Phylogenetic analysis of eastern Asian and eastern North American disjunct *Lespedeza* (Fabaceae) inferred from nuclear ribosomal ITS and plastid region sequences

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Lespedeza (tribe Desmodieae, Fabaceae) follows a disjunct distribution in eastern Asia and eastern North America. Phylogenetic relationships among its species and related taxa were inferred from nuclear ribosomal internal transcribed spacer (ITS) and plastid sequences (*trnH-psbA, psbK-psbI, trnK-matK* and *rpoC1*). We examined 35 species of *Lespedeza*, two of *Kummerowia* and one of *Campylotropis*, the sole constituents of the Lespedeza group. An analysis of these data revealed that the genus *Campylotropis* is sister to the other two genera. However, we were unable to resolve the relationships between *Kummerowia* and *Lespedeza* in the strict consensus trees of parsimony analyses based on plastid and combined DNA data. In the genus *Lespedeza*, the Old World subgenus *Macrolespedeza* is monophyletic, whereas the transcontinental subgenus *Lespedeza* is paraphyletic. Monophyly of eastern Asian species and of North American species is strongly supported. Although inconsistent with the traditional classification, this phylogenetic finding is consistent with seedling morphology. Three subgroups recognized in subgenus *Macrolespedeza* were unresolved in our phylogenetic trees. An incongruence length difference (ILD) test indicated that the two partitions (nuclear ITS and plastid sequences) were significantly incongruent, perhaps because of hybridization between species in *Lespedeza*. Most of the primary clades of tribe Desmodieae are Asian, implying that the relatively few New World ones, such as those in *Lespedeza*, are more recently derived from Asia. © 2010 The Linnean Society of London, Botanical Journal of the Linnean Society, 2010, **164**, 221–235.

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INTRODUCTION

The genus *Lespedeza* Michaux is included in subtribe Lespedezinae Michx., tribe Desmodieae Benth. & Hook.f., of Fabaceae (Ohashi, Polhill & Schubert, 1981). The subtribe is characterized by one-seeded fruits compared with other members of the tribe, which have jointed fruits and multiple seeds or loments. Based mainly on molecular data, the subtribe has been called 'the Lespedeza group' (Ohashi, 2005) and was recently described as comprising only three genera: *Lespedeza*, *Campylotropis* Bunge and *Kummerowia* Schindl. The first two are distinguished from each other by the following characteristics: keel petals straight and blunt vs. falcate and acute; primary bracts on the inflorescence axis subtending two

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flowers, i.e. 'pseudoraceme' (Lackey, 1981; Tucker, 1987) vs. one flower (simple raceme); and bracteoles persistent vs. deciduous. In contrast, *Kummerowia* differs from those two genera as follows: lateral veins extending to the margin vs. looped within the margin; annual herbs vs. perennial shrubs or herbs; and flowers and fruits subtended by several bract-like structures vs. not (Ohashi *et al.*, 1981). The intergeneric relationship among these three genera has been debated (Table 1). Akiyama & Ohba (1985) suggested, based on inflorescence morphology, that *Lespedeza* is more similar to *Campylotropis* than to *Kummerowia*. However, a closer relationship between *Lespedeza* and *Kummerowia* has been suggested based on floral structure (Nemoto & Ohashi, 1988) and inflorescence morphology (Nemoto & Ohashi, 1990, 1993a). Moreover, Nemoto, Ohashi & Tamate (1995) confirmed this relationship using restriction fragment length polymorphism (RFLP) of plastid DNA. In addition, Nemoto & Ohashi (1996) regarded the characteristic racemose inflorescence of *Campylotropis* as an extremely reduced form of the pseudoracemes of *Lespedeza* and *Kummerowia*. Although that is based on organographic and developmental observations, *Campylotropis* is distinguished from the latter by its inflorescence.

Lespedeza comprises approximately 40 species, of which 30 are found in eastern Asia, with the remainder occurring in eastern North America (Ohashi *et al.*, 1981). Historically, Maximowicz (1873) recognized two sections in subgenus *Lespedeza* (the present genus *Lespedeza*) (Table 1): (1) section *Macrolespedeza* Maxim., containing only Asian species which produce only flowers with corollas (chasmogamous); and (2) section *Eulespedeza* Maxim. [= section *Lespedeza*], comprising both American and Asian species that bear flowers with and without corollas (cleistogamous). Schindler (1913) and Nakai (1927) followed this traditional treatment and Nakai (1939) introduced a new section, *Heterolespedeza* Nakai, based on bud phyllotaxy, that contained two species, *Lespedeza buergeri* Miq. and *Lespedeza maximowiczii* C.K.Schneid. Ohashi (1982a) later divided *Lespedeza* into two subgenera, *Lespedeza* and *Macrolespedeza*, with section *Heterolespedeza* within subgenus *Macrolespedeza*. Akiyama (1988) further divided section *Macrolespedeza* into three series [*Macrolespedeza* Maxim., *Formosae* S.Akiyama & H.Ohba and *Heterolespedeza* (Nakai) S.Akiyama & H.Ohba]. *Heterolespedeza* is distinguished from *Macrolespedeza* by its distichously arranged winter bud scales that are arranged spirally. *Macrolespedeza* differs from *Formosae* in having standard petals with an attenuated base, whereas the latter has petals with a clawed base.

The genus *Lespedeza* exhibits a well-known disjunct distribution between eastern Asia and eastern North America. Maximowicz (1873) recognized four groups as series (*Violaceae* Maxim., *Junceae* Maxim., *Lespedezariae* (Torr. & A. Gray) Maxim. and *Pilosa* Maxim.) in his section *Lespedeza* (the present subgenus *Lespedeza*) (Table 1) and grouped disjunct Asian and North American species within series *Violaceae* and *Lespedezariae*. In contrast, Schindler (1913) recognized three lineages in subgenus *Lespedeza*. One comprised 14 Asian species, the second, only North American species and, the third, three North American and one Asian species (*L. tomentosa* (Thunb.) Siebold ex Maxim.). Nemoto & Ohashi (1993b) suggested a monophyletic origin for North American species from an Asian ancestor based on seedling morphology. All Asian species, in both subgenera *Macrolespedeza* and *Lespedeza,* develop two opposite unifoliolate eophylls subsequent to cotyledons, followed by alternate trifoliolate metaphylls, whereas the North American species have only one unifoliolate eophyll between cotyledons and alternate trifoliolate metaphylls. Floristic relationships and similarity between eastern Asian and eastern North American species have been reviewed several times (Li, 1952; Tiffney, 1985; Boufford, 1998; Wen, 1998, 1999) and most of those disjunct genera have been considered monophyletic (Wen, 2001). Despite the long-standing interest, phylogenetic relationships among taxa displaying the disjunction have remained obscure (Xiang, Soltis & Soltis, 1998) and these taxa are still not well studied, perhaps because of their wide geographical range (Wen, 1999).

