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MONOPHYLY OF ANEUPLOID ASTRAGALUS (FABACEAE): EVIDENCE FROM NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER SEQUENCES[1]

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Evolutionary relationships within *Astragalus* L. (Fabaceae) were inferred from nucleotide sequence variation in nuclear ribosomal DNA of both New World and Old World species. The internal transcribed spacer regions (ITS) of 18S–26S nuclear ribosomal DNA from representatives of 26 species of *Astragalus,* three species of *Oxytropis* DC., and two outgroup taxa were analyzed by polymerase chain reaction amplification and direct DNA sequencing. The length of the ITS 1 region within these taxa varied from 221 to 231 bp, while ITS 2 varied in length from 207 to 217 bp. Of the aligned, unambiguous positions, approximately 34% were variable in each spacer region. In pairwise comparisons among *Astragalus* species and outgroup taxa, sequence divergence at these sites ranged from 0 to 18.8% in ITS 1 and from 0 to 21.7% in ITS 2. Parsimony analyses of these sequences resulted in a well-resolved phylogeny that is highly concordant with previous cytogenetic and chloroplast DNA evidence for a major phylogenetic division in the genus. These data suggest that the New World aneuploid species of *Astragalus* form a monophyletic but morphologically cryptic group derived from euploid species of Old World (Eurasian) origin, which are consequently paraphyletic.

With more than 2,500 species worldwide, *Astragalus* L. (Fabaceae) is one of the largest genera of flowering plants (even in its restricted sense, excluding *Astracantha* Podl.; Podlech, 1986; Lock and Simpson, 1991). It is distributed primarily in arid and semiarid mountainous regions of the Northern Hemisphere, but is also found along the Andes of South America and to a limited extent in east Africa. The genus is most diverse in the Irano-Turkish region of southwestern Asia, the Sino-Himalayan Plateau of southcentral Asia, and the Great Basin and Colorado Plateau of western North America (Polhill, 1981; Podlech, 1986). According to the most recent morphological classification of the papilionoid tribe Galegeae (Polhill, 1981), the closest relatives of *Astragalus* include *Oxytropis* DC., *Caragana* Fabr., *Chesneya* Lindl. ex Endl., *Alhagi* Adans., *Biserrula* L. (included in *Astragalus* by Barneby, 1964), *Gueldenstaedtia* Fisch., *Halimodendron* Fisch. ex DC., and *Calophaca* Fisch., all members of the subtribe Astragalinae.

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Attempts to deal with the size and diversity of *Astragalus* by division of the genus into meaningful taxonomic units have been numerous. Recognition of over 150 sections in Old World *Astragalus* (Podlech, 1986) and 93 in North America (Barneby, 1964) gives an indication of the degree of morphological diversification and taxonomic complexity within the genus. Furthermore, concepts of taxa above the level of section have been especially fluid. The number of subgenera in treatments of Old World *Astragalus* has varied from seven (Bunge, 1868, 1869) to nine (Goncharov et al., 1965) until Podlech (1982, 1983) combined these subgenera into two and recognized the segregate genus *Astracantha* which corresponds closely to subgenus *Tragacantha* Bunge. In North America, Gray (1864) recognized two subgenera divided into 27 sections while Rydberg (1929) erected 28 genera within the traditional limits of the genus. These were later replaced, at least provisionally, with seven informal higher taxa ("phalanxes") within *Astragalus* by Barneby (1964), four of which are considered equivalent to Old World subgenera (see Table 1). The remaining three phalanxes–Homaloloboid, Orophaca, and Piptoloboid—represent the endemic North American groups. Recently, Isely (1983) resurrected the segregate genus *Orophaca* Rydb., which is equivalent to Barneby's phalanx *Orophaca.* In South America, no subgeneric/sectional classification exists for the 100 or more species there (Johnston, 1947; Gomez-Sosa, 1979).

Cytological evidence has indicated a distinction between Old and New World *Astragalus.* Senn (1938) established that the Old World species of *Astragalus,* like the rest of the tribe Galegeae (with the exception of *Gueldenstaedtia* with $n = 7$), have a "basic" chromosome number of $n = 8$ and that euploidy (i.e., $n = 8$, and even multiples thereof) is common. Subsequent cytotaxonomic studies on New World species (Vilkomerson, 1943; Head, 1957; Ledingham, 1957, 1960; Ledingham and Rever, 1963; Ledingham and Fahselt, 1964; Ledingham and Pepper, 1973; Spellenberg, 1976) confirmed Senn's work but

suggested that the bulk of the 500+ species of *Astragalus* found in the New World possess chromosome numbers in an aneuploid series of $n = 11–15$. Based on a recent survey of the 450–500 Old World species for which chromosome numbers have been determined (Wojciechowski and Sanderson, unpublished data), counts of $n = 12$, 13, 14, or 15 have been reported for only 23 species (Ledingham and Rever, 1963; Fedorov, 1974; Martinez, 1974; Maassoumi, 1987, 1989; Goldblatt and Johnson, 1991, and references therein), and some of these are questionable due to discrepancies in samples from the same species and among authors. These presumed aneuploid species are sporadically distributed both geographically and taxonomically among disparate Old World groups, and their closest relatives appear to be Old World euploids. No species having $n = 11$ are known from the Old World, even though this is the most common number in the New World. Recently, Ashraf and Gohil (1988) have reported a chromosome count of $n = 6$ for the Himalayan species *Astragalus melanostachys* although they suggest that it is derived from the base number $n = 8$.

In contrast, the 13 species of *Astragalus* with chromosome counts of $n = 8$, 16, and 24 found in the New World are plants of circumboreal distribution that are restricted to northern North America and/or higher elevations of the Rocky Mountains. These species are thought to be North American representatives of Old World, primarily Asian, subgenera, corresponding to Barneby's Phacoid, Cercidothrix, and Hypoglottis phalanxes (Barneby, 1964). Of the additional 200+ species and varieties from North America that have been surveyed to date, including all species examined from the three endemic North American phalanxes, there are only two species for which euploid counts based on $n = 8$ have been reported. For both of these species, however, aneuploid counts of $2n = 24$ have also been reported. Furthermore, none of the 32 South American species (of approximately 100 described species) for which chromosome numbers have been reported are euploid (Ledingham and Pepper, 1973).

