Phylogenetic Inferences in Antennaria (Asteraceae: Gnaphalieae: Cassiniinae) Based on Sequences from Nuclear Ribosomal DNA Internal Transcribed Spacers (ITS)

Randall J. Bayer, Douglas E. Soltis, Pamela S. Soltis

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PHYLOGENETIC INFERENCES IN *ANTENNARIA* (ASTERACEAE: GNAPHALIEAE: CASSINIINAE) BASED ON SEQUENCES FROM NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACERS (ITS)[1]

RANDALL J. BAYER,[2,4] DOUGLAS E. SOLTIS,[3] AND PAMELA S. SOLTIS[3]

[2]Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada; and
[3]Department of Botany, Washington State University, Pullman, Washington 99164-4238

The phylogenetic relationships among sexually reproducing species of *Antennaria* (Asteraceae) are poorly understood. An earlier cladistic analysis based on morphology did not fully resolve the phylogeny of these taxa and therefore a different approach using molecular data was explored. The internal transcribed spacer regions (ITS-1 and ITS-2) of nuclear ribosomal DNA were sequenced for 30 species of *Antennaria* and one species from each of the outgroup genera *Anaphalis, Ewartia, Leontopodium,* and *Pseudognaphalium.* The ITS-1 sequence in *Antennaria* ranged from 253 to 260 base pairs (bp) in length, and the proportion of nucleotide differences between pairs of species of *Antennaria* ranged from 1 to 14%. For ITS-2, the divergence between pairs of species of *Antennaria* ranged from 0 to 8%. ITS-2 is shorter than ITS-1, ranging from 213 to 219 bp. Phylogenetic analysis indicates that, relative to the outgroups included, *Antennaria* is a well-supported monophyletic group. Based on the genera surveyed, *Leontopodium* appears to be the sister genus of *Antennaria.* The general topology of the molecular trees agrees with that based on previous morphological analyses and indicates that *Antennaria* is composed of six clades of equal rank, corresponding to the traditionally recognized informal groups, the Geyeriae, Argenteae, Arcuatae, Dimorphae, Pulcherrimae, and Catipes. Sequence and morphological data indicate that the Alpinae and Dioicae are unnatural, polyphyletic units that should be abandoned and redefined as the monophyletic Catipes group. Phylogenetic analysis of ITS sequences also suggests the dissociation of *A. stenophylla* from the Dimorphae, where it is traditionally placed, and its affiliation with the Argenteae, as well as the placement of *A. arcuata* in its own group.

Key words: *Antennaria*; Asteraceae; internal transcribed spacer regions (ITS); nuclear ribosomal DNA; phylogenetic analysis.

Antennaria Gaertner is a genus of dioecious, perennial herbs that is distributed throughout temperate to arctic regions of the northern hemisphere with three species occurring in the Andes of South America. The genus consists of 33 known sexual diploid/tetraploid species and at least five large polymorphic polyploid agamic complexes (Bayer, 1990a). *Antennaria* has long been known for its taxonomic complexity, this being caused by the presence of numerous apomictic clones, or agamospecies, that have been recognized as distinct species. Much of the taxonomic confusion in *Antennaria* has been clarified by investigations into the origins and evolutionary history of the polyploid complexes in the genus (Bayer, 1985a, b, 1987, 1990b; Bayer and Crawford, 1986), but comparatively little is known about the phylogenetic relationships among the amphimictic taxa, i.e., those taxa that produce seed sexually. Only some 16 of the 33 amphimictic species of *Antennaria* appear to have been involved in the ancestry of the polyploid agamic complexes (Bayer, 1990a), and the evolutionary relationships of those taxa to the other amphimictic species is in need of exploration. Investigations into the phylogeny of those amphimictic taxa that gave rise to the polyploid complexes can provide information on the evolution of polyploidy and agamospermy within genera, such as *Antennaria,* where polyploidy tends to be disproportionately distributed among the taxa. A morphology-based cladistic analysis (Bayer, 1990a) provided the first phylogenetic hypothesis for the sexually reproducing species, but a lack of suitable characters left much of the topology unresolved. Other independent tests of the phylogenetic relationships among these taxa were therefore sought to reconstruct the phylogeny.

The internal transcribed spacer region (ITS) of the 18S-26S ribosomal DNA cistron has been used successfully to reconstruct phylogenies at the generic and species levels with numerous examples from the Asteraceae (in *Calycadenia, Argyroxiphium, Dubautia, Wilkesia, Adenothamnus, Madia, Raillardella,* and *Railardiopsis,* Baldwin, 1992, 1993; in *Krigia,* Kim and Jansen, 1994; in *Ratibida, Dracopsis,* and *Rudbeckia,* Urbatsch and Baldwin, 1993; and in the Cardueae, Susanna et al., 1995). ITS has several advantages that make it an ideal region to sequence for phylogenetic analysis of congeneric species: (1) its rate of evolution is appropriate for studies at the specific and generic levels; (2) it is phylogenetically interpretable, i.e., the sequences are relatively easy to

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[4] Author for correspondence.

align because there tends to be very little length variation at the generic level in flowering plants; (3) it is large enough to offer potentially enough characters for phylogenetic reconstruction; and (4) it is flanked by regions that are highly conserved within genera, making polymerase chain reaction (PCR) amplification and sequencing straightforward.

The primary goal of this study was to reconstruct the phylogeny of all (30+) amphimictic *Antennaria* species based on sequence divergence in ITS-1 and ITS-2. It was hoped that many of the relationships that remained uncertain in earlier studies (Bayer, 1990a; Bayer, unpublished cpDNA RFLP data) could be resolved through sequence analysis. Also, ITS provides an independent data set for comparison with the phylogenetic hypotheses that were produced in the earlier studies.

MATERIALS AND METHODS

Thirty species of *Antennaria* (Table 1) were used, and this includes all but three of the known sexually reproducing species in the genus. Material of *A. linearifolia* Wedd., *A. eucosma* Fern., and *A. sleumeri* Cabrera from Peru, Newfoundland, and Argentina, respectively, was not available. Taxonomic circumscriptions used herein for *Antennaria* follow Bayer and Stebbins (1993) for North America and Urbanska (1983a, b) for the European members of the Pulcherrimae group (= Carpaticae sensu Urbanska). As a result of the morphology-based cladistic studies of Bremer (1987) and Anderberg (1989) the tribe Inuleae as classically recognized (sensu Merxmüller, Leins, and Roessler, 1977) is paraphyletic, although molecular data do not support this result (Bremer et al., 1992). Consequently, *Antennaria* belongs to the recently redefined tribe Gnaphalieae Rydb., subtribe Cassiniinae A. Anderb. (Anderberg, 1991). Following Anderberg's (1991) classification four taxa were chosen to represent the outgroup; two of these are from the proposed sister group of *Antennaria, Anaphalis margaritacea* (L.) Benth. and Hook. f. and *Ewartia catipes* (DC) Beauverd, and two are from an adjoining clade of gnaphaloid composites, *Leontopodium alpinum* Cass. and *Pseudognaphalium microcephalum* (Nutt.) A. Anderb.