Our main foci here were: (1) phylogenetic relationships among the species of *Lespedeza* and related taxa and (2) phytogeographical implications in relation to the disjunct distribution between eastern Asia and eastern North America that arise from analyses of nuclear ribosomal and plastid DNA data.

MATERIAL AND METHODS

TAXON SAMPLING

In this study, we sampled 35 species of the genus *Lespedeza*, 25 from east Asia and 10 from eastern North America (Table 2). To investigate other relationships, we also examined both species of *Kummerowia* and one of the 37 species of *Campylotropis*. To assess the disjunct distribution between those two continents, we sampled 13 species of subgenus *Macrolespedeza* and 22 species of subgenus *Lespedeza*, 12 from east Asia and 10 from North America. Two species from *Hylodesmum* H.Ohashi & R.R.Mill were included as an outgroup, because members of that genus (previously included in *Desmodium* Desv.) are sister to the Lespedeza group, as inferred from molecular phylogenetic research of plastid sequences for almost all legumes (Kajita *et al.*, 2001) or phaseo-

Table 1. Previous taxonomic treatments of *Lespedeza* and related taxa

Maximowicz (1873)	Schindler (1913)	Nakai (1927, 1939*)	Ohashi (1982a), Ohashi *et al.* (2009a & b)	Akiyama (1988)
Genus *Lespedeza* Michaux Subgenus *Campylotropis* (Bunge) Maxim.	Genus *Campylotropis* Bunge	Genus *Lespedeza* Michaux Section *Campylotropis* (Bunge) Bentham		
Subgenus *Lespedeza* Section *Macrolespedeza* Maxim.	Genus *Lespedeza* Michaux Section *Macrolespedeza* Maxim.	Section *Macrolespedeza* Maxim.	Genus *Lespedeza* Michaux Subgenus *Macrolespedeza* (Maxim.) H.Ohashi Section *Macrolespedeza*	Genus *Lespedeza* Michaux Section *Macrolespedeza* Maxim. Series *Macrolespedeza* Series *Formosae* S.Akiyama & H.Ohba
		Section *Heterolespedeza* Nakai*	Section *Heterolespedeza* Nakai	Series *Heterolespedeza* (Nakai) S.Akiyama & H.Ohba
Section *Eulespedeza* Series *Violaceae* Maxim. Series *Junceae* Maxim. Series *Lespedezariae* (Torr. & A.Gray) Maxim. Series *Pilosa* Maxim.	Section *Eulespedeza*	Section *Eulespedeza*	Subgenus *Lespedeza*	Section *Lespedeza*
Subgenus *Microlespedeza* Maxim.	Genus *Kummerowia* Schindl			

Table 2. Voucher specimens and GenBank accession numbers of the materials examined for nuclear ribosomal internal transcribed spacer (ITS) and plastid sequences

Taxon	Voucher	ITS	*trnK-matK*	*psbK-psbI*	*trnH-psbA*	*rpoC1*
Lespedeza angustifolia (Pursh) Elliott	USA; H. Ohashi *et al*. 61079 (TUS)	GU572169	GU572328		GU572289	GU572249
	USA; A.F. Clewell & R.K Godfrey 2067 (TUS)			GU572209		
L. bicolor Turcz.	Korea; C.Y. Yoon *s.n.* (IUI)	GU572170	GU572329	GU572210	GU572290	GU572250
L. buergeri Miq.	Japan; B.H. Choi *et al*. 9811 (IUI)	GU572171	GU572330	GU572211	GU572291	GU572251
L. capitata Michx.	USA; K.H. Chung 479 (IUI)	GU572172	GU572331	GU572212	GU572292	GU572252
L. caraganae Bunge	China; H. Ohashi & T. Nemoto 62004 (TUS)	GU572173	GU572332	GU572213	GU572293	GU572253
L. chinensis G.Don	Taiwan; T. Nemoto 2392 (TUS)	GU572174	GU572333	GU572214	GU572294	GU572254
L. cuneata (Dumon de Courset) G.Don	Korea; J.E. Han 092 (IUI)	GU572175	GU572334	GU572215	GU572295	GU572255
L. cyrtobotrya Miq.	Korea; B.H. Choi *et al*. 9813 (IUI)	GU572176	GU572335	GU572216	GU572296	GU572256
L. davidii Franch.	Korea; J.S. Han *s.n.* (IUI)	GU572178	GU572337	GU572218	GU572298	GU572258
L. daurica (Laxm.) Schindl.	Korea; J.E. Han 105 (IUI)	GU572177	GU572336	GU572217	GU572297	GU572257
L. floribunda Bunge	Korea; J.E. Han 618 (IUI)	GU572179	GU572338	GU572219	GU572299	GU572259
L. formosa (Vogel) Koehne subsp. *velutina* (Nakai) S.Akiyama & H.Ohba	Korea; B.H. Choi *et al*. 9814 (IUI)	GU572180	GU572339	GU572220	GU572300	GU572260
L. forrestii Schindl.	China; T. Nemoto *et al*. 96803001 (TUS)	GU572181	GU572340	GU572221	GU572301	GU572261
L. hirta (L.) Hornem.	USA; T. Nemoto & J. Yokoyama 95817001 (TUS)	GU572182	GU572341	GU572222	GU572302	GU572262
L. homoloba Nakai	Japan; B.H. Choi *et al*. 9815 (IUI)	GU572183	GU572342	GU572223	GU572303	GU572263
L. inschanica (Maxim.) Schindl.	Korea; J.E. Han 311 (IUI)	GU572184	GU572343	GU572224	GU572304	GU572264
L. intermedia (S.Watson) Britton	USA; B.H. Choi 9816 (IUI)	GU572185	GU572344	GU572225	GU572305	GU572265
L. japonica L.H.Bailey	Japan; B.H. Choi *et al*. 9817 (IUI)	GU572186	GU572345	GU572226	GU572306	GU572266
L. juncea (L.f.) Pers.	Korea; J.E. Han 093 (IUI)	GU572187	GU572346	GU572227	GU572307	GU572267
L. lichiyuniae T.Nemoto, H.Ohahi & T.Itoh	Korea; J.E. Han 199 (IUI)	GU572189	GU572348	GU572229	GU572309	GU572269
L. leptostachya Engelm.	USA; P. Frana & M. J. Leoschke 37 (ISC)	GU572188	GU572347	GU572228	GU572308	GU572268
L. maritima Nakai	Korea; B.H. Choi *et al*. 9707 (IUI)	GU572190	GU572349	GU572230	GU572310	GU572270

