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Molecular phylogeny of the subgenus *Ceratotropis* (genus *Vigna*, Leguminosae) reveals three eco-geographical groups and Late Pliocene–Pleistocene diversification: evidence from four plastid DNA region sequences

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• *Background and Aims* The subgenus *Ceratotropis* in the genus *Vigna* is widely distributed from the Himalayan highlands to South, Southeast and East Asia. However, the interspecific and geographical relationships of its members are poorly understood. This study investigates the phylogeny and biogeography of the subgenus *Ceratotropis* using chloroplast DNA sequence data.

• *Methods* Sequence data from four intergenic spacer regions (*pet*A-*psb*J, *psb*D-*trn*T, *trn*T-*trn*E and *trn*T-*trn*L) of chloroplast DNA, alone and in combination, were analysed using Bayesian and parsimony methods. Divergence times for major clades were estimated with penalized likelihood. Character evolution was examined by means of parsimony optimization and MacClade.

• *Key Results* Parsimony and Bayesian phylogenetic analyses on the combined data demonstrated well-resolved species relationships in which 18 *Vigna* species were divided into two major geographical clades: the East Asia–Southeast Asian clade and the Indian subcontinent clade. Within these two clades, three well-supported eco-geographical groups, temperate and subtropical (the East Asia–Southeast Asian clade) and tropical (the Indian subcontinent clade), are recognized. The temperate group consists of *V. minima*, *V. nepalensis* and *V. angularis*. The subtropical group comprises the *V. nakashimae–V. riukiuensis–V. minima* subgroup and the *V. hirtella–V. exilis–V. umbellata* subgroup. The tropical group contains two subgroups: the *V. trinervia–V. reflexo-pilosa–V. trilobata* subgroup and the *V. mungo–V. grandiflora* subgroup. An evolutionary rate analysis estimated the divergence time between the East Asia–Southeast Asia clade and the Indian subcontinent clade as 3·62 ± 0·3 million years, and that between the temperate and subtropical groups as 2·0 ± 0·2 million years.

• *Conclusions* The findings provide an improved understanding of the interspecific relationships, and ecological and geographical phylogenetic structure of the subgenus *Ceratotropis.* The quaternary diversification of the subgenus *Ceratotropis* implicates its geographical dispersal in the south-eastern part of Asia involving adaptation to climatic condition after the collision of the Indian subcontinent with the Asian plate. The phylogenetic results indicate that the epigeal germination is plesiomorphic, and the germination type evolved independently multiple times in this subgenus, implying its limited taxonomic utility.

Key words: Subgenus *Ceratotropis*, *Vigna*, Leguminosae, diversification, intergenic spacer, germination type.

INTRODUCTION

The genus *Vigna* Savi (Leguminosae) comprises >80 species which are distributed throughout the Old World and New World. The genus is divided into six subgenera, *Ceratotropis* (Piper) Verdc., *Haydonia* (Wilczek) Verdc., *Lasiospron* (Benth.) Verdc., *Plectotropis* (Schum.) Baker, *Sigmoidotropis* (Piper) Verdc. and *Vigna* Savi (Verdcourt, 1970; Maréchal *et al.*, 1978), and the subgenus *Macrorhynchus* Verdc. previously placed in genus *Vigna*, was transferred to genus *Wajira* Thulin (Thulin *et al.*, 2004).

Subgenus *Ceratotropis*, one of the most economically important groups in the genus *Vigna* for food, forage and cover crops, contains five well-known domesticated species (Baudoin and Maréchal, 1988; Smartt, 1990; Lumpkin and McClary, 1994; Tomooka *et al.*, 2002*b*). It is originally circumscribed as a group of the Asian *Vigna* (Verdcourt, 1970) and distinguished from the other subgenera by having peltate stipule, a pocket on the left keel petal, style extending beyond the stigma as a beak, keel petals curved to the left in the upper part, and pollen grains with a coarse reticulate sculpture (Verdcourt, 1970; Maréchal *et al.*, 1978).

The species of subgenus *Ceratotropis* are widely distributed in South Asia, the Himalayan highlands, Southeast Asia, and East Asia (Maréchal *et al.*, 1978; Tateishi, 1985, 1996; Tateishi and Ohashi, 1990; Tomooka *et al.*, 2002*b*), while the members of other subgenera are endemic to Africa [*Plectotropis* (except *V. vexillata* (L.) A. Rich], Africa and Madagascar (*Haydonia*), or America (*Lasiospron* and *Sigmoidotropis*). The subgenus *Vigna* is found throughout sub-Saharan Africa, with representatives present in tropical Asia and the Americas (Maréchal *et al.*, 1978).

The 21 known wild species in the subgenus *Ceratotropis* inhabit coastal sandy soil, limestone hills, forest margins and open fields (Tateishi, 1983, 1985; Tomooka *et al.*, 2002*a*, *b*). East Asian and Southeast Asian species of the subgenus *Ceratotropis* occur naturally in temperate and subtropical regions [e.g. *V. angularis* (Willd.) Ohwi & H. Ohashi var. *nipponensis* (Ohwi) Ohwi & H. Ohashi and *V. nepalensis* Tateishi & Maxted in temperate regions and *V. nakashimae* (Ohwi) Ohwi & H. Ohashi, *V. umbellata* (Thunb.) Ohwi & H. Ohashi and *V. tenuicaulis* N. Tomooka & Maxted in subtropical regions], while Indian subcontinental species [i.e. *V. mungo* (L.) Hepper var. *silvestris* Lukoki, Maréchal & Otoul, *V. radiata* (L.) R. Wilczek var. *sublobata* (Roxb.) Verdc., *V. trilobata* (L.) Verdc., and *V. aridicola* N.Tomooka & Maxted] are mainly confined to tropical regions.

All of the species in the subgenus *Ceratotropis* are diploid ($2n = 2x = 22$; Maréchal *et al.*, 1978; Tateishi, 1985; Tomooka *et al.*, 2002*b*) except tetraploid *V. reflexo-pilosa* Hayata ($2n = 4x = 44$; Swindell *et al.*, 1973; Egawa *et al.*, 1988). The ancestral donor of *V. reflexo-pilosa* has been assumed to be *V. exilis* Tateishi & Maxted or *V. minima* (Roxb.) Ohwi & H. Ohashi based on isozyme, interspecific hybridization (Tateishi, 1985; Egawa *et al.*, 1996; Konarev *et al.*, 2002) or *V. trinervia* (B. Heyne ex Wight & Arn.) Tateishi & Maxted as the maternal donor based on plastid DNA phylogeny (Yano *et al.*, 2004; Ye Tun Tun and Yamaguchi, 2007).

In Leguminosae, seedling germination type [hypogeal (cotyledons may remain underground) vs. epigeal (cotyledons emerge above the soil surface following germination)] shows no variation in some tribes, while both types are found within the Trifolieae and Phaseoleae (Gates, 1951; Polhill, 1981; Endo and Ohashi, 1997) and papilionoid genera such as *Phaseolus* and *Vigna* (Gates, 1951; Polhill, 1981; Tomooka *et al.*, 2002*b*). In some genera, epigeal and hypogeal germination are distinguished by different subgenera (Essig, 1992). In the subgenus *Ceratotropis*, seedling germination has been focused on as one of the diagnostic characters for inferring relationships among species of the subgenus (Maekawa, 1955; Baudoin and Maréchal, 1988; Tomooka *et al.*, 1991; Tateishi, 1996). These studies recognized two morphological groups within the subgenus *Ceratotropis*, the azuki bean group (*angularis*–*umbellata*) with hypogeal germination having petiolate first and second leaves and the mung bean group (*radiata*–*mungo*) with epigeal germination having sessile first and second leaves, and ungrouped species showing epigeal germination with petiolate first and second leaves (Baudet, 1974) which later was considered as the intermediate group, *aconitifoila*–*trilobata* (Tomooka *et al.*, 1991). Recently, three groups within the subgenus *Ceratotropis* were proposed as sections *Angulares* N.Tomooka & Maxted (azuki bean group), *Ceratotropis* N.Tomooka & Maxetd (mung bean group) and *Aconitifoliae* N.Tomooka & Maxted (Intermediate group), based on seedling characteristics, size of floral parts and growth habit (Tomooka *et al.*, 2002*a*). In spite of the importance of morphological characters that have been used traditionally to define taxonomic relationships within the subgenus *Ceratotropis*, few works have evaluated them in a phylogenetic context. The only cladistic study in the subgenus *Ceratotropis* was conducted by Taeishi (1996) who considered hypogeal germination as the primitive state in the subgenus based on morphological data.