DNA isolation and PCR amplification—All of the DNAs were isolated from plants collected from natural populations, except *Leontopodium alpinum,* which was purchased from a commercial plant nursery and subsequently cultivated in the greenhouse. Voucher specimens are deposited at the University of Alberta Vascular Plant Herbarium (ALTA).

Total DNA was isolated from 0.7 to 1.5 g of fresh leaf material using a modified CTAB method (Doyle and Doyle, 1987), with 1.0% β-mercaptoethanol (instead of 0.2%) used in the extraction buffer. In most cases, RNA in the resulting samples was digested with RNAase A (Sigma R-9009, Sigma Chemical Corp., St. Louis, MO) according to the manufacturer's instructions. DNA was reprecipitated with ice-cold 95% ethanol, washed in 70% ethanol, and resuspended in TE.

The ITS region was amplified via the polymerase chain reaction (PCR) using Replitherm® DNA polymerase (Epicentre Technologies, Madison, WI). The PCR reaction mixture consisted of 5 μL of 20X reaction buffer, 6 μL of 25 mmol/L magnesium chloride solution, 16 μL of a 1.25 mmol/L dNTP solution in equimolar ratio, 25 pmol of each primer, 10–50 ng of template DNA, and 0.5 unit of Replitherm, all in a total volume of 100 μL. The PCR samples were heated to 94°C for 2 min prior to the addition of Replitherm to denature proteases and nucleases. The double-stranded PCR products were produced via 30 cycles of denaturation (94°C for 1.5 min), primer annealing (55°C for 2 min), and extension (72°C for 3 min). A 15-min final extension at 72°C followed cycle 30.

The two ITS sequences were amplified separately. ITS-1 was amplified using the primers 1407F (D. Nickrent, Southern Ill. University, Carbondale, IL, personal communication) and ITS2 (White et al., 1990) in equal proportions to produce double-stranded product (Fig. 1), whereas ITS3 (White et al., 1990) and 307R (D. Nickrent, personal communication) were used to amplify the ITS-2 region. Double-stranded products were then used as templates to produce single-stranded DNA using the ITS2 primer to produce single-stranded DNA of the ITS-1 region and ITS3 to produce single-stranded DNA of the ITS-2 region (Fig. 1). PCR reactions to produce single-stranded DNA were the same as for double-stranded except only one primer (25 pmol) was used in the amplifications. The single-stranded DNA was precipitated with 20% PEG/2.5 mol/L NaCl, washed in 70% EtOH, washed a second time in 95% EtOH, and then resuspended in 7 μL of TE (Morgan and Soltis, 1993) prior to sequencing. Often, a second set of single-stranded products was produced for use in a second sequencing reaction using manganese to increase the yield of short fragments (per U.S. Biochemical Corp., Cleveland, OH).

Sequencing the single-stranded DNA—The single-stranded DNAs were sequenced using the dideoxy chain termination method (Sanger, Nicklen, and Coulson, 1977) with the use of the Sequenase® version 2.0 kit (U.S. Biochemical, Cleveland, OH) and ^{35}S-dATP initially without the addition of manganese. The ITS1 primer (White et al., 1990) was used to sequence the ITS-1 region, and the ITS4 (White et al., 1990) primer was used to sequence the ITS-2 region (Fig. 1). Fragments were separated in 6.0% polyacrylamide gels (0.4 mm thickness; 1X TBE buffer) at 2 000 V/80 W. The gels were fixed in 10% acetic acid for 20 min, washed in distilled water, and allowed to air dry. They were then used to expose Kodak X-Omat AR film for 24–36 h.

Sequence analysis and phylogenetic reconstruction—The ITS sequences were aligned visually, and the alignment of the sequences required interpretation of several small (1-2 bp) insertion/deletion (indel) events and one seven-bp indel in ITS-1 (Appendix 1). Alignment of the ITS-2 sequences required the interpretation of several single-bp indels, and one each of three-, four- and five-bp indels (Appendix 1). The proportion of nucleotide differences among pairs of species was calculated using the Kimura two-parameter model and the MEGA program (Kumar, Tamura, and Nei, 1993). A total of 81 potentially phylogenetically informative nucleotide substitutions in ITS-1 and ITS-2 was used in the analysis of the 34 taxa. The entire sequence of *Pseudognaphalium microcephalum* (Appendix 1) is given as a reference sequence. The sequences for the remaining 33 taxa have been submitted to the Genome Sequence Data Base and the GSDB accession numbers are given in Table 1.

Phylogenetic reconstruction was performed using PAUP version 3.1.1 (Swofford, 1991) on unweighted characters by heuristic searches using Fitch parsimony and stepwise SIMPLE addition of data. The outgroup in the analyses included the four taxa mentioned above. Invariant sites and strictly autapomorphous base changes were also ignored in the phylogenetic reconstruction ("ignore uninformative characters" option). Indels were coded as missing data following the recommendation of Wojciechowski et al. (1993) and therefore ignored in the analysis. They were later mapped on the phylogenetic reconstructions to assess their phylogenetic utility (Fig. 2). The "Tree-Bisection-Reconnection" (TBR) branch swapping option in conjunction with saving all minimal trees (MULPARS) and accelerated transformation (ACCTRAN) were used to search for the shortest topologies. Branches of zero length were collapsed to reduce the number of equally parsimonious trees. Heuristic searches employing 100 replicates of a stepwise random (RANDOM) addition of taxa were conducted to search for other groups of trees (i.e., islands; Maddison, 1991) that are equal to in length or shorter than the most parsimonious trees. Two analyses were conducted using the options described above; in the first, the monophyly of *Antennaria* was tested, and its potential sister-group relationships were explored. The outgroup was initially rooted using the "basal polytomy" option and

TABLE 1. Populations of *Antennaria, Anaphalis, Ewartia, Leontopodium,* and *Pseudognaphalium* used in the ITS sequencing study. Presented are species, voucher numbers (assigned DNA codes), [Genome Sequence Data Base accession numbers for ITS-1 and ITS-2 sequences, respectively], and place of origin. Voucher specimens are deposited at ALTA, with duplicates of some (**) at WS. The voucher for *Ewartia catipes* is Breitwieser and Vogt #724 at the University of Canterbury, Christchurch, New Zealand (CANU).