Table 2. *Continued*

Taxon	Voucher	ITS	*trnK-matK*	*psbK-psbI*	*trnH-psbA*	*rpoC1*
L. maximowiczii C.K.Schneid.	Korea; J.E. Han 547 (IUI)	GU572191	GU572350	GU572231	GU572311	GU572271
L. melanantha Nakai	Korea; K.H. Chung 336 (IUI)	GU572192	GU572351	GU572232	GU572312	GU572272
L. nipponica Nakai	Japan; B.H. Choi *et al.* 9820 (IUI)	GU572193	GU572352	GU572233	GU572313	GU572273
L. patens Nakai	Japan; B.H. Choi *et al.* 9821 (IUI)	GU572194	GU572353	GU572234	GU572314	GU572274
L. pilosa (Thunb.) Siebold & Zucc.	Korea; J.E. Han 318 (IUI)	GU572195	GU572354	GU572235	GU572315	GU572275
L. procumbens Michx.	USA; H. Ohashi *et al.* 60412 (TUS)	GU572196	GU572355	GU572236	GU572316	GU572276
L. repens (L.) Barton	USA; T. Nemoto & J. Yokoyama 95816027 (TUS)	GU572197	GU572356	GU572237	GU572317	GU572277
L. stuevei Nutt.	USA; H. Ohashi *et al.* 61092 (TUS)	GU572198	GU572357	GU572238		GU572278
L. thunbergii (DC.) Nakai	Japan; B.H. Choi *et al.* 9823 (IUI)	GU572199	GU572358	GU572239	GU572318	GU572279
L. tomentosa (Thunb.) Siebold ex Maxim.	Korea; J.E. Han 240 (IUI)	GU572200	GU572359	GU572240	GU572319	GU572280
L. violacea (L.) Pers.	Japan; B.H. Choi 9824 (IUI)	GU572202				
	Japan; B.H. Choi 9828 (IUI)		GU572361	GU572242	GU572321	GU572282
L. virgata (Thunb.) DC.	Korea; J.E. Han 53 (IUI)	GU572203	GU572362	GU572243	GU572322	GU572283
L. virginica (L.) Britton	USA: T. Nemoto & J. Yokoyama 95817018	GU572201	GU572360	GU572241	GU572320	GU572281
Campylotropis macrocarpa (Bunge) Rehder	Japan; B.H. Choi 9808 (IUI)	GU572164	GU572323	GU572204	GU572284	GU572244
Kummerowia stipulacea (Maxim.) Makino	Korea; B.H. Choi *et al.* 9826 (IUI)	GU572167	GU572326	GU572207	GU572287	GU572247
K. striata (Thunb.) Schindl.	Korea; B.H. Choi *et al.* 9827 (IUI)	GU572168	GU572327	GU572208	GU572288	GU572248
Hylodesmum oldhamii (Oliv.) H.Ohashi & R.R.Mill	Korea; B.H. Choi 9809 (IUI)	GU572165	GU572324	GU572205	GU572285	GU572245
H. podocarpum (DC.) H.Ohashi & R.R.Mill	Korea; B.H. Choi 9810 (IUI)	GU572166	GU572325	GU572206	GU572286	GU572246

loid legumes (Stefanović *et al.*, 2009). Voucher specimens of the samples collected were deposited in herbaria at Inha University (IUI) and Tohoku University (TUS).

MOLECULAR TECHNIQUES

Total DNA was extracted from either silica gel-dried leaves or dry leaves from herbarium samples, using a modified cetyl trimethylammonium bromide (CTAB) procedure (Doyle & Doyle, 1987) and a G-spin IIp for Plant Genomic DNA Extraction Kit (Intron Bio Co., Korea). The internal transcribed spacer (ITS) of nuclear ribosomal DNA and plastid sequences have been widely applied for phylogenetic studies to resolve relationships between closely related genera in Fabaceae (Wojciechowski, Lavin & Sanderson, 2004; Ribeiro *et al.*, 2007; Saslis-Lagoudakis *et al.*,

Table 3. Sequences of primers used for PCR amplification and sequencing of nuclear ribosomal internal transcribed spacer (ITS) and four plastid regions

Region	Primer	Sequence (5′–3′)
ITS	ITS4	TCCTCCGCTTATTGATATGC
	ITS5	GGAAGTAAAAGTCGTAACAAGG
trnK-matK	matK6	TGGGTTGCTAACTCAATGG
	matKAR	CTGTTGATACATTCGA
psbK-psbI	psbK	GTTTTCCCAGTCACGACTTAGCCTTTGTTTGGCAAG
	psbI	CAGGAAACAGCTATGACAGAGTTTGAGAGTAAGCAT
trnH-psbA	trnH	GTTTTCCCAGTCACGACCGCGCATGGTGGATTCACAATCC
	psbA	CAGGAAACAGCTATGACGTTATGCATGAACGTAATGCTC
rpoC1	rpoC1F	GTTTTCCCAGTCACGACGTGGATACACTTCTTGATAATGG
	rpoC1R	CAGGAAACAGCTATGACCCATAAGCATATCTTGAGTTGG

2008; Stefanović *et al.*, 2009). Moreover, combined analyses of ITS and plastid sequences have been conducted to address phytogeographic and phylogenetic questions relating to other plant taxa that show disjunct distributions between east Asia and North America (Xiang *et al.*, 1998; Nie *et al.*, 2006; Jiao & Li, 2007; Havill *et al.*, 2008). Here, we analysed the nuclear ribosomal ITS and four plastid regions (*trnH-psbA, trnK-matK, psbK-psbI* and *rpoC1*), because these have been used successfully for phylogenetic studies of closely related plants (Shaw *et al.*, 2005) and/or plant DNA barcoding studies (Kress *et al.*, 2005; Ford *et al.*, 2009; Clerc-Blain *et al.*, 2010). We conducted PCR amplifications of the complete ITS regions and plastid regions from template DNA following the protocols of Choi & Kim (1997) and Miller & Bayer (2001), respectively. Primer sequences for each region are presented in Table 3.