In recent phylogenetic studies on the subgenus *Ceratotropis*, relationships among the species remain in dispute. A phylogenetic tree based on DNA sequences from the ITS and *atp*B-*rbc*L regions showed a close relationship between *V. reflexo-pilosa* and the species of section *Angulares* such as *V. umbellata*, *V. hirtella* and *V. exilis* (Doi *et al.*, 2002); however, the *trn*T-F sequence data indicated a closer relationship between *V. reflexo-pilosa* to *V. trinervia*, consisting of some species of section *Ceratotropis* (Yano *et al.*, 2004; Ye Tun Tun and Yamaguchi, 2007). A molecular phylogeny by the cpDNA and nuclear ITS sequence data recognized three lineages corresponding to the three sections (*Aconitifoliae*, *Angulares* and *Ceratotropis*) in the subgenus *Ceratotropis* (Doi *et al.*, 2002), but its clades are not well supported. In contrast, phylogenetic analyses based on DNA sequences of nuclear ribosomal ITS (Goel *et al.*, 2002) and plastid DNA phylogenies (Yano *et al.*, 2004; Ye Tun Tun and Yamaguchi, 2007) revealed two main groups in the subgenus *Ceratotropis.* The phylogenetic analysis using 5S IGS divided the ten *Vigna* species of subgenus *Ceratotropis* into two weakly supported clades: clade I which included the most species of sections *Ceratotropis* and *Aconitifoliae* and clade II consisting of some of the species in section *Angulares* (Saini and Jawali, 2009).

The biogeographic history of the subgenus *Ceratotropis* could be inferred from a phylogenetic analysis of the subgenus. However, previous studies have attempted to determine its molecular phylogenetic relationships with representative species from the limited geographical regions, e.g. samples mainly from Thailand based on AFLP marker (Seehalak *et al.*, 2006) and those from Myanmar using *trn*T-F noncoding regions of chloroplast genome (Ye Tun Tun and Yamaguchi, 2007). These studies did not resolve the geographical relationships or interspecific relationships in the subgenus *Ceratotropis*. Furthermore, due to the limited molecular data, a low amount of variation and weak bootstrap support were found within each of the recognized taxonomic groups.

This study aimed to provide evidence for advancing our understanding of the phylogenetic relationships and historical biogeography of the subgenus *Ceratotropis* by using substantially increased molecular sequence data and improved species sampling in comparison to that of previous studies, and also to elucidate evolutional patterns of the seedling germination type on the molecular tree and to consider its taxonomic implication as well. To achieve the objectives, 18 species with four outgroups were selected and sequence data used from four plastid intergenic spacer regions, *psb*D-*trn*T, *trn*T-*trn*L, *trn*T-*trn*E and *pet*A-*psb*J, all of which are reported to include higher information content for phylogenetic analyses at lower taxonomic levels (Shaw *et al.*, 2005, 2007).

MATERIALS AND METHODS

Plant materials

Out of 21 species of the subgenus *Ceratotropis*, 18 were included, representing the three taxonomical groups described

by Tomooka *et al.* (2002*a*, *b*); material of the three exceptions, *V. khandalensis* (Santapau) Sundararagh. & Wadhwa, *V. dalzelliana* (Kuntze) Verdc. and *V. subramaniana* (Babu ex Raizada) M. Sharma, was not accessible at the time of the study. For those 18 species, accessions were obtained from varied sources, and included multiple accessions whenever possible (Table 1). Four outgroup taxa, *V. unguiculata* (L.) Walp. var. *unguiculata* and *V. marina* (Burm.) Merr. (subgenus *Vigna*), *V. kirkii* (Baker f.) J.B. Gillett (subgenus *Plectotropis*) and *V. venulosa* Baker (subgenus *Haydonia*), were selected (Table 1) based on previous molecular studies (Maréchal *et al.*, 1978; Fatokun *et al.*, 1993; Yasuda and Yamaguchi, 1996; Goel *et al.*, 2002; Yano *et al.*, 2004; Ye Tun Tun and Yamaguchi, 2007).

DNA analysis

Total DNA was extracted from fresh leaves using the modified CTAB method (Doyle and Doyle, 1987) or obtained from herbarium samples from the Royal Botanic Gardens, Kew (Table 1). Primers used for amplification and sequencing of the *psb*D-*trn*T and *trn*T-*trn*E intergenic spacer regions were taken from a previous study (Ye Tun Tun and Yamaguchi, 2008). Those of the *trn*T-*trn*L region were taken from Taberlet *et al.* (1991), and those for the *pet*A-*psb*J intergenic spacer region were designed at the conservative region of alignment sequences of *Meidcago truncatula* L. (AC093544) and *Lotus japonicus* L. (AP002983) obtained from the GeneBank (Table 2). For the *trn*T-L spacer region, sequences were included from a previous study (Ye Tun Tun and Yamaguchi, 2007), and those for the 13 accessions of the ingroup and two outgroups were sequenced in this study (Table 1). PCR reactions were performed in a volume of 25 μL containing 2·5 μL of 10 × reaction buffer, 2·5 μL of 25 mM $MgCl_2$, 2·5 μL of 1·25 mM dNTPs, 1·25 μL of each 10 μM primer, 0·1 μL of Takara r*Taq* (Takara Shuzo Co., Ltd, Japan), and 1·0 μL of 5–10 ng μL^{-1} genomic DNA. Amplifications were performed using a thermal cycler (GeneAmp® PCR System 2700; Applied Biosystems, Foster City, CA, USA) as follows: one cycle of 5 min at 95 °C, 35 cycles of 45 s at 95 °C, 45–60 s at 55 °C for *trn*T-*trn*E, 53 °C for *pet*A-*psb*J, 52 °C for *psb*D-*trn*T, 57 °C for *trn*T-*trn*L; 2 min at 68 °C; and finally one cycle of 5 min at 68 °C. Clean PCR products were sequenced with the BigDye Terminator v3·1 Cycle Sequencing Kit (Applied Biosystems), and cycle sequencing was performed according to the manufacturer's protocols. Sequencing was carried out on an ABI PRISM 3100 automated sequencer (Applied Biosystems).

Phylogenetic analyses

Sequence alignment was initially performed using Clustal X v.1·83 (Thompson *et al.*, 1997) and manually adjusted using MEGA v. 4 (Tamura *et al.*, 2007). Phylogenetic analyses were performed using Bayesian and maximum-parsimony approaches. Maximum-parsimony analyses involved a heuristic search strategy with 1000 replicates of random addition of sequences, in combination with ACCTRAN character optimization, MULPARS + TBR branch swapping and STEEPEST DESCENT options off in PAUP* 4·0b10 (Swofford, 2002). All character states were treated as unordered and equally weighted. Informative insertions and deletions (indels) were coded as binary characters (0, 1) according to Graham *et al.* (2000). The strict consensus tree was constructed from the most-parsimonious trees. Support for individual branches or clades was estimated using resampling bootstrap analysis (Felsenstein, 1985). Bootstrap values were estimated with 1000 replicates, random addition sequence and TBR branch swapping.

Incongruence length difference (ILD) tests (Farris *et al.*, 1995) as implemented under a partition homogeneity test in the PAUP* version 4·0b10 (Swofford, 2002), were conducted among pairwise combinations of the four datasets to determine whether the four regions were statistically incongruent. The ILD test was performed using 100 replications of heuristic searches with 100 random addition analyses and TBR branch swapping, holding ten trees per step, and using STEEPEST DESCENT off, with the MULTREES option enabled.

Bayesian inference of phylogeny using Monte Carlo Markov Chains (MCMC) was done with MrBayes 3·1·2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) under the optimal model of evolution. The dataset was partitioned into data partitions (*trn*T-*trn*E, *pet*A-*psb*J, *psb*D-*trn*T and *trn*T-*trn*L) and all non-partitioned combined datasets. Models of sequence evolution for each of the partitions and for all combined datasets were determined using the program ModelTest 3·7 (Posada and Crandall, 1998) based on the Akaike information criterion (AIC) (Posada and Buckley, 2004). The indels were treated as a 'standard' data type in MrBayes. For comparing the partitioned and non-partitioned datasets, Bayes factors were employed. Bayes factors measure the relative performance of two analyses as the ratio of their marginal likelihoods, i.e. the likelihood of the data under the model (Kass and Raftery, 1995; Nylander *et al.*, 2004). The marginal likelihood may be approximated by the harmonic mean of likelihood values of evolutionary hypotheses sampled from the posterior distribution (Nylander *et al.*, 2004), which is calculated by the sump command in MrBayes 3·1·2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). A Bayes factor of >10 provides strong evidence against the alternative hypothesis (Kass and Raftery, 1995; Brandley *et al.*, 2005). For each analysis, four MCMC chains were run for 3×10^6 permutations of tree parameters and sampled every 3×10^4 permutation, such that the sampling yielded 100 Bayesian trees which excluded the burn-in and autocorrelated trees (Lavin *et al.*, 2005; Delgado-Salinas *et al.*, 2006). The trees were imported into PAUP* 4·0b10 to construct the 50 % majority rule consensus tree.

