1] and 8 in *trn*L-F [Fig. 2a]) support the separation of Winteraceae from Canellaceae. Three ITS indels support the monophyly of *Pseudowintera*, and three different ITS indels support the monophyly of the *Zygogynum* s.l. (*Bubbia*, *Belliolum*, *Exospermum*, and *Zygogynum*) assemblage. One indel each in both nuclear and chloroplast sequences (Figs. 1 and 2a, respectively) support inclusion of *Drimys* in the *Zygogynum* s.l. + *Pseudowintera* clade, and thus provide additional evidence of its distinctness from *Tasmannia*. Within Canellaceae, four ITS indels support the sister relationship between *Cinnamodendron* and *Pleodendron*, whereas a single ITS indel supports a sister relationship between African *Warburgia* and Malagasy *Cinnamosma*. The basal position of *Canella* in the family is further suggested by three ITS indels that support the monophyly of the remaining genera of Canellaceae. None of the indels found in the *trn*L-F Canellaceae sequences were phylogenetically informative. In total, 139 indels were scored overall and 114 (82%) were consistent with the trees generated from the sequence data. The remaining 25 were homoplastic and in some cases difficult to score. For example, in *trn*L-F sequences, long adenine runs occurred at varying numbers in closely related taxa. The length of these runs was not consistent when mapped on the tree, and delimiting homology was difficult when scoring this region. No indels were detected in either ITS or *trn*L-F that bear on the position of *Takhtajania* as sister to the remainder of Winteraceae.

Zygogynum s.l., *Pseudowintera*, and *Takhtajania* exhibit a short, early-rupturing involucrum (the congenitally fused two, or rarely three, outermost perianth parts, which could as well be considered the "calyx") that is persistent in fruit vs. the condition in both *Drimys* and *Tasmannia* where the ultimately caducous, late-rupturing involucrum becomes as long as the fully developed next inner tepal whorl, and therefore completely encloses the flower just prior to anthesis (Doust, 2000 this issue). A persistent, short calyx occurs in Canellaceae that is similar to the ruptured, persistent involucrum of basal *Takhtajania*, suggesting that *Pseudowintera* and *Zygogynum* s.l. have retained the ancestral plesiomorphic condition of an early-rupturing involucrum. In conjunction with the hypothesized phylogenetic positions of *Drimys* and *Tasmannia*, the differing ontogenetic patterns of tepal initiation and development exhibited by these two genera (Doust, 2000) imply that the late-rupturing, caducous involucrum has evolved twice independently. Similarly, deep red tepal color, present in *Canella* and other Canellaceae, as well as *Takhtajania* and many *Zygogynum* s.l. species, may be the ancestral condition, with the evolution of white tepals through the loss of pigmentation and/or the acquisition of an optical tapetum (Endress et al., 2000 this issue) occurring a number of times independently.

The historical biogeographical implications of *Takhtajania* in a basal position sister to the remaining genera of the Winteraceae have been discussed in the context of the distribution of fossil Winteraceae (Doyle, 2000 this issue) and ecophysiological constraints (Feild et al., 2000 this issue). It is reasonable to assume that the basal branch leading to *Takhtajania* became isolated in Madagascar after reaching there from continental Africa, and also that Madagascar may have played a prominent role in the migration of the family to Australasia and South America via Antarctica during the mid-Cretaceous. The presence of two different winteraceous pollen types in the Miocene of the southwestern Cape possibly referable to *Tasmannia* and the most advanced *Zygogynum* s.l. clade is more puzzling and problematic (Coetzee & Praglowski, 1988). With South Africa relatively isolated from both South America and Antarctica from the mid-Cretaceous onward (Smith et al., 1994), either all of the currently extant clades of Winteraceae had already evolved prior to that point in time, or Madagascar may have continued to serve as a conduit for migration of more advanced Winteraceae back into South Africa from Australasia. In comparison to the Southern Gondwanan pattern of radiation in Winteraceae, the preliminary phylogenetic hypothesis of infrafamilial relationships within Canellaceae suggests an "inverted" Northern Gondwanan biogeographical history. Basal clades of Canellaceae are centered in northern South America and the West Indies, with a more advanced clade exhibiting a southeastern Brazil/African–Malagasy split related to the middle Cretaceous sepa-

ration of South America and Africa (Goldblatt, 1993) and a subsequent dispersal event to Madagascar. Thus, the modern-day sympatric occurrence of the two Malagasy endemic genera *Cinnamosma* (Canellaceae) and *Takhtajania* (Winteraceae) may well be the end result of vastly divergent biogeographic histories.

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