Sequences from both strands were obtained under BigDyeTM terminator cycling conditions. The products were purified via ethanol precipitation and run on an Automatic Sequencer (Model 3730xl; Applied Biosystems, USA). Sequences were aligned by CLUSTAL X (1.81) and were visually adjusted to resolve minor conflicts.

PHYLOGENETIC ANALYSES

Sequence data were analysed by maximum parsimony (MP) and Bayesian approaches. These analyses were performed separately for the nuclear ITS and four plastid regions and the two DNA sequences were also concatenated in a combined analysis. Congruence between the combined DNA data sets was assessed by an incongruence length difference (ILD) test (Farris *et al.*, 1994). We implemented the partition homogeneity test in PAUP* 4.0b10 (Swofford, 2003) with an heuristic search and random addition sequences. MP analyses were performed in PAUP* 4.0b10. Heuristic

searches were conducted with 1000 random addition replicates and the tree–bisection–reconnection (TBR) branch-swapping algorithm, holding 10 trees at each step with MulTrees on. All characters were equally weighted and gaps were treated as missing data. Bootstrap analyses were performed with 1000 replicates to estimate the support values for clades. Bayesian analyses were performed with MrBayes 3.1.1 (Huelsenbeck & Ronquist, 2001). We ran four simultaneous Monte Carlo Markov Chain searches for 3 million generations. The chains were sampled every 1000 iterations. Burn-in time was estimated from the plot of likelihoods generated using the 'sump' command in MrBayes. Posterior probabilities were based on analysis of post-burn-in trees. The best-fit models were determined using the Akaike information criterion (AIC) as implemented by MrModeltest 2.3 (Nylander, 2004). The model HKY + G was selected for ITS and GTR + I + G was used for plastid and combined sequence data.

RESULTS

Sequence characteristics of ITS, plastid and combined DNA data sets are shown in Table 4. For our complete ITS region analysis, sequence alignment required 28 indels. One of the 282 most parsimonious trees from MP analysis is shown in Figure 1, with bootstrap percentages and Bayesian posterior probabilities (PP). The Bayesian tree topology (tree not shown) was similar to that from MP (Fig. 1).

MP and Bayesian trees show that *Campylotropis* is sister to *Lespedeza* plus *Kummerowia*, with strong support (97% bootstrap, 100% PP). The MP tree weakly supports monophyly of *Lespedeza* (60% bootstrap), which is sister to *Kummerowia*. The latter forms a polytomy, together with the subgeneric groups of genus *Lespedeza* in the Bayesian analysis.

Table 4. Values and statistics from parsimony analyses of internal transcribed spacer (ITS), plastid and combined DNA data

	Nuclear	Plastid	Combined
Unaligned length of sequences (bp)	643–660	2202–2334	2851–3013
Aligned length of sequences (bp)	685	2517	3201
No. of variable characters	220	307	523
No. of potentially parsimony informative characters (%)	153 (22.3)	154 (6.1)	294 (9.2)
No. of trees retained	282	> 10 000	565
Tree length	391	391	799
Consistency index (CI)	0.70	0.87	0.77
Retention index (RI)	0.86	0.89	0.85

In genus *Lespedeza*, subgenus *Macrolespedeza* is monophyletic (100% bootstrap, 100% PP) in MP and Bayesian analyses, but subgenus *Lespedeza* is paraphyletic. Among members of subgenus *Lespedeza*, the North American species form a monophyletic group (90% bootstrap, 100% PP), whereas the eastern Asian species are paraphyletic with respect to *Macrolespedeza*. The North American clade can be divided into two monophyletic groups, with strong support. Among the Asian subgenus *Lespedeza*, four species (*L. chinensis* G.Don, *L. floribunda* Bunge, *L. tomentosa* and *L. virgata* (Thunb.) DC.) are sister to subgenus *Macrolespedeza* (Fig. 1). All species of Asian *Lespedeza*, including subgenera *Lespedeza* and *Macrolespedeza*, form a monophyletic clade with weak support (50% bootstrap, 57% PP); this clade is sister to North American species of subgenus *Lespedeza* in both trees.

The sequence matrix for the four combined plastid regions was 2517 bp long. Each region contributed to that length as follows: *trnK-matK*, 896 characters (867–880 bp); *psbK-psbI*, 556 characters (449–551 bp); *trnH-psbA*, 387 characters (276–382 bp); and *rpoC1*, 574 characters (574 bp). All regions had different levels of potentially informative characters, with *trnH-psbA* being the most informative (10.7%), followed by *psbK-psbI* (7.2%), *trnK-matK* (5.9%) and *rpoC1* (1.9%). The number of indel events scored was 10 from *psbK-psbI*, 21 from *trnH-psbA* and 22 from *trnK-matK*. No events were noted for the *rpoC1* region. One of most parsimonious trees from MP analysis with bootstrap percentages is shown in Figure 2. Topology of the parsimony tree was almost identical to that for the Bayesian (tree not shown).

Based on plastid DNA data, the MP and Bayesian trees revealed that genus *Campylotropis* is sister to *Lespedeza* plus *Kummerowia* (67% bootstrap, 100% PP). However, the relationships between *Kummerowia* and *Lespedeza* were not resolved in the MP strict consensus and Bayesian trees. According to our plastid sequence data, the subgeneric relationships of genus *Lespedeza* are highly congruent with those noted in the ITS analyses. The plastid DNA tree strongly supports monophyly of subgenus *Macrolespedeza* (92% bootstrap, 100% PP), but species within subgenus *Lespedeza* form a paraphyletic group in both trees. North American species of subgenus *Lespedeza* form a clade (68% bootstrap, 100% PP), which is then further divided into two monophyletic groups that are the same as those of the ITS trees. In contrast, the Asian species of subgenus *Lespedeza* are sister to the Asian subgenus *Macrolespedeza* and not to the North American species of subgenus *Lespedeza*. Among those Asian species from subgenus *Lespedeza*, three species (*L. chinensis*, *L. floribunda* and *L. virgata*) are sister to the other species and *Lespedeza pilosa* (Thunb.) Siebold & Zucc. is also separated from the other Asian species (Fig. 2). Monophyly of all Asian species from genus *Lespedeza* is supported by parsimony (91% bootstrap) and Bayesian trees (99% PP).