Molecular calibration and age estimation

A recent study of ages and evolutionary rates of Leguminosae (Lavin *et al.*, 2005) provides estimated ages for many crown and stem clades which can serve as calibration points for legume groups lacking a fossil record. The hypothesis of rate constancy was evaluated with a likelihood ratio (LR) test comparing the likelihood scores from unconstrained and clock-constrained analyses (Felsenstein, 1981,

TABLE 1. *List of plant materials, inversion type in the* psb*D*-trn*T region, and seed germination*

Taxon	Accession number* (code)	Country	Inversion type†	Seed germination
Ingroup				
Subgenus *Ceratotropis* (Piper) Verdc.				
Section *Angulares* N.Tomooka & Maxted				
Vigna angularis (Willd.) Ohwi & H.Ohashi	Azn/90-J-41 (Van-2)	Japan	I	Hypogeal
V. angularis (Willd.) Ohwi & H.Ohashi var. *angularis*	Aza/88-J-26 (Van-1)	Japan	I	Hypogeal
V. angularis (Willd.) Ohwi & H.Ohashi var. *nipponensis* (Ohwi) Ohwi & H.Ohashi	Azn/91-J-14 (Van-3)	Japan	I	Hypogeal
	Azn/96-B-02 (Van-4)	Bhutan	I	Hypogeal
V. aff. *angularis* (Willd.) Ohwi & H.Ohashi var. *nipponensis* (Ohwi) Ohwi & H.Ohashi	Azn/01-My-05 (Van-5)	Myanmar	I	Hypogeal
V. nepalensis Tateishi & Maxted	NI 971 (Vne-1)	India	I	Hypogeal
	NI 1704 (Vne-2)	Nepal	I	Hypogeal
V. tenuicaulis N. Tomooka & Maxted	Azte/01-My-8 (Vten-1)‡	Myanmar	I	Hypogeal
	Azte/01-My-9 (Vten-2)‡	Myanmar	I	Hypogeal
V. aff. *minima* (Roxb.) Ohwi & H.Ohashi	Azr/01-My-01 (Vmn-1)	Myanmar	I	Hypogeal
V. aff. *minima* (Roxb.) Ohwi & H.Ohashi	Azr/01-My-04 (Vmn-2)	Myanmar	I	Hypogeal
V. minima (Roxb.) Ohwi & H.Ohashi	NI 1363 (Vmn-3)‡	Indonesia	I	Hypogeal
V. nakashimae (Ohwi) Ohwi & H.Ohashi	Azm/01-My-04 (Vnk-1)	Myanmar	I	Hypogeal
	Azm/96-J-01 (Vnk-2)	Japan	I	Hypogeal
V. riukiuensis (Ohwi) Ohwi & H.Ohashi	Azr/91-O-01 (Vri-1)	Japan	I	Hypogeal
	NI 1635 (Vri-2)	Japan	I	Hypogeal
V. hirtella Ridley	Azh/01-My-03 (Vhi-1)	Myanmar	I	Hypogeal
	Azh/00-My-01 (Vhi-2)	Myanmar	I	Hypogeal
	NI 1394 (Vhi-3)‡	Thailand	I	Hypogeal
	Azh/06-C-01 (Vhi-4)‡	China	I	Hypogeal
	Azh/06-C-04 (Vhi-5)‡	China	I	Hypogeal
V. umbellata (Thunb.) Ohwi & H.Ohashi	Azu/03-Th-01 (Vum-1)	Thailand	I	Hypogeal
	Azu/01-My-04 (Vum-2)	Myanmar	I	Hypogeal
V. exilis Tateishi & Maxted	Aze/01-My-01 (Vxi-1)	Myanmar	I	Hypogeal
	Aze/01-My-02 (Vxi-2)	Myanmar	I	Hypogeal
V. trinervia (B. Heyne ex Wight & Arn.) Tateishi & Maxted	Aztr/01-My-09 (Vtrn)	Myanmar	II	Hypogeal
V. reflexo-pilosa Hayata var. *reflexo-pilosa*	Azp/92-O-01 (Vrp-1)	Japan	II-i	Hypogeal
V. reflexo-pilosa Hayata var. *glabra* (Roxb.) N.Tomooka & Maxted	Azp/03-V-02 (Vrp-2)	Vietnam	II-i	Hypogeal
Section *Ceratotropis* N.Tomooka & Maxted				
V. radiata (L.) R. Wilczek var. *radiata*	Azd/My-004178 (Vrd-1)	Myanmar	II	Epigeal
V. radiata (L.) R. Wilczek var. *sublobata* (Roxb.) Verdc.	Azd/00-My-01 (Vrd-2)	Myanmar	II	Epigeal
	NI634 (Vrd-3)‡	India	II	Epigeal
V. mungo (L.) Hepper var. *mungo*	Azg/My-003935 (Vmg-1)	Myanmar	II	Epigeal
V. mungo (L.) Hepper var. *silvestris* Lukoki, Maréchal & Otoul	NI 969 (Vmg-2)‡	India	II	Epigeal
V. grandiflora (Prain) Tateishi & Maxted	NI 1721 (Vgrd-1)‡	Thailand	II-ii	Epigeal
	Azgr/06-Th-01 (Vgrd-2)‡	Thailand	II-ii	Epigeal
	Azgr/06-Th-02 (Vgrd-3)‡	Thailand	II-ii	Epigeal
Section *Aconitifoliae* N.Tomooka & Maxted				
V. aridicola N.Tomooka & Maxted	3227/Kew (Vari)‡	Sri Lanka	II	Epigeal
V. aconitifolia (Jacq.) Maréchal	Azc/95-I-01 (Vac)	India	II-iii	Epigeal
V. stipulacea Kuntze	Azt/01-My-02 (Vst-1)	Myanmar	II	Hypogeal
	Azt/01-My-04 (Vst-2)	Myanmar	II	Hypogeal
V. trilobata (L.) Verdc.	Azt/96-I-01 (Vtb-1)	India	II	Epigeal
	NI 453 (Vtb-2)‡	Sri Lanka	I	Epigeal
Outgroup				
Subgenus *Vigna* Savi				
V. unguiculata (L.) Walp var. *unguiculata*	Vuc/My-4208 (Out-1)	Myanmar	II-ii	Epigeal
V. marina (Burm.) Merr.	Vgm/90-O-01 (Out-2)	Japan	I	Epigeal
Subgenus *Haydonia* (Wilczek) Verdc.				
V. venulosa Baker	NI 548 (Out-3)‡	Liberia	I	Epigeal
Subgenus *Plectotropis* (Schum.) Baker				
V. kirkii (Baker f.) J.B.Gillett	NI 448 (Out-4)‡	Congo	I	Epigeal

* NI, National Botanical Garden of Belgium; Kew, Royal Botanical Garden, Kew.
† Inversion type – see text. In the *psb*D-*trn*T region.
‡ Sequencing of the *trn*T-*trn*L region in this study.