The consistent difference in chromosome number and the almost total absence of euploidy (and polyploidy) in the endemic New World *Astragalus* provide a notable distinction from the Old World species. Although this has been considered evidence of a major phylogenetic split in the genus (Ledingham, 1957, 1960), the division of *Astragalus* into New World and Old World groups is not supported by independent morphological evidence, nor is there any evidence to suggest whether either or both groups are monophyletic. Preliminary molecular evidence for this phylogenetic division between New and Old World *Astragalus* was recently obtained in a study of restriction site variation in the chloroplast DNA (cpDNA) of 58 aneuploid North American *Astragalus* species, two euploid species of *Astragalus,* and one outgroup species of *Oxytropis* (Sanderson and Doyle, in press). In a study focusing on cpDNA variation in annual members of the North American aneuploid group, Liston (1992) also found evidence for the distinctiveness of the aneuploids, although sampling of the aneuploid clade was limited to only three sections. Here we present a comparative analysis of nucleotide sequences of the internal transcribed spacers of 18S–26S nuclear ribosomal DNA from 26 representatives of North American aneuploid and Old World euploid *Astragalus* species and five representatives of outgroup genera. Our results provide substantial molecular evidence for the monophyly of the New World aneuploid species of *Astragalus.*

TABLE 1. *Subgeneric classification of* Astragalus[a]

Goncharov et al. (1965) (Old World)	Barneby (1964) (North America and circumboreal)
Phaca[b] (19 sect.)	Phacoid* (6 sect., 13 species)
*Caprinus** (17 sect.)	**Homaloboid** (46 sect., 194 species)
*Hypoglottis** (3 sect.)	Cercidothrix* (2 sect., 4 species)
*Trimeniaeus** (13 sect.)	Hypoglottis* (1 sect., 2 species)
*Tragacantha** (3 sect.)	**Piptoloboid** (35 sect., 192 species)
*Calycophysa** (9 sect.)	Trimeniaeus (1 sect., 1 species[b])
*Cercidothrix** (31 sect.)	**Orophaca** (2 sect., 7 species)
*Epiglottis** (1 sect.)	
*Calycocystis** (7 sect.)	

[a] Boldfaced names are groups completely restricted to North America.
[b] Recent molecular evidence (Liston, 1992) suggests this species is actually a North American member of the Piptoloboid phalanx.
* Species in subdivision possess primarily or entirely euploid chromosome numbers.

MATERIALS AND METHODS

Taxa—Twenty-six species of *Astragalus,* including 14 species of endemic North American aneuploids (representatives of 12 sections, three phalanxes; Barneby, 1964), eight species of endemic Old World euploids (representing seven sections and five subgenera; Goncharov et al., 1965), and four species of North American euploids (representing four sections, three phalanxes; Barneby, 1964) were included in the present study (Table 1). The North American euploids include *A. adsurgens, A. agrestis, A. alpinus,* and *A. canadensis,* species that in most cases are circumboreal and also included in Old World sections (Barneby, 1964; Goncharov et al., 1965). The euploid *A. cicer* is an Old World species (subgenus *Hypoglottis,* sect. *Eu-Hypoglottis*) that has been introduced into North America and included in the Hypoglottis phalanx (Barneby, 1964). No Old World aneuploids were available for sampling. Outgroups included three North American or circumboreal species of *Oxytropis,* the genus believed to be most closely related to *Astragalus* (Barneby, 1952, 1964), and one species each from two other genera in Galegeae, *Caragana* and *Clianthus* (Polhill, 1981). The classification and nomenclature used here for *Astragalus* and *Oxytropis* are based upon Barneby (1952, 1964, 1989a, b) in the New World and Goncharov et al. (1965); Goncharov, Vasilchenko, and Fedchenko, 1972) in the Old World.

Plant samples—Total genomic DNA was isolated from either field-collected (kept on ice for 1–7 days, then stored at −80 C) or greenhouse-grown leaf material of individual plants (Table 1) using the 2X CTAB (hexadecyltrimethylammonium bromide) procedure described by Doyle and Doyle (1987). DNAs were further purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis, Fritsch, and Sambrook, 1982). The country of origin, accession numbers, and taxonomic classification for all taxa are provided in Table 2. Seed accessions were grown under greenhouse conditions until flowering/fruiting and were identified using published keys

TABLE 2. *Species of* Astragalus *and related genera included in nrDNA-ITS sequence analysis*

Higher Taxon/ Species[a]	Section	Geographic origin	Accession[b]	*n*[c]	Distribution[d]
Phacoid Phalanx					
Astragalus alpinus L.	Astragalus	Wyoming	USDA 232536, W&S 183	8	NW, OW
Homaloboid Phalanx					
A. bisulcatus var. *haydenianus* (Gray ex Brand.) Barneby	Bisulcati	Colorado, Montezuma Co.	W&S 133	12	NW
A. brandegei T. C. Porter	Quinqueflori	Arizona, Apache Co.	W&S 157	11	NW
A. hallii Gray var. fallax (Wats.) Barneby	Scytocarpi	Arizona, Coconino Co.	Sanderson 900	11	NW
A. oöcalycis Jones	Oocalyces	Colorado, La Plata Co.	W&S 141	12	NW
A. scopulorum T. C. Porter	Tiopsidei	Colorado, Montezuma Co.	W&S 135	11	NW
Cercidothrix Phalanx					
A. adsurgens Pall. var. *robustior* Hook.	Onobrychoidei	Canada	USDA 236749, W&S 186	16	NW, OW
A. canadensis L.	Uliginosi	Canada	USDA 232539, W&S 184	8	NW, OW
Hypoglottis Phalanx					
A. agrestis Dougl. ex G. Don.	Hypoglottoidei	Wyoming, Sweetwater Co.	Sanderson 917	8	NW, OW
A. cicer L.	Hypoglottoidei	Turkey	USDA 206405, W&S 160	8	NW, OW
Piptoloboid Phalanx					
A. cremnophylax var. *hevronii* Barneby	Humillimi	Arizona, Coconino Co.	S&W 1152 (Hevron 1085)	11	NW
A. humillimus Gray ex Brand.	Humillimi	New Mexico, San Juan Co.	S&W 1151 (Hevron)	11	NW
A. lentiginosus Dougl. ex Hook. var. *australis* Barneby	Diphysi	Arizona, Pima Co.	Wojciechowski 103	11	NW
A. monumentalis Barneby	Desperati	Utah, San Juan Co.	W&S 158	12	NW
A. nuttallianus var. *nuttallianus* DC.	Leptocarpi	Arizona, Pima Co.	Wojciechowski 102	11	NW
A. purshii Dougl. ex Hook. var. *tinctus* Jones	Argophylli	California, Kern Co.	Sanderson 986	11	NW
A. thurberi Gray	Inflati	Arizona, Pima Co.	Sanderson 955	11	NW
A. utahensis (Torr.) Torr. & Gray	Argophylli	Nevada, Lincoln Co.	Sanderson 1005	11	NW
Orophaca Phalanx					
A. aretioides (Jones) Barneby	Sericoleuci	Utah, Dagget Co.	W&S 250	12*	NW
Subgenus Caprinus					
A. lobophorus Boiss.	Myobroma	Iran	USDA 330696, W&S 170	8	OW
Subgenus Hypoglottis					
A. pulchellus Boiss.	Malacothrix	Iran	USDA 384778, W&S 168	8	OW
Subgenus Trimeniaeus					
A. asterias Stev. ex Ledeb.	Oxyglottis	Morocco	USDA 516491, W&S 169	8	OW
A. corrugatus Bertol.	Harpilobus	Iran	USDA 227441, W&S 164	16	OW
A. tribuloides Del.	Oxyglottis	Afghanistan	USDA 220085, W&S 172	8	OW
Subgenus Cercidothrix					
A. chaborasicus Boiss. et Hausskn.	Onobrychium	Iran	USDA 330693, W&S 162	16*	OW
Subgenus Epiglottis					
A. hamosus L.	Buceras	Iran	USDA 226627, W&S 166	8	OW

TABLE 2. *Continued*

Higher Taxon/ Species[a]	Section	Geographic origin	Accession[b]	*n*[c]	Distribution[d]
Caragana arborescens Lam.		USSR	USDA 310390	8	OW
Clianthus puniceus (G. Don) Lindley		New Zealand	T&M 7140 (A. Liston)	16	OW
Oxytropis campestris var. *johannensis* (L.) DC.		Maine	USDA 504535, W&S 174	8, 16	NW, OW
O. deflexa (Pall.) DC. var. *sericea* Torr. & Gray		Colorado, San Miguel Co.	W&S 132	8	NW, OW
O. lambertii Pursh.		Utah, Kane Co.	Sanderson 909	24	NW

[a] Phalanx, subgenus, and section names for *Astragalus* species follow the treatment of Barneby (1964) for North America, and Goncharov et al. (1965) and Townsend and Guest (1974) for Old World.