Species	Voucher [ITS-1/ITS-2]	Country: State/Province: County/Topographic Quad/Place Name
A. anaphaloides Rydb.	UT-91005(59) [L40770/L40857]	U.S.A.: Utah: Uintah Co.
A. arcuata Cronq.	WY-90022(45) [L40771/L40858]	U.S.A.: Wyoming: Fremont Co.
A. argentea Benth.	CA-91012(70) [L40772/L40859]	U.S.A.: California: Sierra Co.
A. aromatica Evert	M-628(228) [L40773/L40860]	U.S.A.: Montana: Gallatin Co.
A. carpatica (Wahl.) Bl. & Fingerh.	SZ-91002(78) [L40774/L40861]	Switzerland: Swiss Alps
A. corymbosa E. Nels.	CO-91001(61) [L40775/L40862]	U.S.A.: Colorado: Gunnison Co.
A. densifolia A. E. Pors.	YK-10(183) [L40776/L40863]	Canada: Yukon: Klondike Quad
A. dimorpha (Nutt.) T. & G.	NV-90007(39) [L40777/L40864]	U.S.A.: Nevada: Elko Co.
A. dioica (L.) Gaertn.	G-702(52) [L40778/L40865]	Germany: Bavaria: Bayreuth
A. flagellaris (Gray) Gray	OR-91006(64) [L40779/L40866]	U.S.A.: Oregon: Crook Co.
A. friesiana (Trautv.) Ekman ssp. *alaskana* (Malte) Hult.	YK-89082(13) [L40766/L40856]	Canada: Yukon: Porcupine River Quad
neoalaskana (A. E. Pors.) Bayer & Stebbins	NWT-89029(12) [L40794/L40897]	Canada: Northwest Territories: Porcupine River
A. geyeri Gray	CA-91011(81) [L40780/L40867]	U.S.A.: California: Sierra Co.
A. lanata (Hook.) Greene	MT-92053(148) [L40781/L40868]	U.S.A.: Montana: Madison Co.
A. luzuloides Torr. & Gray	WA-90001(41) [L40782/L40869]	U.S.A.: Washington: Spokane Co.
A. marginata Greene	NM-93009(258) [L40790/L40870]	U.S.A.: New Mexico: Sante Fe Co.
A. microphylla Rydb.	MT-92016(130) [L40791/L40895]	U.S.A.: Montana: Meagher Co.
A. monocephala DC.	AK-89173(1) [L40792/L40896]	U.S.A.: Alaska: Mt. Hayes Quad
A. neglecta Greene**	OH-94001(303) [L40793/L40899]	U.S.A.: Ohio: Delaware Co.
A. nordhageniana Rune & Rönning	AN2(306) [L40795/L40900]	Norway: Finmark Province
A. plantaginifolia (L.) Richards.	MC-43(135) [L40842/L40901]	U.S.A.: Kentucky: Meade Co.
A. pulchella Greene	CA-93009(278) [L40843/L40902]	U.S.A.: California: Inyo Co.
A. pulcherrima (Hook.) Greene	CO-91012(87) [L40844/L40903]	U.S.A.: Colorado: Gunnison Co.
A. racemosa Hook.	MT-92011(151) [L40845/L40924]	U.S.A.: Montana: Cascade Co.
A. rosulata Rydb.	AZ-93010 (272) [L40846/L40925]	U.S.A.: Arizona: Coconino Co.
A. solitaria Rydb.	ASOL-9106(57) [L40847/L40926]	U.S.A.: Tennessee: Wayne Co.
A. stenophylla (Gray) Gray**	WA-94002(305) [L40848/L40927]	U.S.A.: Washington: Lincoln Co.
A. suffrutescens Greene	CA-91002(62) [L40849/L40928]	U.S.A.: California: Humboldt Co.
A. umbrinella Rydb.	M-600(71) [L40850/L40929]	U.S.A.: Montana: Ravalli Co.
A. virginica Stebbins**	WV-94001(298) [L40851/L40930]	U.S.A.: West Virginia: Pendleton Co.
Outgroups:		
Anaphalis margaritacea (L.) Benth. & Hook.	CO-90028(16) [L40940/L40853]	U.S.A.: Colorado: San Juan Co.

TABLE 1. Continued.

Species	Voucher [ITS-1/ITS-2]	Country: State/Province: County/Topographic Quad/Place Name
Ewartia catipes (DC.) Beauvard**	724(302) [L40931/L40854]	Australia: Tasmania: Ben Lomond National Park
Leontopodium alpinum Cass.**	GH-94001(312) [L40765/L40855]	Europe: (Cultivated material obtained from: Forest Farm, Williams, Oregon)
Pseudognaphalium microcephalum (Nutt.) A. Anderb.	GH-92001(124) [L40639/L40852]	U.S.A.: California: Yolo Co.

because the first analysis clearly showed *Antennaria* to be monophyletic relative to the outgroup included, a second analysis, in which the ingroup (*Antennaria*) was considered monophyletic and the outgroup was considered as paraphyletic, was conducted to examine further the possible sister group of *Antennaria*. A strict consensus tree was constructed from the first analysis and a 50% majority-rule consensus tree (Margush and McMorris, 1981) was produced (showing all compatible groups) from the second. Consensus trees were inspected using MacClade (Maddison and Maddison, 1992). In addition, a reanalysis of morphology (data matrix modified from Bayer, 1990a) for only those species of *Antennaria* also analyzed for ITS sequences was performed for comparison with the current study. Although a combined data set (ITS sequences and morphology) for cladistic analysis was considered, it was not performed because some controversy exists as to whether such combined analysis is appropriate.

The relative support for the various clades was determined by bootstrap analysis (Felsenstein, 1985) employing 100 replicates. To avert the problems of memory exhaustion and unrealistically long analyses, a maximum of 2 500 trees was saved during each bootstrap replicate. Additionally, a decay analysis (Bremer, 1988; Donoghue et al., 1992) was performed following the general methods of Johnson and Soltis (1994) to assess the strength of each clade.

RESULTS

Structure of the ITS region in Antennaria—The length of the ITS-1 region in *Antennaria* ranged from 253 to 260 bp. The proportion of nucleotide differences between pairs of species of *Antennaria* for this region ranged from 1 to 14%; between species of *Antennaria* and the outgroup taxa it ranged from 7 to 19%. With respect to the ITS-2 region, the pairwise divergence ranged from 0 to 8% within *Antennaria* and from 6 to 14% between the ingroup and outgroup taxa. ITS-2 is shorter than ITS-1, ranging from 213 to 219 bp in *Antennaria*. Taking both ITS-1 and ITS-2 into consideration, the pairwise divergence between species of *Antennaria* ranged from 1 to 10%, whereas values between *Antennaria* and the outgroups ranged from 7 to 17%. Eighty-one sites (17%) from the combined ITS-1 and ITS-2 regions have the potential to provide phylogenetic information. The remaining sites (83%) are either invariant or are autapomorphous.

For the most part, the indels in the ITS sequences are autapomorphous (Fig. 2) and were therefore phylogenetically uninformative. The phylogenetically informative indels include a five-bp insertion ("PQRST"; Fig. 2) in ITS-2 (Appendix 1), which is a synapomorphy that defines the *Ewartia–Leontopodium–Antennaria* clade, two single-base insertions ("I,J"; Fig. 2) in ITS-1 (Appendix 1), which are synapomorphies that define the *A. rosulata–A. marginata* clade, and a single-bp insertion ("I"; Fig. 2) in ITS-1 (Appendix 1), which is a synapomorphy that defines the *A. densifolia–A. corymbosa* clade.

Phylogenetic reconstruction of Antennaria—The 50% majority-rule tree presented (length = 212) (Fig. 3) is identical to one of the 2 118 most parsimonious trees.