The combined matrices of the nuclear ribosomal and plastid DNA sequences were 3201 bp long, of which 294 bp (9.2%) were potentially parsimony informative characters. Analysis produced 565 equally most parsimonious trees with a score of 799, with consistency index = 0.77 and retention index = 0.85. One of the most parsimonious trees, with bootstrap percentages, is shown in Figure 3. For the Bayesian analysis, we used the same matrix as for MP. The Bayesian tree topology (tree not shown) was similar to the parsimony one (Fig. 3). Bayesian posterior probabilities are also shown in Figure 3.

Our ILD test indicated that the two partitions (nuclear ITS and plastid DNA) are significantly incongruent (*P* = 0.001). The phylogenetic relationships among Asian species in each of the two subgenera show incongruence between the nuclear (Fig. 1) and plastid (Fig. 2) DNA data and no clade is common between the two MP strict consensus trees in either subgenus. The relationships among the North American species are also incongruent between the two data sets, except that the North American clade is first divided into two monophyletic groups and *Les-*
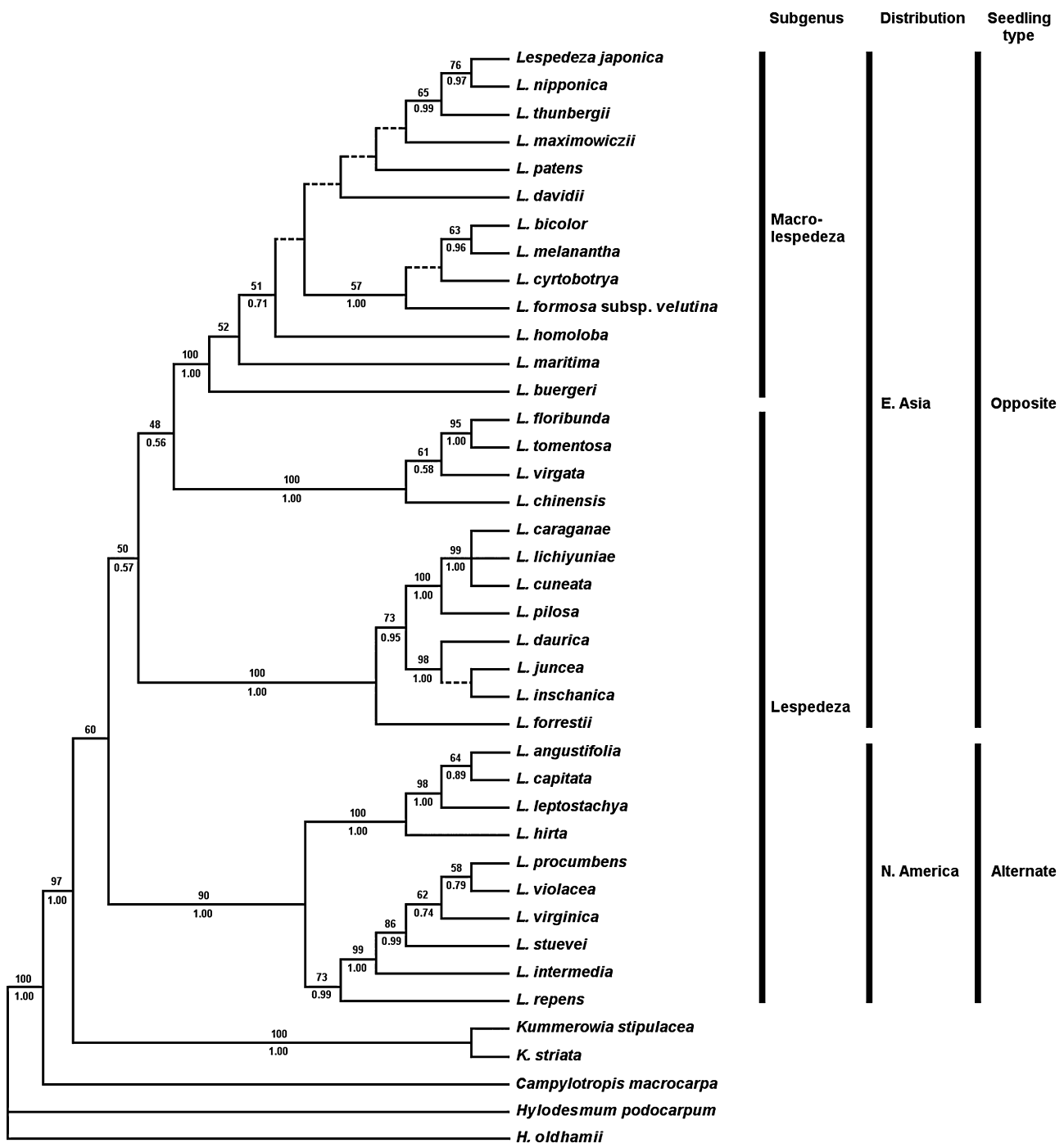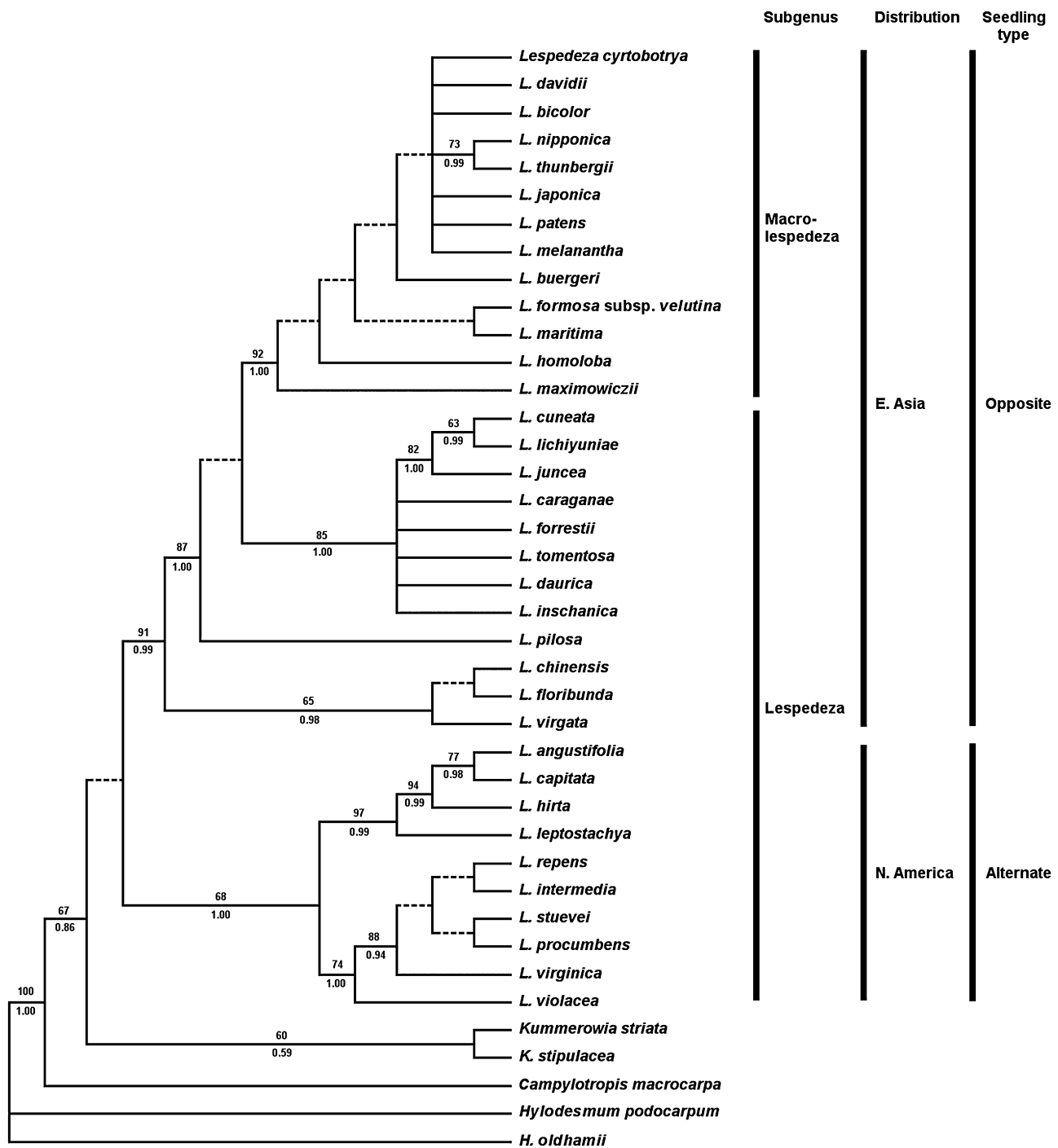
Figure 1. One of 282 most parsimonious trees from the parsimony analysis [1000 random addition replicates, tree–bisection–reconnection (TBR) branch swapping] of nuclear ribosomal internal transcribed spacer (ITS) 1 and 2 regions for the Lespedeza group. Bootstrap values are above branches and Bayesian posterior probabilities below. Dotted lines indicate branches that collapse in the strict consensus. Classification by subgenus, geographical distribution and seedling type is indicated at right.