TABLE 2. *Primers used for amplification and sequencing of the four chloroplast intergenic spacer regions*

Region/primer	Sequence (5'-3')	Use	Reference
*psb*D-*trn*T			
psbD-F	TCAACTACTTCAACCATTTCC	Amplification/sequencing	Ye Tun Tun and Yamaguchi (2008)
trnT-R	TGGTAAGGCGTAAGTCATCG	Amplification/sequencing	Ye Tun Tun and Yamaguchi (2008)
DT-F2	TGGTGGAACTTGAAATTGGT	Sequencing	Ye Tun Tun and Yamaguchi (2008)
DT-R2	ACCAATTTCAAGTTCCACCA	Sequencing	Ye Tun Tun and Yamaguchi (2008)
*trn*T-*trn*E			
trnT-F	CGATGACTTACGCCTTACC	Amplification/sequencing	Ye Tun Tun and Yamaguchi (2008)
trnE-R	AGAGAGATGTCCTGAACCAC	Amplification/sequencing	Ye Tun Tun and Yamaguchi (2008)
*pet*A-*psb*J			
petA-F	GTTACGTGTCCAAGGTCTC	Amplification/sequencing	This study
psbJ-R	CCGATACTACTGGAAGGATT	Amplification/sequencing	This study
*trn*T-*trn*L			
trn-a	CATTACAAATGCGATGCTCT	Amplification/sequencing	Taberlet *et al.* (1991)
trn-b	TCTACCGATTTCGCCATATC	Amplification/sequencing	Taberlet *et al.* (1991)

1988; Huelsenbeck and Crandall, 1997), where $LR = 2$ ($\ln L_{clock} - \ln L_{no\ clock}$) was assumed to be χ^2 distributed with the degrees of freedom equal to *n* taxa minus two. The molecular clock was rejected because constrained and unconstrained analyses differed significantly [$(-\ln L = 2(7399{\cdot}779 - 7359{\cdot}102) = 81{\cdot}534$, d.f. = 44, $P < 0{\cdot}001$]. Therefore, the penalized likelihood method with the truncated Newton algorithm (Sanderson, 2002) was used in the rate smoothing program r8s, version 1·70 (Sanderson, 2003) to estimate the ages of the groups, as described by Lavin *et al.* (2005), using multiple trees with branch lengths estimated by Bayesian analyses (Huelsenbeck and Ronquist, 2001; Huelsenbeck *et al.*, 2001). The smoothing parameters for the penalized likelihood analyses were determined using cross-validation tests. The outgroups *V. kirkii* and *V. marina* were pruned prior to analysis using r8s (Sanderson, 2003; M. Lavin, Montana State University, USA, pers. comm.). Based on an analysis with multiple calibration points across the legumes (Lavin *et al.*, 2005), the age of the root node on the trees was fixed at a maximum of 8·0 million years (Mya) which represents the root of the Old World *Vigna* clade (i.e. all of the endemic African *Vigna* lineages in which members of *Ceratotropis* are nested; node 68 in Lavin *et al.*, 2005, which had a maximum age estimate of 10·4 Mya, minimum of 6·4, and a mean of 8·0; M. Lavin, pers. comm.). Ages were biased old by assigning the mean age estimate of 8·0 Mya rather than the youngest ages within the 95 % confidence interval. This was done for emphasis regardless of a bias in older estimates, and young ages were still estimated. Analyses using 8·0 Mya as the root age estimated 32 as the optimal smoothing value. Means and standard deviations of ages of specified clades were obtained from the input of 100 Bayesian trees (Lavin *et al.*, 2005; Delgado-Salinas *et al.*, 2006; Javadi *et al.*, 2007).

Character state evolution was examined for seedling germination type (Table 1) by mapping character states onto the strict consensus tree of the most-parsimonious phylogenetic trees of the combined data with MacClade 4·06 (Maddison and Maddison, 2000). Both ACCTRAN (maximizing the proportion of the homoplasy accounted for parallelism) and DELTRAN (maximizing the proportion accounted for reversal) optimizations were considered and analysed.

RESULTS

Sequence characteristics of the four plastid regions

The length of the *psb*D-*trn*T region ranged from 1085 bp (*V. mungo* var. *sylvestris*) to 1122 bp (*V. stipulacea*) in the subgenus *Ceratotropis*. The total aligned length for *psb*D-*trn*T was 1189 bp in ingroup taxa, which included 93 variable and 72 (6·0 %) parsimony-informative characters (Table 3). Pairwise sequence divergence varied from 0·00 to 0·0517 within the ingroup. Three unique indels (insertion/deletion), 27 bp, 20 bp and 7 bp, were found in *psb*D-*trn*T: the 27-bp insertion in two accessions of *V. stipulacea* (Vst-1 and Vst-2), the 20-bp insertion in two accessions of *V. tenuicaulis* (Vten-1 and Vten-2), and the 7-bp deletion in two accessions of *V. mungo* (Vmg-1 and Vmg-2). There was a 42-bp inversion in this region. The inversion was bordered by a pair of inverted repeat sequences 14 bp long. Two types of 42-bp inversions were recognized: type I occurred in *V. angularis*, *V. nepalensis*, *V. hirtella*, *V. exilis*, *V. umbellata*, *V. minima*, *V. riukiuensis*, *V. tenuicaulis*, *V. nakashimae* and Sri Lankan accession of *V. trilobata*; and type II occurred in the remaining ingroup taxa, while single nucleotide substitutions were found in type II which further includes three subtypes, II-i, II-ii and II-iii (Fig. 1 and Table 1). The orientation of the loop in Type II was reverse complementary to that of type I (Fig. 1). Subtypes II-i, II-ii and II-iii differed from type II by one nucleotide substitution and from each other by two nucleotide substitutions (i.e. type II-i, *V. reflexo-pilosa*; type II-ii, *V. grandiflora*; type II-iii, *V. aconitifolia*) (Fig. 1 and Table 1).

The length of the *trn*T-*trn*L region ranged from 796 bp (*V. trinervia*) to 849 bp (*V. grandiflora*) within the ingroup taxa. The total aligned length for *trn*T-*trn*L was 903 bp in ingroup taxa, which included 90 variable and 60 (6·6 %) parsimony-informative characters (Table 3). Pairwise sequence divergence ranged from 0·00 to 0·0323 among taxa in the subgenus *Ceratotropis*. Two unique insertions (19 bp and 17 bp) were observed in *V. grandiflora* in this region.

Within the subgenus *Ceratotropis*, the length of the *trn*T-*trn*E region ranged from 754 bp (*V. radiata*) to 795 bp (*V. hirtella*). The total aligned length for *trn*T-*trn*E was 801 bp in ingroup taxa, which included 84 variable and 54

TABLE 3. *Sequence characteristics of four plastid regions in the subgenus* Ceratotropis

Character	*trn*T-*trn*L	*psb*D-*trn*T	*pet*A-*psb*J	*trn*T-*trn*E
Length range (bp)				
Ingroup	796–849	1085–1122	527–594	754–795
Outgroup included	754–789	1044–1089	509–553	733–800
Aligned length (bp)				
Ingroup	903	1189	615	801
Outgroup included	957	1193	655	830
Number of variable characters (%)				
Ingroup	90 (9·9)	93 (7·8)	44 (7·2)	84 (10·5)
Outgroup included	156 (16·3)	167 (13·9)	96 (14·6)	131 (15·7)
Parsimony-informative characters (%)				
Ingroup	60 (6·6)	72 (6·0)	28 (4·5)	54 (6·7)
Outgroup included	75 (7·8)	91 (7·6)	35 (5·3)	61 (7·3)

(6·7 %) parsimony-informative characters (Table 3). Pairwise sequence divergence ranged from 0·00 to 0·0295 within the ingroup. Two unique gaps were found in this region, a 13-bp insertion shared by four out of five accessions of *V. hirtella* (except for accession Vhi-5, Table 1) and a 28-bp deletion shared by three accessions of *V. radiata*.

The length of *pet*A-*psb*J ranged from 527 bp to 594 bp within the ingroup. The total aligned length for *pet*A-*psb*J was 615 bp in ingroup taxa, which included 44 variable and 28 (4·5 %) parsimony-informative characters (Table 3). Pairwise sequence divergence varied from 0·00 to 0·029 among taxa in this subgenus. Within the *pet*A-*psb*J data, a 67-bp insertion was found in one accession of *V. exilis* (Vxi-1; Table 1).

Phylogenetic analysis

The suitability of combined phylogenetic analysis was conducted using ILD tests. Pairwise ILD tests indicated that there were no conflicts among any of the four chloroplast regions (Table 4). Therefore, phylogenetic analyses based on the combined dataset were conducted. For the phylogenetic analysis, the inversion was excluded, and potentially informative indels were added to the data matrix. The combined dataset comprised 2832 bp, of which 465 were variable and 224 were parsimony-informative. Maximum-parsimony analysis resulted in 15 most-parsimonious trees (TL = 613, CI = 0·809, RI = 0·901), and the strict consensus tree of the 15 most-parsimonious trees is shown in Fig. 2I.

Bayesian analyses were also performed for individual DNA regions; the best fitting models and parameter values are shown in Table 5. The optimal models identified were TIM (four rate classes) for the *pet*A-*psb*J, TIM + G (four rate classes following a gamma distribution) for the *trn*T-*trn*L, TVM + G (five rate classes following a gamma distribution) for the *trn*T-*trn*E, K81uf + I + G (three rate classes following a gamma distribution and invariant sites) for the *psb*D-*trn*T, and TVM + I + G (five rate classes following a gamma distribution and invariant sites) for the combined datasets. Since MrBayes only allows the choices of 1, 2 or 6 rate categories, all of the 3+ rate category submodels of the general time reversible model were placed in the six rate classes. Bayesian analysis using a single evolutionary model across all DNA regions and the partitioned analysis of each cpDNA region produced trees with $-\ln L = 17\,111{\cdot}61$ and $-\ln L = 18\,949{\cdot}80$, respectively. The Bayes factor between the single and partitioned models was therefore 1838·19, indicating that application of a single evolutionary model was more appropriate than partitioned analysis. The results presented here are therefore based on homogeneous Bayesian inference.