[b] Abbreviations used: S&W, Sanderson and Wojciechowski; USDA, U.S. Department of Agriculture Plant Introduction (PI) accession numbers; W&S, Wojciechowski and Sanderson.

[c] Chromosome numbers (*n*) taken from Barneby (1964), Federov (1974), Goldblatt and Johnson (1991), Ledingham (1957, 1960), Ledingham and Fahselt (1964), Ledingham and Rever (1963), and Spellenberg (1976); * denotes species for which chromosome numbers are not available, but numbers listed here are based on published counts for species from same section.

[d] New World (NW)—Old World (OW) geographic distribution.

(Barneby, 1964, 1989a, b; Goncharov et al., 1965; Townsend and Guest, 1974). Vouchers for these species will be deposited in the University of Nevada, Reno herbarium (RENO); further information is available upon request from M. Sanderson or M. Wojciechowski.

Nuclear ribosomal DNA—The 18S–26S nuclear ribosomal RNA (nrDNA) gene family has proven to be a valuable tool for phylogeny reconstruction in plants (for reviews, see Hillis and Dixon, 1991; Hamby and Zimmer, 1992), especially at the family and higher taxonomic levels by DNA sequencing (Hamby and Zimmer, 1992) as well as among closely related genera or species by restriction site variation analysis (e.g., Sytsma and Schaal, 1985; Rieseberg, Soltis, and Palmer, 1988; Kim and Mabry, 1991). The nuclear genes that code for ribosomal DNA are arranged in a tandemly repeated unit that is found in high and variable copy number at the interspecific and intraspecific levels as well as within populations and single individuals (Rogers and Bendich, 1987; Schaal and Learn, 1988). The nrDNA units in angiosperms, separated by a large intergenic spacer (IGS), consist of a single transcribed region containing the external transcribed spacer (ETS), the 17–18S gene, an internal transcribed spacer (ITS 1), the 5.8S gene, a second internal transcribed spacer (ITS 2), and the 26S gene. The ubiquity of rRNA genes in nature and considerable evidence that the repeated unit consists of regions that have different rates of sequence divergence accounts for its phylogenetic utility. Recently, sequencing of the ITS regions has provided a new source of nuclear DNA characters for inferring intra- and intergeneric evolutionary relationships in the Compositae subtribe Madiinae (Baldwin, 1992), the plant pathogen *Phytophthora* (Lee and Taylor, 1992), and hominids (Gonzalez et al., 1990).

PCR and DNA sequencing—Polymerase chain reaction (PCR) amplification and dideoxy termination sequencing of the internal transcribed spacers of the nuclear ribosomal region in genomic DNA followed procedures described by Baldwin (1992; see his Fig. 1). Single-stranded DNAs of the ITS 1 and ITS 2 regions were amplified directly by 40 cycles of asymmetric PCR (20:1 molar ratio of primers) using the "ITS" primers described by White et al. (1990) (see below). Amplifications were performed in 25-μl reactions containing 10–100 ng genomic DNA, 200 μM deoxyribonucleotide triphosphates (equimolar), 0.5 units *AmpliTag* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), and oligonucleotide primers at 25 to 500 nmol. Initial PCR cycle conditions were 1 min at 97 C for denaturation, 1 min at 48 C for annealing, and 45 sec at 72 C for primer extension. Primer extension times were increased by 4 sec each subsequent cycle, followed by a final 7 min incubation at 72 C to complete the primer-template extensions. PCR products were purified by differential filtration in Millipore Ultrafree-MC tubes (Millipore UFC3 THK00). Purified DNAs were sequenced by the dideoxy chain termination technique using *Tag* DNA Polymerase (TAQuence®, U.S. Biochemical Co., Cleveland, OH), as described by Brow (1990), and [α-^{35}S]dATP (Amersham), with 7-deaza-dGTP substituted for dGTP to prevent base compressions, according to reaction conditions specified by the manufacturers. Samples were resolved by electrophoresis in 5% acrylamide-8 M urea gels. The gels were fixed in 5% methanol/5% glacial acetic acid for 30 min, transferred to Whatman 3MM paper, vacuum dried at 80 C for 1 hr, and exposed to autoradiographic film for at least 12 hr.

Single-stranded DNA containing 5′-18S rDNA-ITS 1-5.8S rDNA-3′ sequences was amplified using primers "ITS5" and "ITS2" (in molar excess) and sequenced using the primer "ITS5." Single-stranded DNA containing 5′-5.8S rDNA-ITS 2-25S rDNA-3′ sequences were amplified using primers "ITS3" and "ITS4" (in molar excess) and sequenced using the primer "ITS3" (White et al., 1990). For most taxa, sequences at the 3′ end of the ITS 2-25S rDNA region were determined by additional PCR amplifications using primer "ITS3" in excess, rather than "ITS4," and sequencing in the reverse direction (i.e., 5′-25S rDNA-5.8S rDNA-3′) using the primer "ITS4." The sequences reported in this study are available from GenBank under accession numbers L10756 through L10817.

Primers for PCR and sequencing reactions, "ITS2" (5′-GCTGCGTTCTTCATCGATGC-3′), "ITS3" (5′-GCATCGATGAAGAACGCAGC-3′), "ITS4" (5′-TCCTTCCGCTTATTGATATGC-3′), and "ITS5" (5′-G-

TABLE 3. *Phylogenetic analyses (unweighted) of ITS sequence data (Fig. 1) using four combinations of codings of indels*

Treatment	Indel coding		Most parsimonious trees		
	Missing[a]	Presence-Absence[b]	Number[c]	Length	Components[d]
1	Yes	No	11	238	A, B
2	No	No	166	223	A, B
3	No	Yes	7	258	A
4	Yes	Yes	2	272	A

[a] Positions in which alignment requires a gap for some taxa were scored as "missing" for those taxa and scored as observed base for remaining taxa (i.e., without gap).

[b] Positions at which alignment requires a gap for some taxa were scored as "0" for those taxa and "1" for taxa without gap.

[c] Number of most parsimonious trees found in heuristic searches.

[d] Types of trees found among the set of equally parsimonious trees. Trees A and B refer to the classes of trees shown in Fig. 3.