pedeza angustifolia (Pursh) Elliott and *L. capitata* Michx. form a clade in both MP strict consensus trees. Nevertheless, the major tree topologies for inter- and subgeneric groups of genus *Lespedeza* are highly congruent among nuclear ITS, plastid and combined DNA analyses (Figs 1–3).

An analysis of the combined DNA data showed that *Campylotropis* is sister to genera *Kummerowia* plus
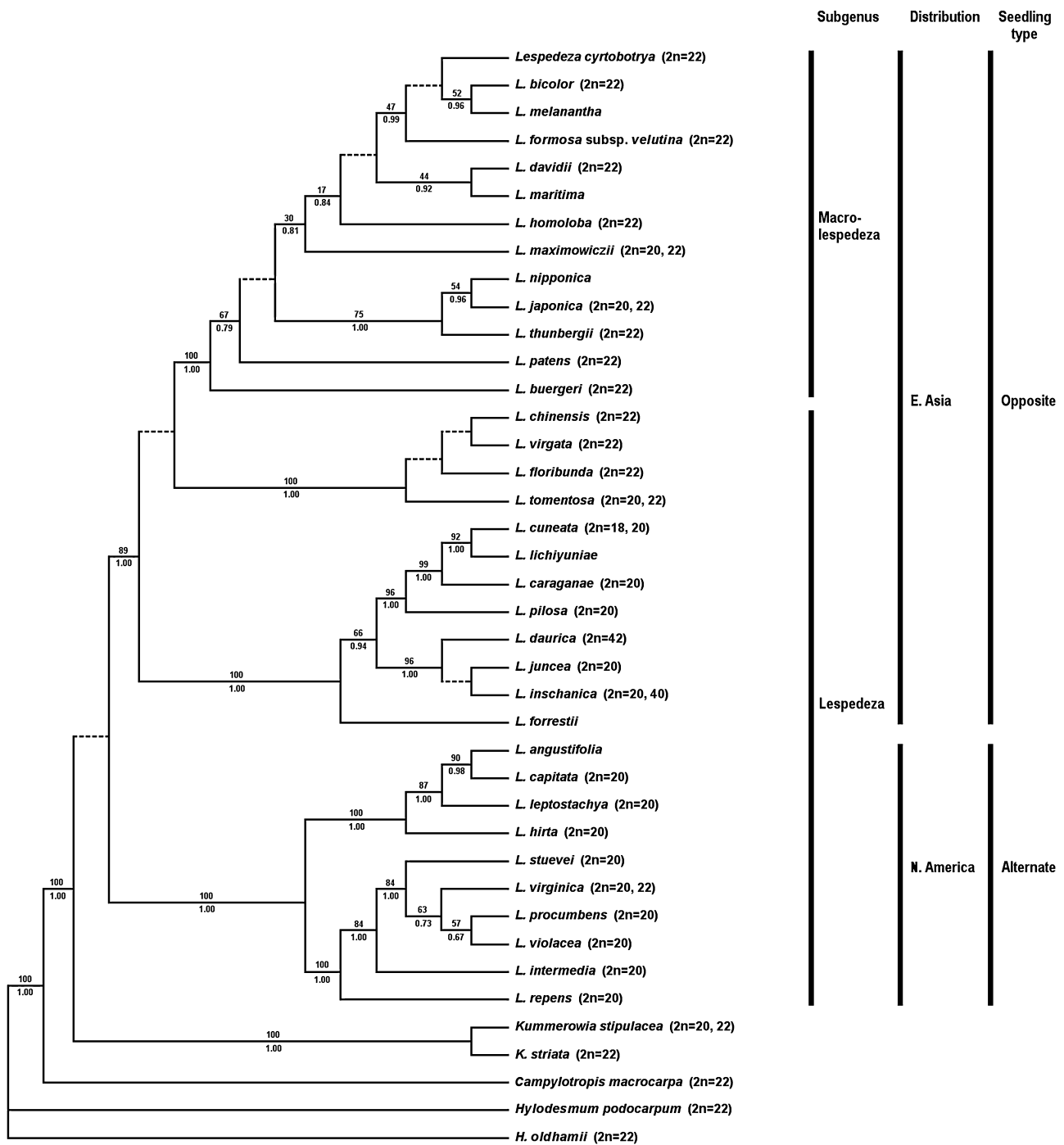
Figure 2. One of > 10 000 most parsimonious trees from the parsimony analysis [1000 random addition replicates, tree–bisection–reconnection (TBR) branch swapping] of four plastid regions for the Lespedeza group. Bootstrap values are above branches and Bayesian posterior probabilities below. Dotted lines indicate branches that collapse in the strict consensus. Classification by subgenus, geographical distribution and seedling type is indicated at right.