The strict consensus tree and Bayesian tree were identical in the topology and revealed two clades with robust support, clade A and clade B (Fig. 2I). Nine taxa of the subgenus *Ceratotropis* formed clade A with high support (BS = 100 %; Fig. 2I) having inversion type I, and they fell into two subclades, A-I and A-II (Fig. 2I). Clade A-I constitutes *V. angularis* (wild, weedy and cultivated forms), two Myanmar accessions of *V.* aff. *minima* (Vmn-1 and Vmn-2; Table 1) and *V. nepalensis* (Fig. 2I) which were collected from the temperate regions of East Asia and the Himalayan highlands. Clade A-II included *V. tenuicaulis*, *V. hirtella*, *V. exilis*, an Indonesian accession of *V. minima* (Vmn-3; Table 1), *V. umbellata*, *V. riukiuensis* and *V. nakashimae* (Fig. 2I) which were mainly found in a subtropical climate. The *V. hirtella*, *V. umbellata* and *V. exilis* clade was highly supported (BS = 86 %) in parsimony but not in Bayesian analysis (Fig. 2I). Twelve taxa of the subgenus *Ceratotropis* form clade B with high support (BS = 100 %; Fig. 2I), including four domesticated and eight wild species of the subgenus *Ceratotropis* which are mainly distributed in the tropical region of the Indian subcontinent and Southeast Asia. This clade B included taxa belonging to three sections of *Ceratotropis* and mainly has inversion type II and its three subtypes, except Sri Lankan accession of *V. trilobata* (Table 1 and Fig. 2I). The species relationships in this clade were not clearly resolved; however, there were two distinct subclades: subclade B-I, including *V. trinervia*, *V. reflexo-pilosa* and an Indian accession of *V. trilobata* (Fig. 2I), and subclade B-II, consisting of *V. mungo* and *V. grandiflora* (Fig. 2I).

Multiple accessions of 13 species were sampled, and most species formed monophyletic clades. Three accessions of *V. minima*, however, fell into two independent clades. The two accessions of *V. trilobata* were paraphyletic (clade B; Fig. 2I). One accession of *V. trilobata* (India, Vtb-1) was sister to *V. reflexo-pilosa* and *V. trinervia* in subclade B-I (Fig. 2I; Table 1). The other accession of *V. trilobata* (Sri Lanka, Vtb-2) was placed in the basal part of clade B (Fig. 2I and Table 1).

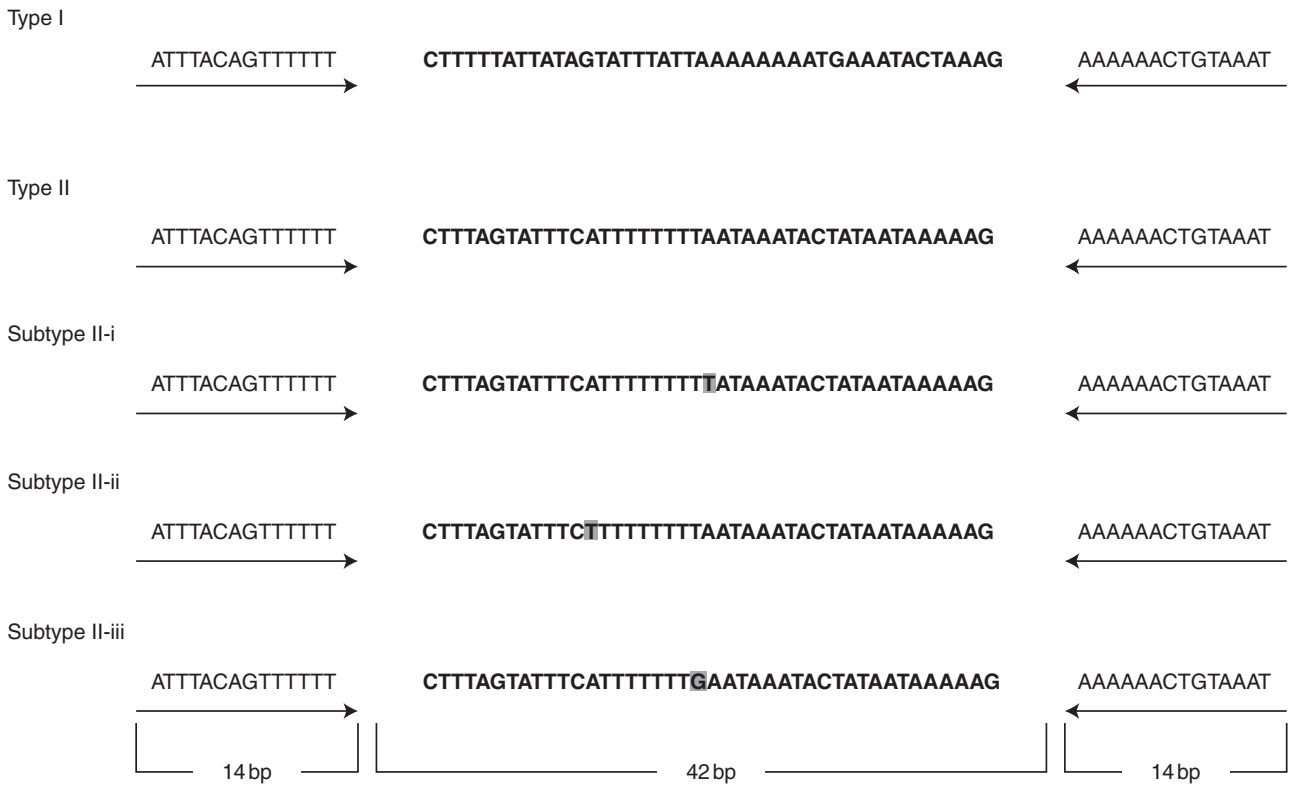

FIG. 1. Inversion types in the *psb*D-*trn*T intergenic spacer region in the *Vigna* subgenus *Ceratotropis*. Arrows below the sequence indicate inverted repeats. Nucleotides bordered by inverted repeats have undergone inversions. The bold letters on a grey background show single nucleotide substitution. The different types of each accession are shown in Table 1 and Fig. 2I.

TABLE 4. *Results of the ILD tests for pairwise comparisons between the data partitions*

Partitions	*pet*A-*psb*J	*trn*T-*trn*L	*trn*T-*trn*E	*psb*D-*trn*T
*pet*A-*psb*J	–			
*trn*T-*trn*L	0·70	–		
*trn*T-*trn*E	0·82	0·99	–	
*psb*D-*trn*T	0·47	0·11	0·11	–

Character state mapping for seedling germination

The character changes and ancestral-state reconstruction on the tree were explored using the total-evidence analysis of plastid sequences. ACCTRAN and DELTRAN optimizations showed similar results, and only results using ACCTRAN are presented. Epigeal seedling germination is polyphyletic according to the trees (Fig. 3). In clade B, *Vigna mungo*, *V. grandiflora*, *V. radiata* and *V. aridicola* are plesiomorphically characterized as having epigeal germination. Two separate gains in hypogeal germination are also inferred in clade B: once in the evolution of *V. stipulacea* and again in the clade containing *V. trinervia* and *V. reflexo-pilosa* (Fig. 3). Hypogeal germination is also a synapomorphy for clade A (Figs 2I and 3). A parsimony interpretation of character evolution reveals that hypogeal germination was derived independently on three occasions from epigeal germination.

Age estimation

By setting the root node on the trees at a maximum of 8·0 Mya, the estimated divergence time for clade A and clade B was 3·62 ± 0·3, and that of two subclades, A-I and A-II, within clade A (Fig. 2I) was 2·0 ± 0·2 Mya. The chronogram showing the range of estimated divergence times is shown in Fig. 2II.

DISCUSSION

Phylogenetic utility of the four plastid regions

The *psb*D-*trn*T, *trn*T-*trn*E and *trn*T-*trn*L intergenic spacer sequences showed a significant amount of variation in *Vigna*, in accordance with that of Shaw *et al.* (2005, 2007). The *psb*D-*trn*T region has a higher substitution rate than those of the three other regions (Table 3). The *trn*T-*trn*E and the *trn*T-*trn*L regions, although evolving more slowly than the *psb*D-*trn*T region, have a higher percentage of phylogenetically informative sites among the variable sites (6·6 % vs. 6·7 %; Table 3). The indels in the *psb*D-*trn*T, *trn*T-*trn*E and *trn*T-*trn*L regions provided phylogenetically informative and species-specific characters, and a few indels showed intraspecific variation. These DNA regions have good potential for phylogenetic analyses at the interspecific level.