GAAGTAAAAGTCGTAACAAGG-3′) were obtained from the Macromolecular Structure Facility of the Division of Biotechnology at the University of Arizona and from Operon Technologies, Inc., Alameda, California.

Phylogenetic analyses—Phylogenetic trees were reconstructed using Fitch parsimony (i.e., assuming unordered character states) as implemented in PAUP 3.0s (Swofford, 1991). In view of the number of taxa included in this study, heuristic search strategies were employed. These included three regimes of addition sequences and branch-swapping: 1) CLOSEST addition sequence and TBR (tree bisection-reconnection) swapping; 2) SIMPLE addition sequence and TBR swapping; and 3) 500 replicates of RANDOM addition sequence with no swapping, followed by TBR swapping on the resulting set of trees. The last strategy is suggested as a means to uncover multiple islands of equally parsimonious trees if they exist (Maddison, 1991).

Character state changes were weighted equally in our analyses, except in one round in which character-state weighted parsimony was implemented (Albert and Mishler, 1992) in which transversions were weighted over transitions by a factor of 10:1 using PAUP's step matrix option. In this case, HEURISTIC searches were conducted using 1) CLOSEST addition and TBR branch-swapping, and 2) 100 RANDOM additions followed by TBR branch-swapping. The somewhat extreme factor of 10:1 was chosen so that, together with the unweighted analyses, runs that bracketed realistic values of transition/transversion biases could be examined.

Sets of equally parsimonious trees were summarized using strict consensus. Bootstrap analysis was employed to estimate the relative robustness (confidence limits) of individual clades. Bootstrapping was implemented in PAUP 3.0s using 500 replicates of heuristic searches that each entailed 50 random addition sequences. Initial searches established that this number of random addition sequences found one of the set of minimal trees found in more exhaustive searches. The initial goal of this study was to evaluate the monophyly of the aneuploid species of *Astragalus* as a test of prior cytotaxonomic and cpDNA hypotheses; hence reported bootstrap values for this group represent an a priori test not subject to the posterior multiple test problem (Felsenstein, 1985; Sanderson, 1989; Faith, 1991).

Alignment and treatment of "indels"—All ITS DNA sequences were aligned manually by sequential pairwise comparisons. This required the introduction of 28 small 1- or 2-bp indels (insertion/deletions) scattered among ITS 1 and 2, and two larger indels, one of 7 bp in ITS 1 (at positions 101–107) and one of 6 bp in ITS 2 (positions 275–280) (Fig. 1). A few small indels (e.g., positions 64–67, 164–165, 226–227) were required in regions in which alternative alignments were possible that could potentially have differing phylogenetic consequences. These positions were deleted from all phylogenetic analyses. The large, potentially synapomorphic, indel in ITS 1 (positions 101–107) was also omitted from the phylogenetic analyses, not because it led to ambiguous alignments, but because it potentially could exert such a major impact on the inferred phylogeny of the group. It was therefore desirable to exclude this indel initially to determine whether sequence evidence *alone* would corroborate the relationship implied by this major insertion/deletion.

Indels can be treated differently by parsimony analysis depending on how they are coded. Each gap can be recorded as a binary presence or absence of a character (the insertion/deletion), or each site can be coded "as is" with question marks (unknown/missing data). The latter method has the advantage that it retains information about substitutions that occur in other taxa in the indel region. It has the disadvantage that it does not convey the information regarding the evolutionary event/transformation involved in the insertion or deletion, and coding of missing data can introduce ambiguities (Platnick, Griswold, and Coddington, 1991). We examined all four combinations of these two coding techniques (Table 3), but focused on the coding of indels as missing data, using question marks (treatment 1 in Table 3), because we feel this scheme retains the important information about nucleotide substitutions in taxa with the insertion. Including the binary coded indels decreases the number of equally parsimonious trees (Table 3), but this may be due to the redundancy involved in having two sets of (somewhat differently coded) characters for the same indel events. Unless specified below, all results refer to coding using the scheme indicated in the first row of Table 3, that is, indels coded as missing data only.

RESULTS

DNA sequence analysis—The boundaries of the internal transcribed spacer and rDNA coding regions in the 31 taxa included here were identified by comparison to those of *Daucus carota, Nicotiana rustica, Vicia faba* (Yokota et al., 1989; Venkateswarlu and Nazar, 1991), and the genera of subtribe Madiinae (Baldwin, 1992). For most taxa, approximately 650 bp of the ITS 1-5.8S–ITS 2-25S rDNA regions were sequenced. However, we included only the ITS 1 and ITS 2 regions (Fig. 1) since sequence data for the 5.8S rDNA gene was incomplete for most taxa and not sufficiently variable to warrant inclusion.

ITS sequence, size, and variation—The length of the ITS 1 region in the taxa surveyed varied in length from 221 to 227 bp within *Astragalus* species to 231 bp in *Caragana*. Similarly, ITS 2 varied in length from 207 to 217 bp. Percent G + C content of ITS 1 sequences varied