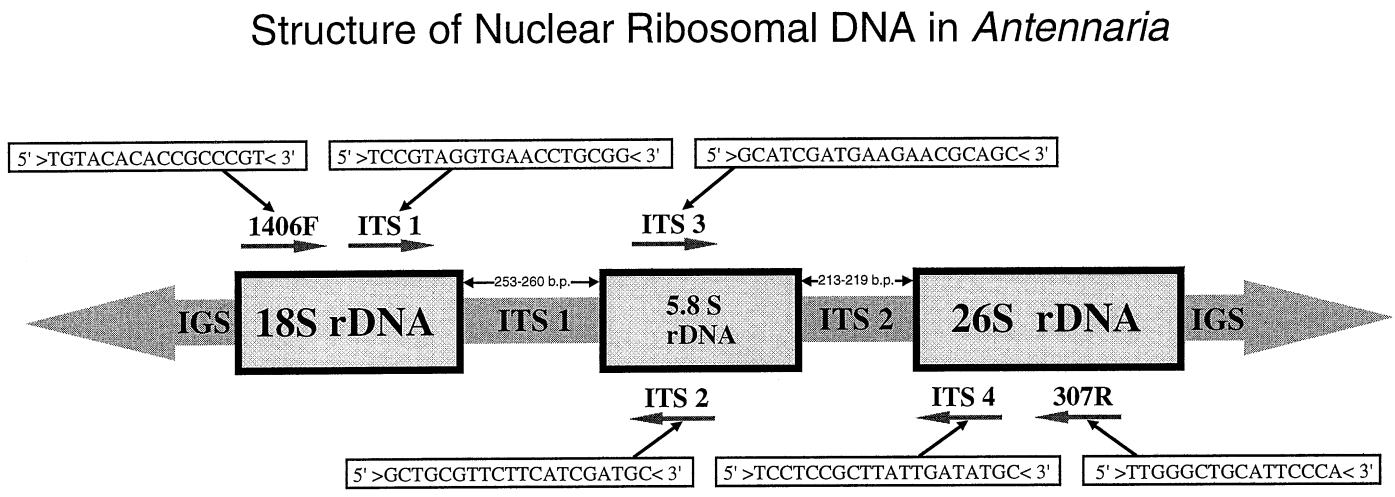

Fig. 1. Structure of nuclear ribosomal DNA in *Antennaria*. Presented are positions of the internal transcribed spacer (ITS) regions relative to the 18S, 5.8S, and 26S rRNA genes and the intergenic spacer (IGS). Relative positions of primers used in PCR and sequencing are indicated, along with their base sequences.

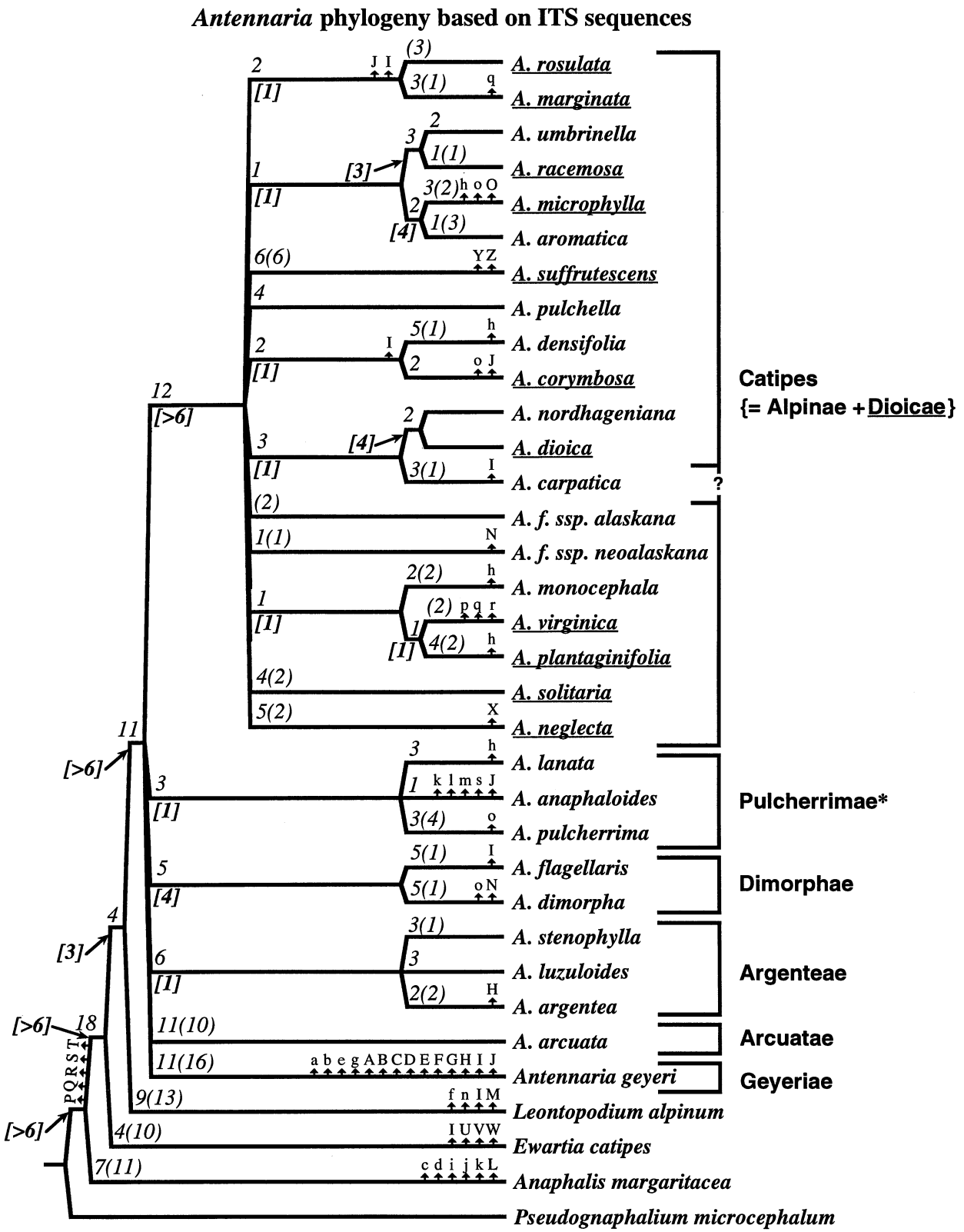

Fig. 2. Strict consensus tree of 2 118 equally parsimonious trees of 30 species of *Antennaria*. Eighty-one phylogenetically informative characters yielded equally parsimonious 212 step trees with consistency indices of 0.72. The strict consensus tree has a length of 231 steps and a consistency index of 0.52. The numbers of unambiguous base-pair changes are indicated above the branches. Numbers in parentheses above each of the branches are the numbers of autapomorphous changes on each terminal branch. Decay indices are given in brackets, and those branches with decay indices > 6 are labelled as such. Indels are indicated above each branch, where uppercase letters indicate base-pair insertions and lowercase letters indicate

A strict consensus tree was also constructed to determine the relative stability of the various clades in the tree (Fig. 2) and to test the monophyly of *Antennaria.* "Trivial" names have been placed on the majority-rule tree (Fig. 3) for ease of referral. In addition, a 50% majority-rule consensus of 135 shortest trees found in a reanalysis of morphology (data matrix modified from Bayer, 1990a) for only those species of *Antennaria* also analyzed for ITS sequencing is presented (Fig 4).