The inversion found in the *psb*D-*trn*T region was bordered by inverted repeat sequences (Fig. 1), suggesting formation

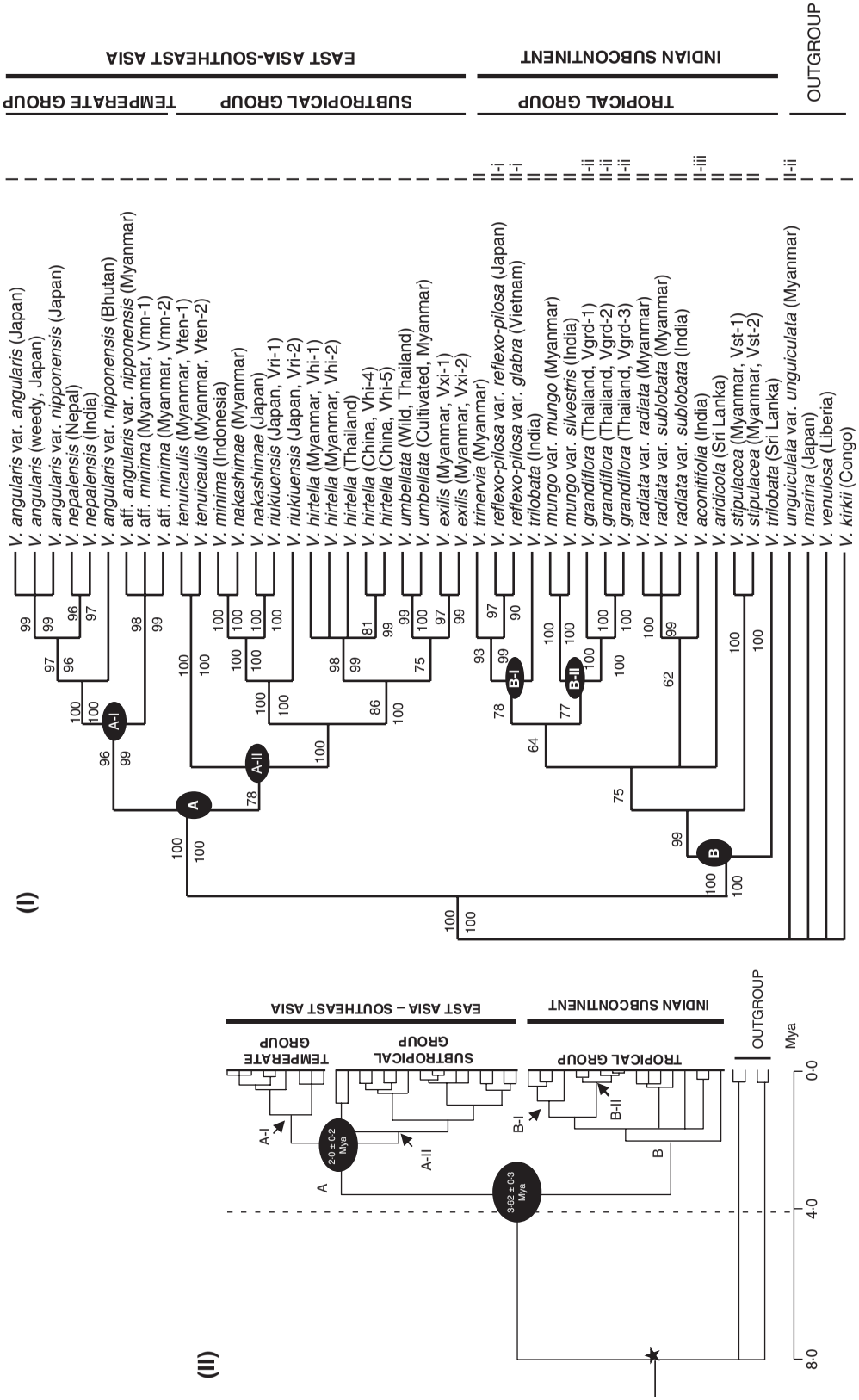

FIG. 2. (I) Strict consensus tree of 15 parsimonious trees inferred from *pet*A-*psb*I, *psb*D-*trn*T, *trn*T-*trn*E, and *trn*T-*trn*L spacer sequences. Numbers above branches refer to bootstrap support in parsimony analysis, and those below branches are Bayesian posterior probability. CI = 0·809, RI = 0·901. Roman numerals following species names represent the inversion type in the *psb*D-*trn*T region. (II) A penalized likelihood chronogram of the Bayesian consensus tree of the combined data. The star indicates the calibration point used (Lavin *et al.*, 2005, for details, see Materials and methods), The time scale is in million years ago (Mya).

TABLE 5. *Best-fitting models and parameter values for separate (*petA-psbJ, trn*T*-trn*L*, trn*T*-trn*E*, psb*D*-trn*T*) and combined datasets in this study*

Region	AIC selected model	Base frequencies				Substitution model (rate matrix)						I*	G*
		A	C	G	T	A–C	A–G	A–T	C–G	C–T	G–T		
petA-psbJ	TIM	0·3623	0·1419	0·1185	0·3773	1·0000	0·8642	0·2735	0·2735	0·5067	1·0000	0	Equal
trnT-trnL	TIM + G	0·4190	0·0884	0·1238	0·3688	1·0000	0·0865	0·0596	0·0596	0·2375	1·0000	0	0·3479
trnT-trnE	TVM + G	0·3709	0·1337	0·1260	0·3695	1·0000	0·5281	0·2382	0·8342	0·5281	1·0000	0	0·6412
psbD-trnT	K81uf + I + G	0·3510	0·1391	0·1247	0·3853	1·0000	0·3110	0·1832	0·1832	0·3110	1·0000	0·5654	0·9177
Combined analyses	TVM + I + G	0·3731	0·1240	0·1254	0·3775	1·1519	0·3672	0·1489	0·4059	0·3672	1·0000	0·3310	0·8910

* I, Proportion of invariable sites; G, gamma distribution.

of stem–loop structures and recombination in the stems at inversion process (Sang *et al.*, 1997). In contrast to some large inversions in cpDNA which provided reliable phylogenetic information at the higher taxonomic levels (i.e. Doyle, 1992; Raubeson and Jansen, 1992), short inversions in the intergenic spacer easily yielded homoplasious information even at the interspecific level (Sang *et al.*, 1997). Thus, sequence data of inversion were not included in the present phylogenetic analyses, following the recommendation of Sang *et al.* (1997). Nevertheless, the inversion might provide additional information on certain levels in the present study, e.g. both clades differed in their inversion type (Fig. 2I).

The tree derived from the combined sequence data of the four chloroplast intergenic spacers strongly supported the interspecific relationships of the subgenus *Ceratotropis* (Fig. 2I). This phylogenetic view is in general agreement with that of previously published subgenus *Ceratotropis* phylogenies (Goel *et al.*, 2002; Yano *et al.*, 2004; Ye Tun Tun and Yamaguchi, 2007), although the results of the present study show that the inclusion of more molecular data under an increasing taxon-sampling scheme was crucial to increase the resolution and support of the phylogenetic tree.

Phylogenetic and biogeographical relationships

The topology of the cpDNA phylogeny clearly indicates geographical and ecological relationships within the subgenus *Ceratotropis* (Fig. 2I). According to known geographical distribution patterns of the subgenus *Ceratotropis* (Tomooka *et al.*, 2002*b*; authors' own field surveys), two major clades in the phylogenetic tree show geographic structure: East Asia–Southeast Asia clade (clade A) and the Indian subcontinent clade (clade B), and the two ecogeographic substructures within clade A, temperate group (subclade A-I) and subtropical group (subclade A-II) (Fig. 2I).