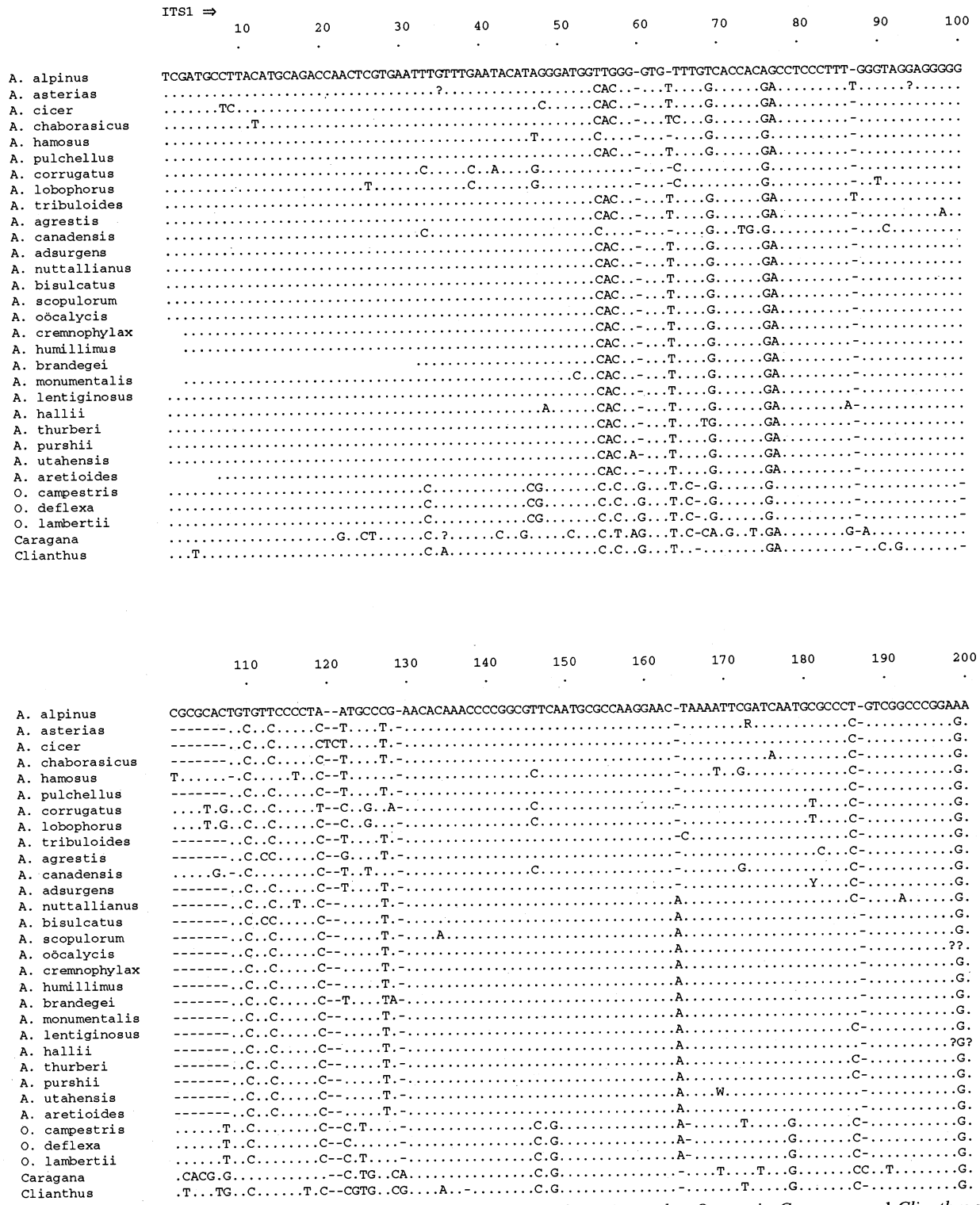

Fig. 1. Aligned nucleotide sequences of nrDNA internal transcribed spacers from *Astragalus, Oxytropis, Caragana,* and *Clianthus* species (see Table 2). Vertical columns represent nucleotide positions, numbered consecutively from 1 to 462 (5′ to 3′), within the nrDNA ITS 1 and ITS 2 spacers only. The beginning of the ITS 1 region (positions 1–237), at position number 1, and the beginning of the ITS 2 region (positions 238–462), at position number 238, are indicated by arrows. *Astragalus alpinus* is used as the reference taxon with only differences or ambiguities in the other taxa indicated at each position. Coding of ambiguous sites follow IUPAC nomenclature; hyphens = gaps; "M" = A or C, "R" = A or G, "S" = C or G, "W" = A or T, "Y" = C or T, "?" = nucleotides of unknown identity, and blanks represent sequence not determined.

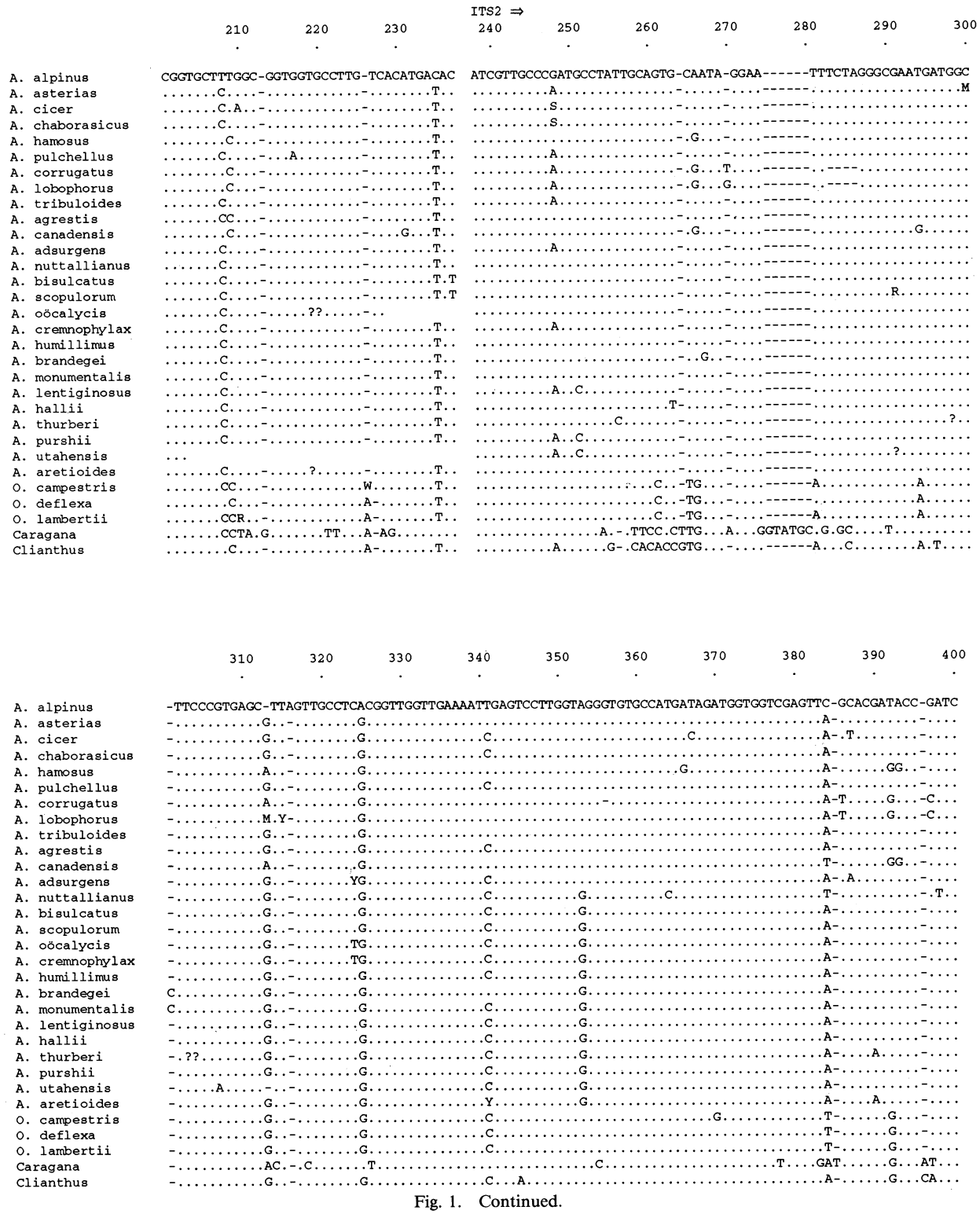

Fig. 1. Continued.

from 54.5% in *A. scopulorum* to 60.4% in *O. deflexa,* and in ITS 2 from 49.8% in *A. cicer* to 54.3% in *Clianthus.* The alignment of ITS 1 sequences for all taxa required one or more gaps at 23 of 237 possible nucleotide positions or 9.7% of the sites, while alignment of ITS 2 sequences required one or more gaps at 10.3% of all sites (23/224).