Based on the taxa analyzed for ITS sequence variation, the strict consensus tree (Fig. 2) indicates that *Antennaria* is monophyletic, a result supported by 11 synapomorphies. The 50% majority-rule consensus tree (Fig. 2) indicates that the possible sister genus of *Antennaria* is *Leontopodium,* and that *Antennaria* is more distantly related to *Ewartia, Anaphalis,* and *Pseudognaphalium.* Using *Leontopodium, Ewartia, Anaphalis,* and *Pseudognaphalium* as the outgroup, *Antennaria* is monophyletic, supported by 6 synapomorphies and with a bootstrap value of 94% (Fig. 3). The strict consensus tree also indicates that *Antennaria* is composed of six clades, the Geyeriae, Arcuatae, Argenteae, Dimorphae, Pulcherrimae, and Catipes (Fig. 2). In the 50% majority-rule tree (Fig. 3), these six groups form two subclades within the genus. One is composed of the Geyeriae, Argenteae, Arcuatae, Dimorphae, and Pulcherrimae here referred to as the "Leontipes" group and is supported by three synapomorphies with a bootstrap value of 39%. The "Leontipes" group is sister to the large Catipes group, which is supported by eight synapomorphies and has 99% bootstrap support. Within the Catipes the "neglecta" group is sister to the remainder of the clade (bootstrap value of 29%). The remaining 19 members share one synapomorphy, which is plesiomorphic in *A. neglecta* and the rest of *Antennaria* (Fig. 3). Among the other 19 species of the Catipes group, two weakly supported clades are evident in the majority-rule tree (Fig. 3): the "friesiana group" and the "corymbosa group". Several smaller monophyletic lineages are evident within the Catipes group (Fig. 2), but most of these are also weakly supported.

DISCUSSION

General characteristics of ITS in Antennaria—The sizes of the ITS regions in *Antennaria* are similar to those that have been reported for other genera of Asteraceae. ITS-1 ranges from 253 to 260 bp in length in *Antennaria,* compared to lengths of 254–257 bp in *Calycadenia* and *Osmadenia* (Baldwin, 1993), 255–261 bp in the Hawaiian silversword alliance, subtribe Madiinae (Baldwin, 1992), 259–267 bp in the Cardueae (Susanna et al., 1995), and 246–253 bp in *Krigia* and its allies in the Microseridinae (Kim and Jansen, 1994). ITS-2 ranges from 213 to 219 bp in length in *Antennaria,* 219–223 bp in *Calycadenia* and *Osmadenia* (Baldwin, 1993), 216–223 bp in the Madiinae (Baldwin, 1992), 213–221 bp in the Cardueae (Susanna et al., 1995), and 218–222 bp in length in the Microseridinae (Kim and Jansen, 1994).

Sequence divergence among species of *Antennaria* (1–14% for ITS-1 and 0–8% for ITS-2) is comparable to sequence divergence values reported in *Calycadenia* and *Osmadenia* (0–11.2% in ITS-1; 0–8.6% in ITS-2) (Baldwin, 1993), in the Madiinae (0.4–19.2% in ITS-1, 0–12.9% in ITS-2) (Baldwin, 1992), and in the Cardueae (1.2–15.9% in ITS-1; 0.9–15.5% in ITS-2) (Susanna et al., 1995). In all these cases ITS-1 is more variable than ITS-2, but both regions are sufficiently variable to make the ITS a useful tool for phylogenetic reconstruction at the level of tribe and below in the Asteraceae.

Circumscription of Antennaria and outgroup relationships—ITS sequences have been very useful in reconstructing the phylogeny of *Antennaria* and also in defining its relationship to the outgroup taxa. Previous cladistic analyses (Fig. 4) did not portray *Antennaria* as a monophyletic group. For example, cpDNA restriction site data (unpublished data) indicated that *Antennaria* was monophyletic only with the inclusion of *Anaphalis.* In contrast, phylogenetic analysis of morphology (Fig. 4) found that *Antennaria* would be monophyletic only if *A. geyeri* were removed from the genus. However, ITS sequence data clearly define *Antennaria,* including *A. geyeri,* as monophyletic, united by 11 synapomorphies (Fig. 2). Furthermore, *Anaphalis* is not a member of *Antennaria* based on ITS sequence data. Therefore, based on these data the recently suggested generic recircumscription of *Antennaria* that *A. dimorpha* be segregated into its own genus (Weber, 1987) may be unwarranted.

The sister-group relationships of *Antennaria* have been unexplored primarily because the tribe Gnaphalieae, to which *Antennaria* belongs, has been largely neglected by taxonomists. Until Anderberg's (1991) cladistic analysis of the Gnaphalieae, phylogenetic relationships among the ≈167 genera of the tribe were uninvestigated. Stebbins (1974) suggested that the "immediate relative" of *Antennaria* was the cosmopolitan genus *Gnaphalium.* However, taxonomists have long recognized that *Gnaphalium* sensu lato (s. l.) was a highly polyphyletic assemblage of species, and the genus has been dismantled recently into smaller segregate genera, such as *Pseudognaphalium, Anaphaloides,* and *Gamochaeta,* among several others (Hilliard and Burtt, 1981; Anderberg, 1991). Anderberg's (1991) analysis showed that the primarily Asian genus *Anaphalis* and the Australia–New Zealand genus *Ewartia* were the sister genera of *Antennaria.* Merxmüller, Leins, and Roessler (1977) included *Antennaria* in the "*Anaphalis* group" closely related to *Anaphalis* and the Eurasian genus *Leontopodium.* All of the cladistic analyses of *Antennaria,* based on morphology, cpDNA, and ITS sequences (Figs. 2–4), have shown that *Gnaphalium* s. l. (*Pseudognaphalium*) is more distantly related to *Antennaria* than is *Anaphalis.* However, based on the genera included here, ITS sequences (Fig. 3) suggest that *Leon-*

←

deletions relative to the sequence of *P. microcephalum.* The "PQRST" five-bp insertion that is a synapomorphy that unites *Antennaria, Leontopodium,* and *Ewartia* is labelled as P-T or PQRST. *Antennaria friesiana* ssp. *alaskana* is labelled as *A. f.* ssp. *alaskana.* Underlined names are those species that belong to the traditionally recognized Dioicae group.