Lespedeza (100% bootstrap, 100% PP). Furthermore, *Kummerowia* is sister to a *Lespedeza* clade, with weak support (51% PP) in the Bayesian analysis and in one of the MP trees (Fig. 3). However, the *Lespedeza* clade collapses in the strict consensus tree.

The combined DNA trees strongly support monophyly of subgenus *Macrolespedeza* (100% bootstrap, 100% PP), whereas subgenus *Lespedeza* is paraphyletic. In the latter, monophyly of the North American species is strongly demonstrated in our parsimony

Figure 3. One of 565 most parsimonious trees obtained from the parsimony analysis [1000 random addition replicates, tree–bisection–reconnection (TBR) branch swapping] of the combined data from nuclear ribosomal internal transcribed spacer (ITS) and four plastid regions for the Lespedeza group. Bootstrap values are above branches and Bayesian posterior probabilities below. Chromosome numbers (2*n*) previously reported are given in parentheses. Dotted lines indicate branches that collapse in the strict consensus. Classification by subgenus, geographical distribution and seeding type is indicated at right.

and Bayesian trees (100% bootstrap, 100% PP). Two monophyletic groups have formed among North American species, with strong support, whereas Asian species are paraphyletic with respect to subgenus *Macrolespedeza*. Four species of Asian subgenus *Lespedeza* (*L. chinensis*, *L. floribunda*, *L. tomentosa* and *L. virgata*) are sister to subgenus *Macrolespedeza*, as also seen in the ITS tree. Our phylogenetic analyses of the combined DNA data revealed strong support for monophyly of all Asian *Lespedeza*, including both subgenera (89% bootstrap, 100% PP).

DISCUSSION

PHYLOGENETIC RELATIONSHIPS AMONG THE LESPEDEZA GROUP

Lespedeza, *Campylotropis* and *Kummerowia* are the sole constituents of the Lespedeza group (Ohashi, 2005). Strong evidence has been presented for that group being monophyletic, through analyses of *rbcL* (Kajita *et al*., 2001) and eight plastid regions (Stefanović *et al*., 2009). Those three genera are so similar to each other morphologically that they were once considered congeneric, either entirely or in part (Maximowicz, 1873; Taubert, 1894; Nakai, 1927; Ohashi, 1971). However, the phylogenetic relationships among *Lespedeza*, *Campylotropis* and *Kummerowia* have been debatable. A sister relationship between *Kummerowia* and *Campylotropis* is weakly supported within a large tree for legumes, based on *rbcL* sequences (Kajita *et al*., 2001), with a single species from each genus. Stefanović *et al*. (2009) recently used three *Lespedeza* spp. and one each from *Campylotropis* and *Kummerowia* to demonstrate a monophyletic relationship between *Lespedeza* and *Kummerowia* using *trnL-F* sequences of 'phaseoloid legumes'. In our analyses with almost all species of *Lespedeza* and *Kummerowia* and one of *Campylotropis*, our results show that *Campylotropis* is sister to the other two genera, with strong support gained from all phylogenetic trees. Nevertheless, the ITS Bayesian tree and strict consensus trees of plastid and combined data do not resolve the relationship between *Kummerowia* and *Lespedeza*, reported for trees obtained from an analysis of *trnL-trnF* data (Nemoto *et al*., 2010). This suggests a closer relationship of *Lespedeza* to *Kummerowia* than to *Campylotropis*. Such a conclusion is consistent with the historical taxonomic treatment of these groups as congeneric (see Isely, 1948; Ohashi, 1982b) and morphological and RFLP analyses of plastid DNA (Nemoto & Ohashi, 1990, 1993a, 1996; Nemoto *et al*., 1995). By contrast, we were able to resolve *Kummerowia* as sister to the *Lespedeza* clade in a MP strict consensus tree of ITS and in each one of most parsimonious trees from our

plastid and combined DNA data. Thus, *Lespedeza* appears to be more closely related to *Kummerowia* than to *Campylotropis*. Nonetheless, the phylogenetic trees are still incongruent with these morphological distinctions and current taxonomic treatments.

PHYLOGENETIC RELATIONSHIPS AMONG THE INFRAGENERIC GROUPS OF *LESPEDEZA*

Maximowicz (1873) previously divided the genus *Lespedeza* into two groups, sections *Macrolespedeza* and *Lespedeza* (as *Eulespedeza*). This treatment was then adopted by Taubert (1894), Schindler (1913), Nakai (1927) and Akiyama (1988). It was further divided into subgenera by Ohashi (1982a) and Ohashi, Nemoto & Ohashi (2009a, b). Subgenus *Macrolespedeza* is restricted to eastern Asia, whereas subgenus *Lespedeza* shows a disjunct distribution between eastern Asia and eastern North America.

All of the phylogenetic trees based on ITS and plastid sequence data strongly demonstrate the monophyly of subgenus *Macrolespedeza*, but subgenus *Lespedeza* is paraphyletic in all trees. For the latter, North American species are strongly supported as monophyletic in all analyses, but the Asian taxa are paraphyletic. All trees provide strong evidence for a monophyletic origin of eastern Asian *Lespedeza* comprising two subgenera. The main clades obtained by our analyses of nuclear and plastid sequence data are highly congruent with those obtained from plastid DNA (*trnL-trnF*) analysis (Nemoto *et al*., 2010).

Moreover, the division of *Lespedeza* into Asian and North American clades is closely related to differences in seedling types between those continents (Nemoto & Ohashi, 1993b), whereas it is inconsistent with the subgeneric classification in genus *Lespedeza*. Although our results more strongly support subgenus *Macrolespedeza* than those from *trnL-trnF* data (Nemoto *et al*., 2010), the phylogenetic relationships among the three species groups, which Akiyama (1988) proposed as series within section *Macrolespedeza*, remain unresolved in all analyses presented here.