The number and distribution of nucleotide sites with variable states were calculated for all possible pairwise combinations of the ITS 1 and ITS 2 sequences presented in Fig. 1. Pairwise nucleotide differences were determined using the Distance Matrix option in PAUP version 3.0s (Swofford, 1991). Unalignable and undetermined sites

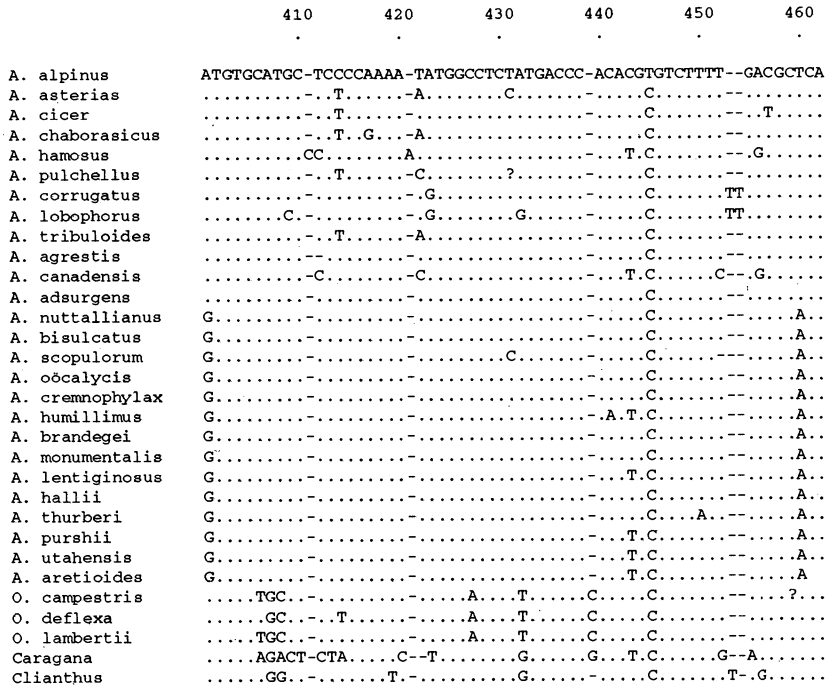

Fig. 1. Continued.

were not included. Of the aligned positions in ITS 1, 81 sites (34.2%) were variable (i.e., possessed one or more nucleotide differences in at least one taxon), while 78 sites in ITS 2 (34.8%) were variable. Within *Astragalus,* ITS 1 sequence divergence between pairs of species ranged from 0.0 to 10.2% (distance matrix not shown), while sequence divergence within *Oxytropis* ranged from only 0.4% to 1.3%. ITS 1 sequence divergence values from pairwise comparisons between *Astragalus* and the outgroups ranged from a minimum of 9.6% (*Clianthus*) to a maximum of 18.8% (*Caragana*). Sequence divergence in ITS 2 ranged from 0.0 to 7.9% within *Astragalus* species, and from 0.5% to 1.9% within *Oxytropis.* ITS 2 sequence divergence values from pairwise comparisons between *Astragalus* and the outgroups ranged from 10.8% for *Clianthus* to 12.7% for *Caragana.* These values are approximately ten times higher than values estimated for the chloroplast genome derived via restriction site mapping (Sanderson and Doyle, in press).

***Phylogenetic analysis*—**The results reported here were obtained when indels were coded as missing values in the phylogenetic analyses (see Materials and Methods and treatment 1 of Table 3). The same set of 11 trees of 238 steps (consistency index = 0.63 for the informative characters) was uncovered in all unweighted searches. The strict consensus (Fig. 2) is highly resolved, and several clades are supported with relatively high bootstrap values. The 11 trees consist of two sets of trees that differ most conspicuously with respect to whether *A. alpinus* is the sister group to the remainder of the genus (four trees), or is nested among *A. hamosus, A. canadensis, A. corrugatus,* and *A. lobophorus* (seven trees). A representative of each of these two classes of trees is shown in Fig. 3, and these trees will be characterized as type "A" and "B," respectively.

Branch lengths are indicated on the trees in Fig. 3 to illustrate divergence levels. The four genera are all quite divergent from one another. Several groups within *Astragalus* are highly divergent, including a major clade composed of the North American aneuploids plus many of the Old World and circumboreal euploids (the clade marked "a" in Figs. 2, 3).

The bootstrap majority rule tree (not shown) is the same as the strict consensus except that several clades in the strict consensus were below 50% and did not appear in the majority rule tree (see Fig. 2). In all analyses, the morphology of the aneuploid *Astragalus* (clade marked "b" in Figs. 2, 3) is one of the best supported results. Bootstrap confidence for this clade is 88%, and the node is supported by three nonhomoplastic substitutions in ITS 2 (positions 353, 401, and 460; Fig. 1), and by one homoplastic single base pair insertion at positive 164 in ITS 1 (Fig. 1).

Low sequence divergence within the aneuploid clade results in poor resolution and low bootstrap levels, but some conclusions emerge. A clade containing *A. lentiginosus, A. utahensis,* and *A. purshii* (Fig. 2) is probably monophyletic (bootstrap confidence level 68%). These three species are representatives of a major taxonomic group within North American *Astragalus,* the large-flowered Piptolobi of Barneby (1964), previously suggested to be monophyletic from an analysis of cpDNA restriction site variation (Sanderson and Doyle, in press). *Astragalus aretioides,* recently placed in the resurrected genus *Orophaca* by Isely (1983), is clearly nested within aneuploid *Astragalus* based upon ITS sequence data. Finally, the hypothesis that *A. nuttallianus,* an annual, is basal in the aneuploid clade, which has been suggested based on

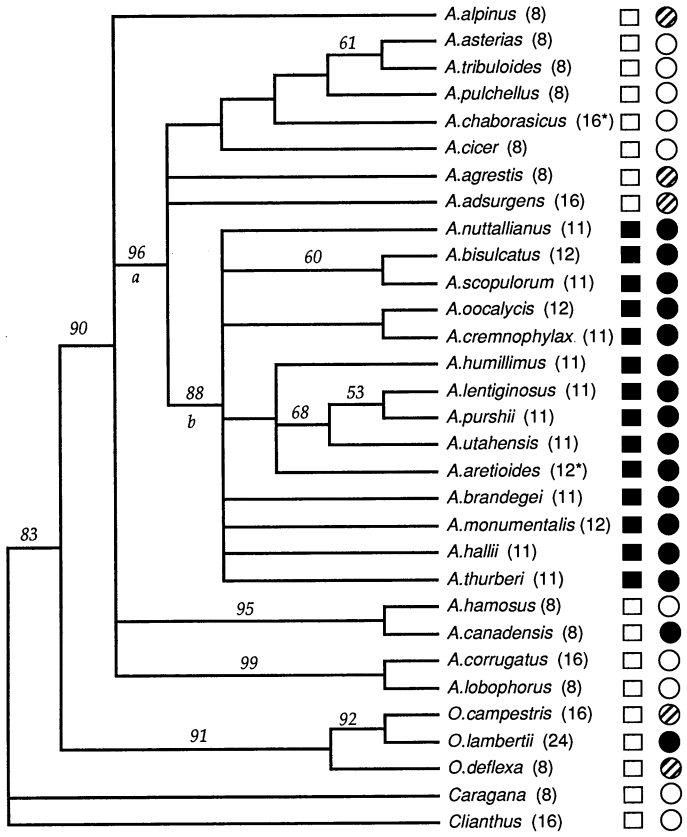

Fig. 2. Strict consensus of the 11 most parsimonious trees of 238 steps derived from heuristic analyses (unweighted) of ITS 1–ITS 2 DNA sequence data (Fig. 1), with indels coded as missing values. *Caragana* and *Clianthus* were designated as outgroups. Numbers above branch points are bootstrap percentage values for clades found in both the strict consensus and bootstrap majority rule trees. Chromosome number(s) follow taxon name: open squares represent species in the euploid series, $n = 8, 16, 24$; filled-in squares represent species in the aneuploid series, $n = 11, 12, 13, 14$, and 15. Old World species are represented by the open circles; filled-in circles represent species restricted to North America; the cross-hatched circles represent species of circumboreal distribution. "a" and "b" = clades referred to in the text; * = species for which chromosome numbers are not available, but numbers listed here are based on published counts for species from same section (see Table 2).