East Asia–Southeast Asia clade (temperate group and subtropical group)

The monophyletic temperate group contains domesticated azuki bean (*V. angularis* var. *angularis*), wild azuki bean (*V. angularis* var. *nipponensis*), weedy azuki (*V. angularis*), *V. nepalensis* and *V.* aff. *minima* (Myanmar accessions) (Fig. 2I), which are distributed throughout the temperate East Asia regions and the cooler parts of the Himalayan foothills. The well-supported monophyly of wild, weedy and cultivated *V. angularis* (Fig. 2I) is congruent with that of previous findings in terms of morphology (Yamaguchi, 1992), cross compatibility (Sawa, 1983), isozyme features (Yasuda and Yamaguchi, 1996), RAPD (Mimura *et al.*, 2000), AFLP (Zong *et al.*, 2003) and cpDNA sequences (Yano *et al.*, 2004; Ye Tun Tun and Yamaguchi, 2007, 2008). Despite the affinity of *V. nepalensis* and *V. tenuicaulis* in AFLP analysis (Tomooka *et al.*, 2002*c*), present and previous studies (Doi *et al.*, 2002; Zong *et al.*, 2003; Ye Tun Tun and Yamaguchi, 2007, 2008) reveal a close relationship between *V. angularis* and *V. nepalensis* (Fig. 2I). These two species have a number of morphological similarities such as flower colour, shape of calyx, hypogeal germination (Tomooka *et al.*, 2002*b*) and share a unique 8-bp insertion in the *trn*T-L

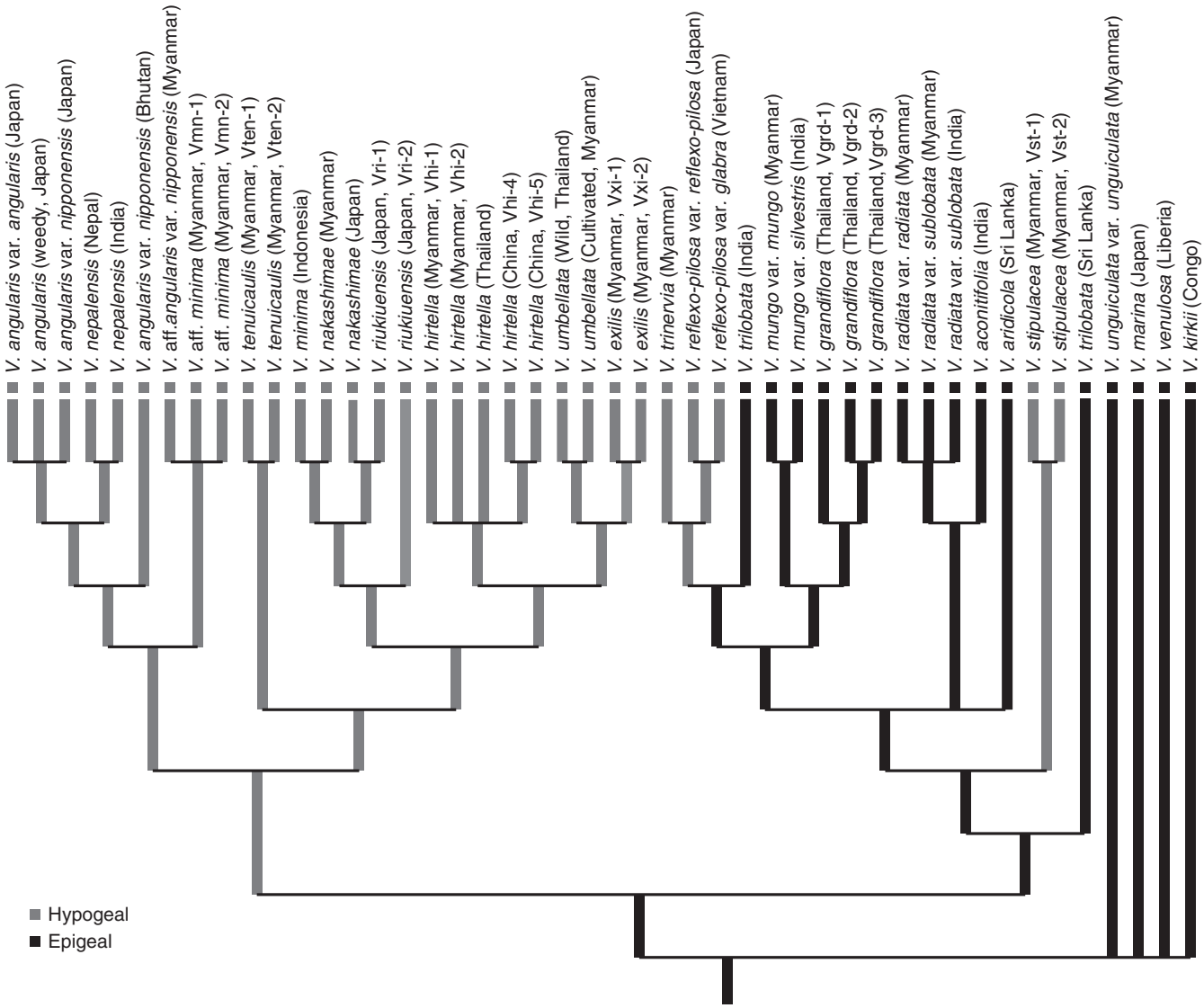

FIG. 3. Mapping of seedling germination type on the strict consensus tree of *Vigna* subgenus *Ceratotropis* generated from the combined DNA dataset using MacClade 4·0 (Maddison and Maddison, 2000).

spacer region. They differ in hairiness of primary and secondary bracts and bracteole (pubescent in *V. angularis* vs. glabrous in *V. nepalensis*) and length of bracteole/calyx (bracteole much longer than calyx in *V. angularis* vs. as long as calyx in *V. nepalensis*) (Tomooka *et al.*, 2002*b*). In this monophyletic temperate group, *V. angularis* aff. var. *nipponensis* (Van-5; Table 1) from the hilly region of northern Myanmar and two accessions of *V.* aff. *minima* from Myanmar are grouped together with high support (BS = 98 %; Fig. 2I) and share three synapomorphic substitutions. This Myanmar accession (Van-5, Table 1) is shorter and has smaller floral parts than the other accessions of *V. angularis* var. *nipponensis*, and they share a 11-bp deletion of the *trn*T-L spacer region with accessions from Bhutan and China (Ye Tun Tun and Yamaguchi, 2007). Since the Myanmar accessions, *V.* aff. *minima* (Vmn-1 and Vmn-2; Table 1) resemble *V. minima* in many key characters, including size of stipule, bract and bracteole, glabrous pod, and well-developed aril (Tomooka *et al.*, 2002*b*), differ from *V. angularis* aff. var. *nipponensis* in a 51-bp insertion of the *trn*L-F spacer region (Ye Tun Tun and Yamaguchi, 2007), and its flower is morphologically similar to that of *V. tenuicaulis*, it is considered to be a distinct taxon in temperate *Ceratotropis*. The relationship between *V.* aff. *minima* and *V. angularis* aff. var. *nipponensis* in the tree reveals that the ecological and geographical aspects of species ranges reflect the phylogenetic relationships and further support the finding that ecological characteristics are an obvious determinant of geographical distribution in *Vigna* and relatives (Lavin and Beyra-Matos, 2008).

The monophyletic subtropical group contains *Vigna minima* (from Indonesia), *V. nakashimae*, *V. riukiuensis*, *V. hirtella*,

V. exillis and *V. umbellata* (Fig. 2I; clade A-II). Almost all the species of this group are found in the plant communities that are marginal to the subtropical rain forests of Southeast Asia except natural populations of *V. nakashimae* in the temperate Far East. The three morphologically close species, *V. nakashimae*, *V. minima* (Indonesia) and *V. riukiuensis*, are included in a well-supported subgroup (Fig. 2I), and they are distinguished by a large golden flower in *V. minima*, small glossy leaflets in *Vigna riukiuensis* and a protruding hilum and a small, pale yellow flower in *V. nakashimae* (Tomooka *et al.* 2002*b*). *Vigna nakashimae* was first described in Japan and has only been reported in East Asia (Tateishi, 1985; Tateishi and Ohashi, 1990; Tomooka *et al.*, 2002*b*); however, it was recently found in the area from the Far East through southern China to the southern part of Myanmar (Mon state; Ye Tun Tun and Yamaguchi, 2007). Accessions of *V. nakashimae* in this study show close similar plastic sequences despite their intraspecific differentiation in AFLP data (Seehalak *et al.*, 2006). Since *V. riukiuensis* is endemic to the southern part of the Ryukyu archipelago (Miyako and Sakishima Islands, Okinawa) and these islands constitute an extended peninsula from Taiwan and the Chinese mainland during the last glacial age, the present results support the hypothesis that the island species *V. riukiuensis* might have evolved from a continental species, *V. nakashimae* (Ye Tun Tun and Yamaguchi, 2007); we further propose that *V. nakashimae*, *V. riukiuensis* and *V. minima* evolved from a common ancestor (Fig. 2I). As *V. nakashimae* and *V. riukiuensis* were treated as distinct taxa (Tomooka *et al.*, 2002*b*) or considered as subspecies/varieties of *V. minima* (Tateishi, 1985), their taxonomic delimitation remains crucial due to a high variation of *Vigna minima*. The Indonesian accession (Vmn-3; Table 1) has the same morphological features of the plants recognized as *V. minima*, representing small stipule, small primary and secondary bracts, small bracteoles, glabrous pod and hilum with well-developed aril. Since the lack of monophyly of even morphologically well-defined species can result from incomplete lineage sorting of shared ancestral polymorphism or contemporary gene flow (Funk and Omland, 2003), the polyphyly of *V. minima* and *V.* aff. *minima* is probably due to the ancestral polymorphism, and thus there is a need for further examination of this group.