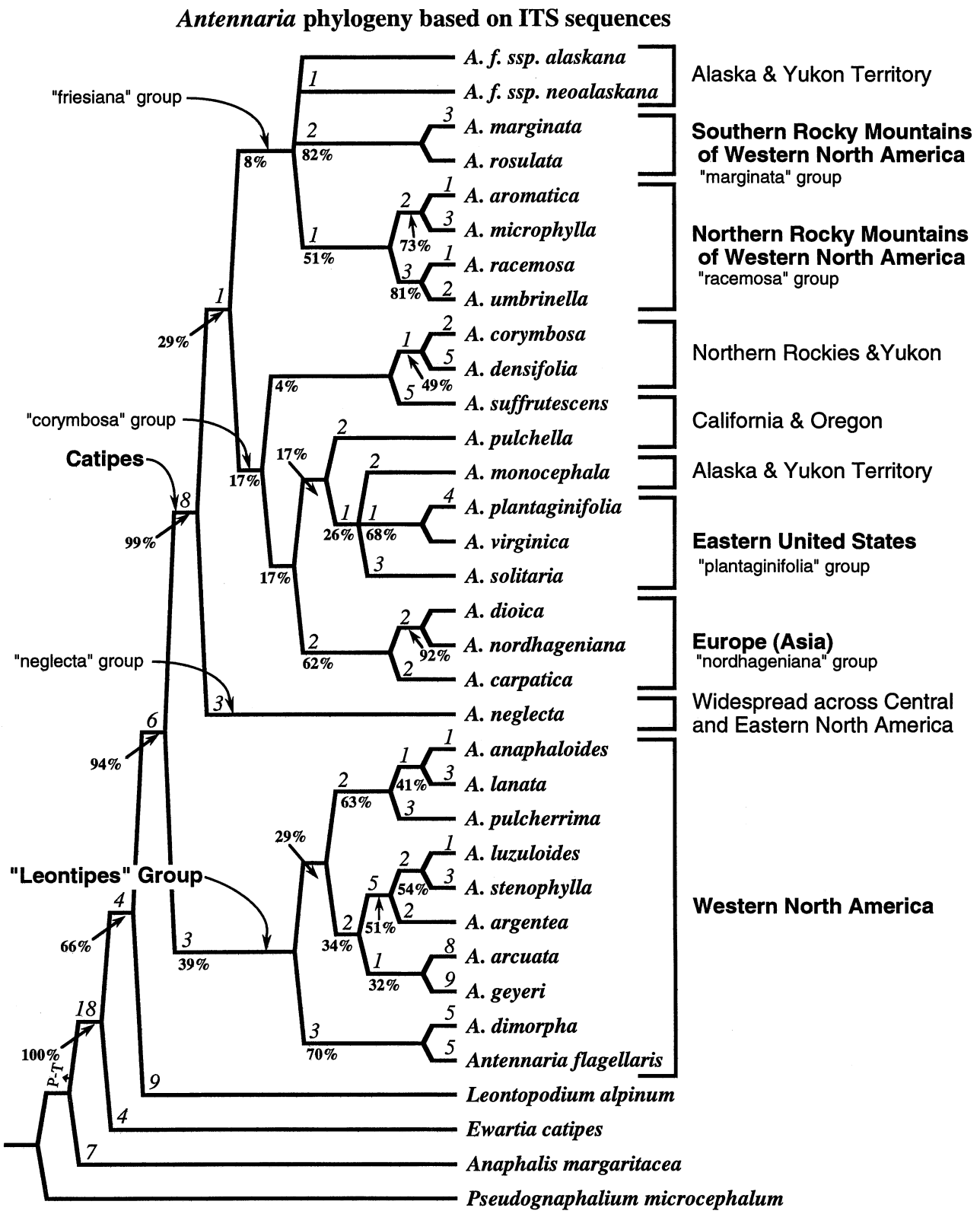

Fig. 3. One of 2 118 equally parsimonious trees that is topologically identical to the 50% majority-rule tree of 30 species of *Antennaria*. Eighty-one phylogenetically informative characters yielded equally parsimonious trees of 212 step trees with consistency indices of 0.72. Bootstrap confidence values (100 replicates) are given below the branches, whereas the number of unambiguous phylogenetically informative base-pair changes along branches are indicated above the branches. *Antennaria friesiana* ssp. *alaskana* is labelled as *A. f.* ssp. *alaskana*. The "PQRST" five-bp insertion that is a synapomorphy that supports the *Antennaria-Leontopodium-Ewartia* clade is labelled as P-T.

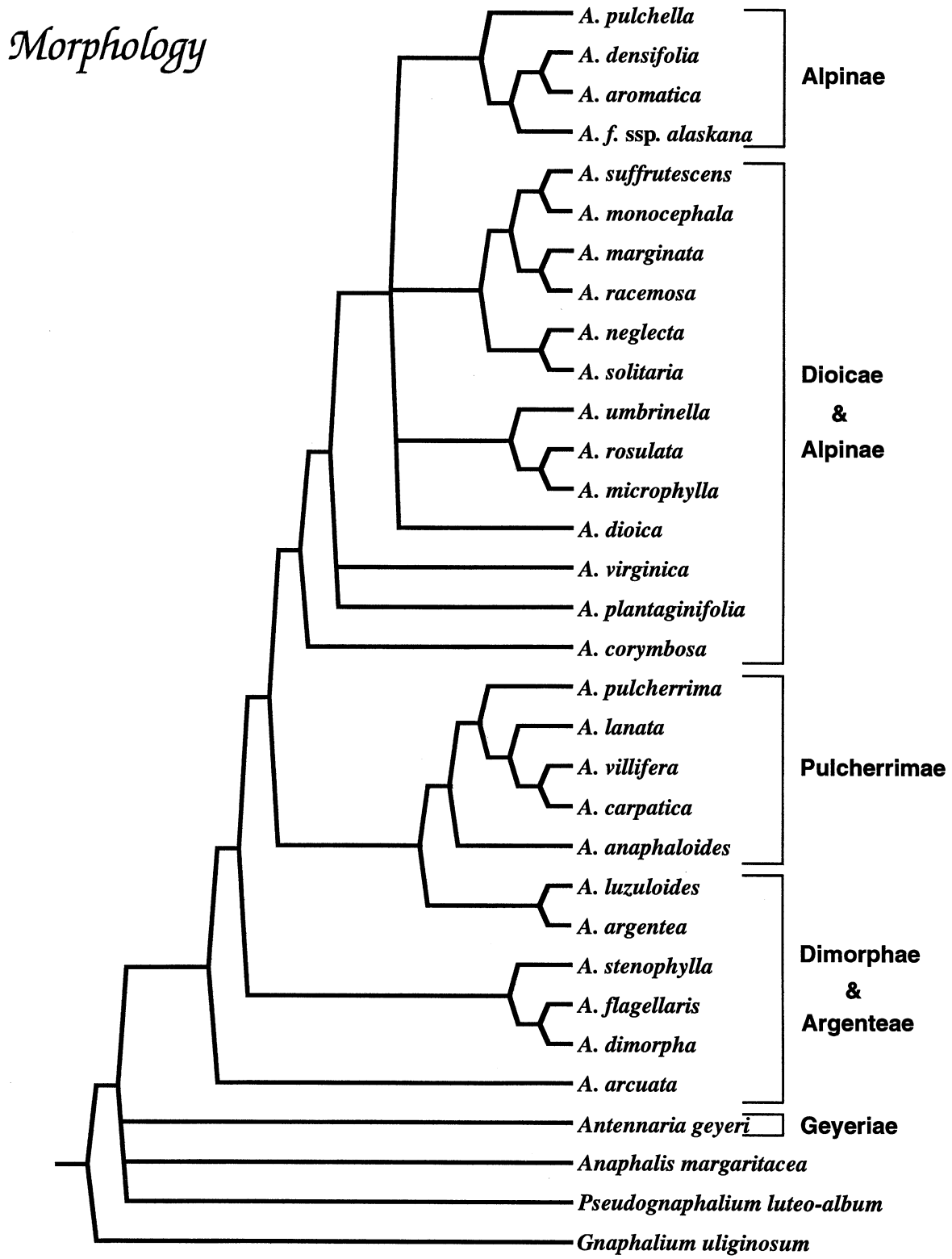

Fig. 4. Morphology-based 50% majority-rule consensus tree of 29 species of *Antennaria* based on the data matrix from Bayer (1990a). Twenty-nine phylogenetically informative characters yielded 135 equally parsimonious trees of 83 steps with consistency indices of 0.35. *Antennaria friesiana* ssp. *alaskana* is labelled *A. f.* ssp. *alaskana*. *Antennaria nordhageniana* and *A. monocephala* are identical with regard to the 29 characters, therefore only *A. monocephala* is shown.