PHYLOGENETIC RELATIONSHIPS AMONG SPECIES OF *LESPEDEZA*

Our study covered two main groups in the genus *Lespedeza* (eastern Asian and eastern North American species). The phylogenetic relationships among Asian species in each of the two subgenera show significant incongruence between the nuclear and plastid DNA data, perhaps a result of hybridization between species (Ferguson & Jansen, 2002; Kim & Donoghue, 2008). The phenomenon of natural hybrids has been reported previously in *Lespedeza* (Lee, 1965; Akiyama & Ohba, 1982; Akiyama, 2003). Within the

Asian clade, the group comprising *L. chinensis*, *L. floribunda*, *L. virgata* and *L. tomentosa* (belonging to subgenus *Lespedeza*) is sister to subgenus *Macrolespedeza*, based on ITS and combined DNA trees. However, those first three species are sister to all other Asian species only in the plastid DNA tree, and their monophyly is also demonstrated in the plastid DNA (*trnL-trnF*) tree presented by Nemoto *et al.* (2010). A hybrid has already been described between *L. tomentosa* and *L. virgata* (Lee, 1965; Ohashi *et al.*, 2009a). Without evidence, Schindler (1913) located *L. chinensis*, *L. floribunda* and *L. virgata* in an early branching position in the Asian lineage, as shown in his phylogenetic diagram of subgenus *Lespedeza* (as section *Lespedeza*). Our current findings are partly consistent with Schindler's determination.

Two sets of chromosome numbers ($2n = 22$, or $n = 11$; and $2n = 20$, or $n = 10$) and a few polyploids or aneuploids, have been reported for *Lespedeza* (Nishikawa, 2008; Index to Plant Chromosome Numbers Database accessed on 8 January 2010; other references summarized by Nemoto *et al.*, 2010). The chromosome numbers $2n = 20$ or $n = 10$ are common throughout subgenus *Lespedeza*, except for *L. chinensis*, *L. floribunda* and *L. virgata* ($2n = 22$ or $n = 11$). Numbers based on $n = 11$ have also been found in subgenus *Macrolespedeza*, and $2n = 22$ ($n = 11$) is common in *Kummerowia* and *Campylotropis* and in other genera attributed to subtribe Desmodiinae Benth. & Hook.f. of tribe Desmodieae and the allied tribe Phaseoleae DC. (Goldblatt, 1981). Therefore, it can be regarded as plesiomorphic, whereas $2n = 20$ ($n = 10$) is apomorphic in *Lespedeza* (Nemoto *et al.*, 2010). These chromosomal data are consistent with the closer positioning of *L. chinensis*, *L. floribunda* and *L. virgata* to subgenus *Macrolespedeza* and the early branching position of the Asian *Lespedeza* clade.

In the Asian clade, *Lespedeza lichiyuniae* T.Nemoto, H.Ohahi & T.Itoh is closely related to *L. cuneata* (Dumon de Courset) G.Don in all trees. Nemoto, Ohashi & Itoh (2007), however, assumed the closer relationships of *L. lichiyuniae* to *L. juncea* and *L. cuneata* based on morphological observations. The plastid DNA trees agree with their assumption better than do the ITS trees. Series *Pilosa* of Maximowicz (1873) is monotypic (only *L. pilosa*) and is separated from the other series in subgenus *Lespedeza* in the plastid DNA tree.

In all analyses of North American species, two main clades have been identified similar to those based on plastid (*trnL-trnF*) data (Nemoto *et al.*, 2010). They are consistent with sections *Lespedeza* (as *Eulespedeza*) and *Lespedezaria* (Torrey & Gray, 1840) or with the series *Violaceae* and *Lespedezariae* (Maximowicz, 1873). Schindler (1913) also recognized these two groups as independent lineages in his phylogenetic

diagram. According to Torrey & Gray (1840) and the keys for identification shown by Isely (1955) and Clewell (1966), these two clades of North American species clearly correspond to diversifications in flower colour, relative length of calyx to pod and inflorescence features. However, the topology of each clade differs between the ITS and plastid DNA trees.

BIOGEOGRAPHICAL IMPLICATIONS FOR THE DISJUNCT DISTRIBUTION OF *LESPEDEZA*

Kummerowia and *Campylotropis* of the Lespedeza group are restricted to Asia. All 11 genera in the Phyllodium group and 14 of 16 genera in the Desmodium group (both tribe Desmodieae) are distributed mostly in Asia, with a few also occurring in Africa and Australia, including New Caledonia (Ohashi, 2005). For the genus *Lespedeza*, all species of subgenus *Macrolespedeza* and many of subgenus *Lespedeza* are naturally distributed in eastern Asia (Ohashi *et al.*, 2009a, b), whereas the rest of subgenus *Lespedeza* (*c.* 11 species) are found only in eastern North America (Clewell, 1966). This bias toward Asia in the allied genera sister to *Lespedeza* suggests that the ancestral area is in Asia.

Phylogenetic analyses using nuclear ribosomal and plastid sequence data revealed two highly distinct clades corresponding to Asia and North America in *Lespedeza*. Furthermore, no sister–species relationships were formed between eastern Asian and North American species in subgenus *Lespedeza*, consistent with the disjunct pattern shown for most genera of flowering plants (Wen, 1999, 2001). This suggests that ancestral species on both continents diversified early in the origin of the genus and that independent speciation occurred afterwards, either through restricted interchange of species between the regions or with extinction of such interchanging species. More than twice as many species of *Lespedeza* are found in eastern Asia than in North America and an ancestral lineage of subgenus *Macrolespedeza* has diverged from the Asian lineage. This greater species diversity in eastern Asia is common within disjunct genera between the two regions (Wen, 1999; Xiang *et al.*, 2004).

Based on plastid sequence data, the estimated time of divergence for the Lespedeza group from the Desmodium group is 10.8–16.5Myr, whereas that of three lineages corresponding to east Asian *Lespedeza*, North American *Lespedeza* and the genus *Kummerowia* is approximately 2.4–4.3 Myr (Stefanović *et al.*, 2009). The Bering land bridge was considered suitable for exchanges of temperate deciduous plants during the Miocene (Tiffney, 1985) and remained available for floristic exchange until the later Tertiary (Tiffney & Manchester, 2001) or approximately

3.5 Myr (Wen, 1999). Based on those estimates, intercontinental migration between eastern Asia and eastern North America appears to have occurred via the Bering land bridge around the time of its closing.

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