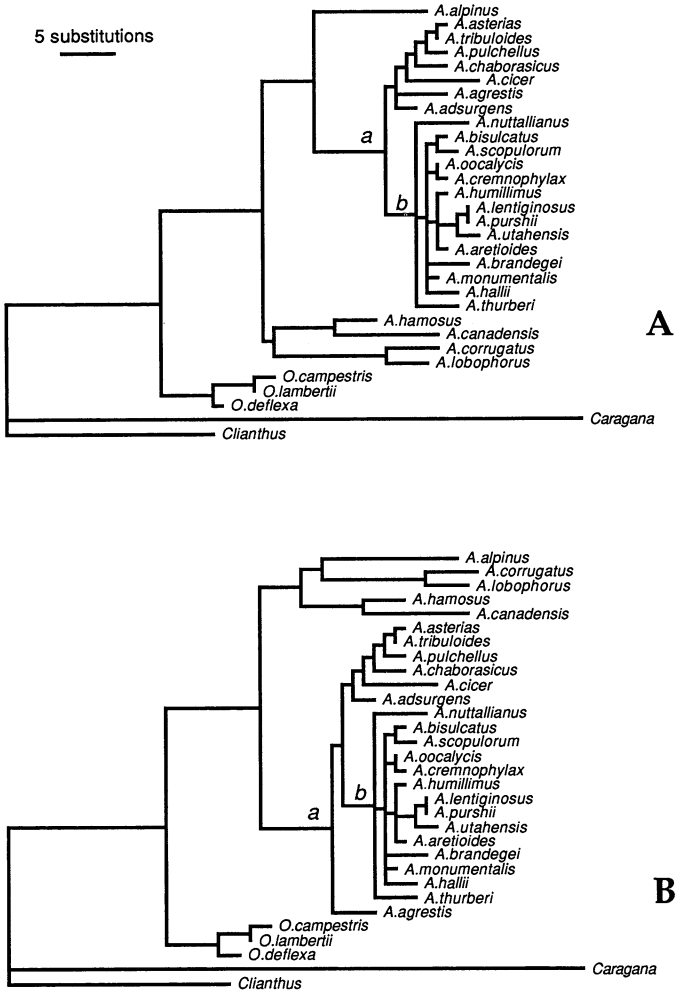

Fig. 3. Representatives of the two sets of trees found among the 11 most parsimonious trees derived from the unweighted analysis of ITS DNA sequence data presented in Fig. 1. These trees differ in the placement of *A. alpinus,* either as sister group to the clade marked "a" containing the aneuploid species (clade marked "b") plus several of the Old World euploids (tree type A), or nested within the clade containing *A. canadensis, A. hamosus, A. lobophorus,* and *A. corrugatus* (tree type B). Lengths of branches are proportional to the number of substitutions inferred.

cpDNA evidence (Liston, 1992; Sanderson and Doyle, in press), is not refuted by the ITS phylogeny, but the lack of resolution does not provide positive support either.

The aneuploid *Astragalus* plus a subset of the remaining euploid *Astragalus* (Fig. 2, clade labeled "a") form a very well-supported clade (bootstrap confidence level 96%). A multibase deletion in ITS 1 (indel at positions 101–107) also supports this group, and can be mapped onto the cladogram with no homoplasy. Included among the possible sister groups to the aneuploids in this clade are circumboreal euploid species, such as the perennials *A. adsurgens* (sect. *Onobrychoidei*) and *A. agrestis* (sect. *Hypoglottoidei*), as well as Old World endemics that include both annual and perennial groups.

Astragalus is monophyletic in the strict consensus and is supported at the 90% level by bootstrap analysis. Likewise, *Oxytropis* appears monophyletic (supported at the 91% confidence level), but in some bootstrap replicates it is found nested among the Old World euploid *Astragalus*—although never within the very highly supported subclade "a" in Figs. 2, 3. The phylogenetic status of these two genera and relationships to their relatives in the tribe Galegeae will be addressed elsewhere (Sanderson and Liston, in press; Sanderson and Wojciechowski, unpublished data).

Character state weighting of the data presented in Fig. 1 produced a set of 36 trees of 1,039 steps (note the lengths of these trees are long because transversions count ten steps). The strict consensus for the weighted trees is basically the type "A" tree shown in Fig. 3, except the aneuploid *A. brandegei* is the sister group to the rest of the aneuploid clade. None of the 36 trees was the same as any of the 11 unweighted trees; in fact all were 238 steps, one step longer than the shortest trees derived from unweighted analyses. Likewise, the lengths of the 11 unweighted trees ranged from 1,047 to 1,065 steps when the

matrix was weighted. The aneuploid group (Fig. 2, clade labeled "b"), the aneuploid + part of the euploid clade (Fig. 2, clade "a"), and the genera *Astragalus* and *Oxytropis* were all well-supported clades in the weighted analysis.

Results under other coding schemes for indels—As noted above, four combinations of coding schemes for the indels in the unweighted analyses were explored (Table 3). The number of trees found in heuristic searches varied from two to 166, but the topologies were always close to either the type A or B tree (or both) shown in Fig. 3. In all trees in these analyses, the North American aneuploid *Astragalus* species were a monophyletic group.

DISCUSSION

Chromosome evolution in Astragalus—Interpretations of the cytogenetic differences between Old and New World *Astragalus* have varied widely (reviewed in Spellenberg, 1976). Ledingham (1960) discussed two explanations for the existence of distinct euploid and aneuploid series: 1) that New World species having $n = 11$, 13, and higher numbers are derived from species with $n = 12$; and 2) that species with $n = 8$ and $n = 11$ are both derived by descending aneuploidy from higher numbers. Turner and Fearing (1959) went so far as to suggest that the two groups were independently derived from a polyphyletic Galegeae, which was in turn derived from several other tribes. More recently, Barneby (1964), Ledingham and Pepper (1973), and Spellenberg (1976) have argued for aneuploid reduction from a tetraploid ($n = 16$) or "hypotetraploid" ($n = 15$) derived from $n = 8$. Two Old World species are reported to have $n = 15$ (Fedorov, 1974; Spellenberg, 1976) even though they are placed in euploid sections, suggesting that hypotetraploidy recurs at some low rate. Similarly, species with $n = 12$ also recur occasionally in the Old World (Ledingham, 1960; Goldblatt and Johnson, 1991).