topodium is the sister genus of *Antennaria,* and together they are the sister genus to *Ewartia.* Additionally, the five-bp insertion (Fig. 3 "P-T") that defines the *Ewartia–Leontopodium–Antennaria* clade supports the monophyly of this group of genera. This is in conflict with Anderberg's analysis (1991), which showed *Leontopodium* as being quite distantly related to the *Antennaria–Anaphalis–Ewartia* clade. Until phylogenetic relationships of more members of the Gnaphalieae can be investigated using molecular techniques, the relationships among genera of the tribe will remain uncertain.

Phylogenetic relationships within Antennaria—*Antennaria* has been informally divided into six phenetic groups (Bayer, 1990a), the Geyeriae, Argenteae, Dimorphae, Pulcherrimae, Dioicae, and Alpinae. The majority-rule consensus tree (Fig. 3) shows that *Antennaria* is composed of two major lineages, the "Leontipes" group, which consists of species that are restricted in their distributions to the western United States, and the Catipes group, comprising the "neglecta," "corymbosa," and "friesiana" groups, occurring throughout the northern hemisphere. The "Leontipes" group, which consists of five smaller groups, the Geyeriae, Arcuatae, Argenteae, Dimorphae, and Pulcherrimae in the strict consensus tree (Fig. 2), is composed of species that are primarily diploid (tetraploids are known only in *A. dimorpha* and *A. pulcherrima,* Bayer and Stebbins, 1987) and as far as is known always amphimictic. Most of the species of the "Leontipes" group lack horizontal stoloniferous growth (exceptions are *A. flagellaris* and *A. arcuata*). Based on morphology the "Leontipes" group is considered the likely basal group in the genus, based on a number of unspecialized morphological features, such as nonstoloniferous growth, lack of extensive polyploidy, and a general lack of well-developed sexual dimorphism. The Catipes group has amphimictic diploids and tetraploids and derived from them are all of the polyploid agamic complexes in the genus. Most of the species of the Catipes group have aggressive horizontal stolons, an effective means of asexual reproduction. Therefore, the Catipes group is considered the more morphologically specialized of the two major groups in *Antennaria.*

The strict consensus tree (Fig. 2) demonstrates that *Antennaria* is composed of six monophyletic groups of equal rank; for the most part these groups correspond to traditionally recognized groups (Bayer, 1990a). The Geyeriae group is monotypic, consisting of *A. geyeri,* a species characterized by a large number (30) of autapomorphous nucleotide substitutions, a four-bp deletion and a large ten-bp insertion (Fig. 2). The tendency toward polygamodioecy in *A. geyeri,* along with its lack of basal leaves, make it more similar morphologically to *Anaphalis* than the remainder of *Antennaria.* Previous studies of morphology (Fig. 4) and cpDNA restriction sites (unpublished data) left unresolved the inclusion of *A. geyeri* within *Antennaria.* It is obvious from the current investigation, however, that *A. geyeri* should remain a member of *Antennaria* (Figs. 2, 3).

Antennaria arcuata, the only member of the newly recognized Arcuatae, also has accumulated a relatively large number (ten) of autapomorphous changes (Fig. 2). Previously, *A. arcuata* had been included in the Argenteae with *A. luzuloides* and *A. argentea* (Bayer, 1990a), but that relationship was always weakly supported. In fact, the Argenteae group is paraphyletic in cladistic analyses of both morphology (Fig. 4) and ITS sequences (Figs. 2, 3) if *A. arcuata* is included in the group. It seems best to recognize that *A. arcuata* belongs to a distinct lineage.

The Argenteae clade is composed of three taxa, *A. argentea, A. luzuloides,* and *A. stenophylla* (Fig. 2), and is supported by six synapomorphies in the strict consensus tree. It is the sister group to the *A. arcuata–A. geyeri* clade in the majority-rule tree (Fig. 3). Morphology (Fig. 4) indicates that *A. argentea* and *A. luzuloides* are sister taxa, whereas ITS sequences indicate that *A. stenophylla* is the sister taxon to *A. luzuloides* (Fig. 3). Moreover, morphology indicates that *A. stenophylla* should be a member of the Dimorphae clade (Fig. 4). The close relationship suggested between *A. stenophylla* and *A. luzuloides* based on ITS sequences is readily acceptable based on gross morphology in that they both have narrow, linear leaves and small flowering heads.

ITS sequence data provide support for a Pulcherrimae clade consisting of *A. pulcherrima, A. anaphaloides,* and *A. lanata* (Fig. 2). However, one surprising feature of the ITS-based tree (Fig. 2) is the inclusion of *A. carpatica* in the Catipes clade as the sister taxon to *A. dioica.* By contrast, morphology (Fig. 4) strongly suggests that *A. carpatica* is a member of the Pulcherrimae. The clade containing *A. carpatica,* the "nordhageniana" group (Fig. 3), is entirely European in distribution. It is also noteworthy that *A. carpatica* and *A. dioica,* also of this "nordhageniana" clade, hybridize in the Alps (Urbanska-Worytkiewicz, 1968), the area that served as the source of our material of *A. carpatica* (Table 1). It could be that past introgressive hybridization between *A. carpatica* and *A. dioica* has effectively transferred the ITS region of *A. dioica* into *A. carpatica* (at least in the material that we used in this study). The Pulcherrimae is a well-defined morphological group, and the suggestion based on sequence data that *A. carpatica* is a member of Catipes rather than the Pulcherrimae is not easily accepted. This result is problematical, and the sequencing of additional material of *A. carpatica* is needed.

The Catipes is a very well-supported group in both the strict (Fig. 2) and majority-rule (Fig. 3) consensus trees, although support for subclades within Catipes is weak. Traditionally, members of Catipes were split into two groups, the Alpinae, distributed in tundra, with black or olivaceous colored phyllaries, and the Dioicae taxa with phyllaries of lighter colors other than black or dark green. Based on ITS sequence data (Fig. 2, 3), as well as morphology (Fig. 4), it is obvious that these two groups are unnatural, polyphyletic groups that should be abandoned. Species with dark phyllaries that grow in arctic and/or alpine tundra are probably the result of convergent evolution under similar environmental conditions (e.g., *A. pulchella* of the Sierra Nevada of the "corymbosa" group and *A. aromatica* of the "friesiana" group). Likewise, taxa with light-colored phyllaries, the Dioicae, are also a paraphyletic group. Lastly, although some have suggested that *A. marginata* and *A. dioica* are conspecific (Jepson, 1925), sequence data suggest that they are only distantly related (Figs. 2, 3).

Amphimixis, apomixis (agamospermy), and the very

high levels of polyploidy (up to dodecaploid; Bayer and Minish, 1993) are prevalent among polyploid derivatives of the Catipes clade. The Catipes clade consists of diploids in which sexual dimorphism is highly evolved and in which an effective means of asexual vegetative reproduction, stoloniferous growth, is well developed (Bayer, 1990a). Additionally, many of the Catipes are specialized as edaphic endemics, such as *A. virginica* on Devonian-age shale barrens (Bayer and Stebbins, 1987, 1993), *A. suffrutescens* on serpentine (Bayer and Stebbins, 1993), and *A. aromatica* and *A. densifolia* on limestone talus (Bayer, 1989). Five polyploid agamic complexes, *A. alpina* (*L.*) Gaertn., *A. howellii* E. L. Greene, *A. parlinii* Fern., *A. parvifolia* Nutt., and *A. rosea* E. L. Greene, have evolved via multiple hybridization among members of the Catipes group (Bayer, 1987). The great success of the Catipes group seems to be correlated with their ability to grow in a diversity of habitats throughout their range from Great Britain across Eurasia and North America to Tierra del Fuego and to their acquisition of characters such as strong sexual dimorphism, aggressive vegetative reproduction (stolons), polyploidy, and agamospermy. Additional investigations, perhaps with a more rapidly evolving nuclear DNA sequence, will be needed, however, before the phylogenetic relationships among members of the Catipes group can be resolved with greater certainty.

Phylogeography—There is some correspondence between geographic patterns and the phylogenetic relationships in *Antennaria* (Fig. 3). The "Leontipes" group, evident in the majority-rule tree, consists of the Geyeriae, Arcuatae, Argenteae, Dimorphae, and Pulcherrimae groups, and is restricted in distribution to the western United States and portions of adjacent Canada (Fig. 3). The "neglecta" group is the sister group to the remainder of the Catipes group in the majority-rule tree and is perhaps the most widespread of the North American Catipes taxa (Fig. 3). Two clades form the "plantaginifolia" group, which are restricted to the eastern United States. The "nordhageniana" clade consists of three Eurasian taxa, although, as discussed above, the inclusion of *A. carpatica* in this group is anomalous (Fig. 3). Within the "friesiana" group are four clades, two of which have some geographic pattern to the relationships. The "marginata" group is composed of two species that have their centers of distribution in the southern Rockies and are largely restricted to Arizona and New Mexico. The four taxa of the "racemosa" group are from the northern Rockies.

Based on this study, the apparent sister genus of *Antennaria* is *Leontopodium* (Fig. 3), a genus most diverse in Chinese, Burmese, and European mountains (Anderberg, 1991). It is likely that the common ancestor that gave rise to both genera was also found in the Northern Hemisphere, perhaps in Asia and western North America. The most logical phytogeographic hypothesis concerning the origin and subsequent divergence of the genus *Antennaria* would maintain that it arose in the southern part of western North America, where the "Leontipes" clade of the genus is still found today (Fig. 3). At a later time, the most specialized clade of *Antennaria,* the Catipes group (Fig. 3), successfully spread into a diverse variety of habitats throughout the Northern Hemisphere. Recent speciation in Catipes appears to have occurred in rather limited geographic regions through adaptive radiation into very different niches. For example, the "racemosa" group contains *A. aromatica, A. microphylla, A. racemosa,* and *A. umbrinella*; all occur in the northern Rockies (Fig. 3), yet each species occupies its own distinct niche within that range (Bayer, Purdy, and Lebedyk, 1991). Similarly, the sister species *A. marginata* and *A. rosulata* (Figs. 2, 3) both occur exclusively in the southern Rockies, but each occurs in its own distinct habitat (Bayer, Purdy, and Lebedyk, 1991).

Conclusions—ITS sequences have been useful in resolving phylogenetic questions in the Cassiniinae. Considering the outgroup taxa that were included in the analysis, *Leontopodium* is the sister genus to *Antennaria,* and *Anaphalis* and *Pseudognaphalium* may be more distantly related to *Antennaria* than previously believed. *Antennaria* is a monophyletic group relative to the outgroups cited, and *A. geyeri* is clearly included within the genus. Within *Antennaria* are six clades, the Geyeriae, Arcuatae, Argenteae, Dimorphae, Pulcherrimae, and Catipes. The first five of these are geographically restricted to western North America and form the "Leontipes" group, in which polyploidy is rare and morphology is less specialized than in Catipes. The geographically widespread Catipes group is morphologically most specialized, and hybridization among these sexual species and subsequent acquisition of polyploidy and agamospermy by the hybrid entities has led to the complex pattern of reticulate evolution that is confined to the Catipes clade.

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APPENDIX 1. Complete ITS-1 and ITS-2 sequences for *Pseudognaphalium microcephalum.* Dashes are deleted base-pairs, and question marks are missing or ambiguous data. The beginning and end points of the ITS sequences are indicated with arrows. Indel positions in various taxa (Fig. 2 for distribution) are indicated below the sequence, where uppercase letters indicate base-pair insertions and lowercase letters indicate deletions relative to the sequence of *P. microcephalum.*

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ITS-1                                1 1111111112 2222222223 3333333334 4444444445 5555555556
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                                TG|TCGAACCC TGCAAAGCGA ACGACCCGTG AACATGT—— ———AACTAC AACT?GGCAA
                           18S←|→ ITS-1
Indels →                                                a                   b ABC DEFG           c
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                           AT—T?GGGAC TG?GCTTTTG TTTGATCCTT AGCTTGCCTT GTCGATGTGT GTCTGAGATT
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                           1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
                           CCTTACGGAT GACTGGATGT CACATTGACA TACTAACCAA CCCCGGCACG —AATGTCG—A
                                        f                                     g       I      hJ
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                           8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
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                           AGGAAATTAA AACTTTAGGA ATGGAAGGTT TCATGATGTC CCGTTTTGCG GTGS?CTATC
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                           4444444445 5555555556 666|6666667 7777777778 8888888889 9999999990
                           1234567890 1234567890 123|4567890 1234567890 1234567890 1234567890
                           AAAACCTTAC TTCTTTGTAA TCA|CAAACGA CTCTCGGCAA CGGATATCTC GGCTCACGCA
                                              ITS-1 ←|→ 5.8S
ITS-2                                 1 1111111|112 2222222223 3333333334 4444444445 5555555556
                           1234567890 1234567|890 1234567890 1234567890 1234567890 1234567890
                           TCTGCCTGGC GTCACGC|ATC GTGTCGCCCC CTACC—ACTC C—TCAAATGG ATGTTTGGTG
                                      5.8S←|→ ITS-2
Indels →                                                                  K      L          i
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                           CGATGGACGC AC—GACTAGT GGTGGTTGCT AAAACCTTCG TCTTTGGTTG TGCATCTTCA
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                           8888888889 9999999990 0000000001 1111111112 2222222223 3333333334|
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                           TTCGTACGGG AAG———CT TAAAGACCCC AATGTGTTGT CTTTTGATGA CACTTCGACC|
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                           |2222222222
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