The ITS sequence-based phylogeny presented here allows us to reject some of these hypotheses but not others. A polyphyletic *Astragalus* is clearly not supported by these data, which support instead a monophyletic *Astragalus* as the sister group to *Oxytropis*. This conclusion is upheld in a broader analysis of the tribe Galegeae (Sanderson and Wojciechowski, unpublished data), which includes outgroups from the Milletieae and several of the northern temperate, herbaceous legume tribes marked by lack of the inverted repeat in the chloroplast genome (Lavin, Doyle, and Palmer, 1990).

The phylogenetic evidence also argues against Ledingham's hypothesis that $n = 8$ could be produced by descending aneuploidy. The direction of change in chromosome number appears to be from $n = 8$ to $n = \{11, 12, \ldots\}$, rather than the reverse. Whether the New World aneuploid series arose by descending aneuploidy from a tetraploid $n = 16$, or by *both* ascending and descending aneuploidy from an $n = 12$ ancestor, remains unclear, however. ITS sequence divergence within the aneuploids is very low, leading to poor resolution and weak support for relationships within this clade. Species with $n = 11$ and $n = 12$ were included in our analysis, but neither state can clearly be assigned to the base of the aneuploid clade (Fig. 2). Inclusion of New World species with higher numbers (i.e., $n = 13$–15) might offer a better test of the directionality of aneuploidy, but it appears unlikely that ITS sequence data alone will provide sufficient resolution to unambiguously determine the ancestral chromosome number in the aneuploid group.

The cladogram in Fig. 2 indicates that the differentiation of the aneuploid clade occurred in the New World after some amount of differentiation of major Old World groups. It does not indicate whether the first aneuploids originated in the New World or were present in the Old World at one time and then dispersed or migrated into the New World.

One issue that may be resolved with additional nrDNA sequence data is whether some or all of the rare Old World aneuploids are part of the New World aneuploid clade, perhaps as their relictual sister groups, or if these species indeed represent independent origins of aneuploidy. The latter seems more likely based on morphology, since each Old World aneuploid identified so far is found in an Old World section that is homogeneously euploid (Goncharov et al., 1965; Chamberlin and Matthews, 1970; Townsend and Guest, 1974; Lock and Simpson, 1991). Furthermore, these sections are not closely related, so it appears likely that multiple origins of aneuploidy in *Astragalus* have occurred, perhaps via nondisjunction in $n = 8$ crosses, hybridization between $n = 8$ and $n = 16$ species, or aneuploid reduction from $n = 16$, as in the case of the $n = 15$ species. However, this does not rule out the possibility that *one* of the Old World aneuploids is indeed the sister group. Because the New World aneuploids are not marked by any obvious set of morphological synapomorphies, morphology is little help in resolving this issue. A definitive test would be to sequence the ITS 2 region of nrDNA for these Old World aneuploids and check for the presence of the three nucleotide synapomorphies that are indicative of the North American aneuploid clade. It would certainly be remarkable if a single relictual species in the Old World is all that remained from a period dating back to the origin of a clade that eventually differentiated to include some 500 species in the New World.

Biogeography of circumboreal euploid species—The phylogenetic distribution of the circumboreal taxa in our analyses is consistent with multiple origins of circumboreal distributions. This would be true even if one of the circumboreal groups were shown to be the sister group to the aneuploid clade. The sister group of the aneuploid clade is unclear partly because of lack of resolution at that node, but potentially includes some of the circumboreal species as well as Old World euploid groups. However, the circumboreal species sampled belong to sections predominantly composed of euploid Old World endemics. A sister group relationship of the New World aneuploid clade to one of these species would therefore require that the entire aneuploid clade be derived from within one of these predominantly Old World sections. This is not impossible, but the implications for the biogeography of the *remainder* of the circumboreal euploids would be unaffected; they would still represent multiple invasions or expansions into the New World.

Radiation of the aneuploid group—It is tempting to regard the New World aneuploid group as a major radiation in the genus, and seek explanations based, for

example, on chromosomal mechanisms such as the adaptive advantages due to reduced recombination (e.g., Spellenberg, 1976, p. 475; Stebbins, 1950). However, recent phylogenetic analyses of adaptive radiation and evolutionary success in a phylogenetic context suggest caution (Doyle and Donoghue, in press; Jensen, 1990; Raikow, 1988). Before explanations are sought for an apparent pattern, the phylogeny must demonstrate that a pattern exists. The mere existence of a "group" with many species does not require an evolutionary explanation, because groups of all sizes exist in the taxonomic hierarchy. Diversity must either be compared relative to some equivalent taxon, such as a sister group, or diversification rate must be estimated absolutely (Sanderson and Bharathan, in press). Among the possible sister groups to the large clade "a" of Fig. 2 are very diverse sections and very small sections. Because the nearest sister group to the New World aneuploids is still uncertain, it is therefore not clear whether the aneuploids differentiated faster or slower than the typical rate found among groups in the genus.

Moreover, the rate of diversification *within* the New World aneuploids may also have been heterogeneous. Groups that diverged early may have remained depauperate while those splitting off later radiated rapidly, generating the bulk of diversity now evident within the aneuploid groups. The single, highly derived (Sanderson and Doyle, in press) Piptoloboid section *Argophylli* (exemplified by *A. purshii* in this work) accounts for some 10% of the species in North America, although it is only one of 93 sections. Clearly, a more resolved phylogeny of the genus will be necessary before hypotheses of evolutionary success can be tested further.

A cryptic clade—It is intriguing that so strongly supported a clade as the aneuploid North American *Astragalus,* supported now by three independent genotypic lines of evidence (chromosomal, nuclear rDNA, and chloroplast DNA), is marked by no major morphological innovations that might have led to its recognition in previous classifications. Such clear cases of mosaic evolution involving nonmorphological characters raise important taxonomic issues. Should well-supported but morphologically indistinct clades be recognized in formal classifications? Of all the authors who have speculated on the implications of the cytogenetic split in the genus, only Ledingham (1957, p. 665; 1960, p. 126) has suggested that the North American aneuploid species (and presumably the closely related South American species), should be placed in a new genus separate from the euploid species of *Astragalus* and *Oxytropis* of the Old World. The molecular evidence presented here suggests that this aneuploid clade will be shown to be at least as well supported as many of the traditional Old World subgenera whose representatives are shown in Fig. 2, and it is much better supported than the informal phalanxes recognized by Barneby in North America (Sanderson, 1991; Sanderson and Doyle, in press). We believe strongly that in the interest of efficient communication of phylogenetic information, we should apply a formal name to this aneuploid clade. At the same time, we recognize this as somewhat premature, because additional sampling of both euploid and aneuploid taxa will identify outgroups and solidify the diagnostic synapomorphies for the aneuploid clade. In the meantime, following Barneby's example of recognizing informal but useful categories in the genus, we suggest the name "Neo-astragalus" for the New World aneuploid clade.

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