The close relationship between the three species, *V. hirtella*, *V. exillis* and *V. umbellata*, is well-resolved and highly supported (BS = 86 %; Fig. 2I) as is also the case in the AFLP study (Seehalak *et al.*, 2006). They share a unique single base-pair deletion in the *trn*T-E region. *Vigna umbellata* and *V. exillis* share some characters such as protruding hilum and well-developed aril in their seed morphology, although these characters are homoplasious in the subgenus *Ceratotropis*. Despite the AFLP results (Seehalak *et al.*, 2006), in *V. hirtella*, five accessions from a wide area (Myanmar, Thailand and China) are monophyletic (BS = 98 %, BI = 99 %; Fig. 2I) and share a 13-bp insertion in the *trn*T-*trn*E region, except for one accession from China (Vhi-5). In this monophyletic of *V. hirtella*, two accessions from China (Vhi-4 and Vhi-5; Table 1 and Fig. 2I) share one unique 1-bp substitution and a single base-pair deletion. The present combined cpDNA tree suggests that AFLP differentiation among *V. hirtella* accessions (Seehalak *et al.*, 2006) might be due to geographical or population differentiation within species but not to a distant relationship.

The wild and cultivated accessions of *V. umbellata* (rice bean) show only two nucleotide substitution differences in the four plastid regions which form a monophyletic clade (Fig. 2I). This result agrees with the findings of the AFLP analysis (Seehalak *et al.*, 2006) and the molecular tree based on the *trn*T-F region (Ye Tun Tun and Yamaguchi, 2007). *Vigna tenuicaulis* has the early branching position within the subtropical group with moderate clade support (BS = 78 %; Fig. 2I), although this species was considered to be close to *V. angularis* and *V. nepalensis* (Tomooka *et al.*, 2002*b*).

Indian subcontinent clade (tropical group)

Vigna species of clade B inhabit hot-dry or sub-humid tropical lowlands mainly on the Indian subcontinent, and this species is considered as the tropical group (Fig. 2I). Although the phylogenetic relationships among members of this group were unresolved in the previous studies (Doi *et al.*, 2002; Konarev *et al.*, 2002; Tomooka *et al.*, 2002*c*; Ye Tun Tun and Yamaguchi, 2007), two well-supported subgroups of the tropical group were recognized in the molecular tree, B-I and B-II (Fig. 2I). The close relationship between *V. trinervia* and *V. reflexo-pilosa* (BS = 93 %, BI = 99 %) and their inclusion in the tropical group is clear as clade B-I which was also the case with the AFLP results (Seehalak *et al.*, 2006), although the two species have been placed as members of the azuki bean group (Maekawa, 1955; Tateishi and Ohashi, 1990) or in section *Angulares* (Tomooka *et al.*, 2002*a*, *b*). *Vigna trinervia* has a distribution pattern in Africa, Asia and the Pacific Islands and shares characters such as rectangular seed and hypogeal germination with *V. reflexo-pilosa* (Tomooka *et al.*, 2002*b*). The placement of *V. reflexo-pilosa* within the Indian subcontinent clade (Fig. 2I) conflicts with the result of the nrDNA ITS tree (Doi *et al.*, 2002). The present results are in agreement with the assumption that *V. trinervia* is a diploid donor (Egawa *et al.*, 1996; Konarev *et al.*, 2002) or a maternal donor (Yano *et al.*, 2004; Ye Tun Tun and Yamaguchi, 2007) of allotetraploid *V. reflexo-pilosa*. Its sister relationship of an Indian accession of *V. trilobata* to *V. reflexo-pilosa* and *V. trinervia* with moderate support (BS = 78 %, BI = 81 %; Fig. 2I) is worth noting, as seen in an earlier analysis based on *trn*T-F data (Ye Tun Tun and Yamaguchi, 2007).

The grouping of *V. grandiflora* and *V. mungo* received moderate support as clade B-II (BS = 77 %, BI = 84 %; Fig. 2I) sharing one synapomorphic substitution and a unique single base-pair deletion in the *trn*T-*trn*L and *pet*A-*psb*J regions, respectively. Although *V. mungo* differs morphologically from *V. grandiflora* by the brightness of its yellow flower, protruding ovate hilum and well-developed aril (Tomooka *et al.*, 2002*b*), a close relationship between *V. grandiflora* and *V. mungo* in the present tree is congruent with that of the nrDNA ITS phylogenetic tree (Doi *et al.*, 2002). *Vigna mungo* var. *silvestris* is widely distributed throughout India, Myanmar and Thailand, while *V. grandiflora* is narrowly distributed in Thailand and Cambodia (Baudoin and Maréchal,

1988; Tateishi, 1996; Tomooka *et al.*, 2002*b*). This phylogeographical pattern leads to the assumption that *V. grandiflora* may have gained its present distribution area after differentiation from *V. mungo* var. *silvestris*. *Vigna aconitifolia* is sister to *V. radiata* in the present plastid tree with low support (BS = 62 %; Fig. 2I), though both of them have one shared synapomorphic character in the *pet*A-*psb*J spacer region. However, these two species differ in numerous morphological characters such as shape of leaflet (deeply five-lobed terminal and deeply four-lobed lateral leaflets in *V. aconitifolia* and ovate terminal and obliquely lateral leaflets in *V. radiata*), flower size and colour (small bright yellow flower in *V. aconitifolia* and large pale greyish yellow flower in *V. radiata*) and seed shape (elliptic in *V. aconitifolia* and rectangular in *V. radiata*), despite their molecular relatedness.

Although the phylogenetic relationship is not so well resolved, the domesticated and wild types of *V. radiata* are monophyletic (Fig. 2I) and their relationship is supported by a 28-bp deletion in the *trn*T-L region. *Vigna radiata* var. *sublobata* is widely distributed in Africa, Asia and the Pacific islands (Tomooka *et al.*, 2002*b*) by its weedy habit (Chandel *et al.*, 1984), and it occupies mostly disturbed habitat such as roadsides (Tomooka *et al.*, 2002*b*) which may contribute to its rapid dispersal. A possible explanation for its low molecular divergence could be that divergence within *V. radiata* occurred recently, such that neutral cpDNA intergenic spacers variation would not have had sufficient time to be fixed in the different taxa. The basal position of *V. stipulacea* in the tropical group is supported by a unique 27-bp insertion in *psb*D-*trn*T and the sister-species relationships of *V. stipulacea* and Sri Lankan *V. trilobata* to the tropical group by 3-bp synapomorphic insertion in the *pet*A-*psb*J region.

The two accessions of *V. trilobata* sampled from Sri Lanka and India are resolved as paraphyletic. *Vigna trilobata* is distributed in Sri Lanka, India and Myanmar, and is characterized by its orbicular to ovate stipule, golden yellow flower, glabrous mature pod, seed with protruding orbicular, and hilum having well-developed aril (Tomooka *et al.*, 2002*b*). The Indian accession of *V. trilobata* has longer hairs on stems and leaves and less protruding hilum in comparison with that of the Sri Lankan accession, and it shares a rectangular seed shape with *V. reflexo-pilosa*. At the molecular level, Sri Lankan and Indian accessions are differentiated by several substitutions and indels, and also type II of the 42-bp inversion in the *pet*A-*psb*D region in Indian accession of *V. trilobata* (Table 1 and Fig. 2I). Because of the apparent frequency of rapidly radiating clades that create conditions favourable for incomplete lineage sorting, plant species paraphyly is a common finding (i.e. Wood and Nakazato, 2009; Fishbein *et al.*, 2010). We suppose that placement of Indian and Sri Lankan accessions of *V. trilobata* in different phylogenetic positions is probably due to the ancient differentiation between the island population (Sri Lanka) and the mainland population (India) or due to delimitation status of *V. trilobata*.

Patterns of character evolution

The well-resolved phylogeny in this study provides an opportunity for an analysis of character evolution that has been used to define groups at various taxonomic levels. Hypogeal and epigeal germination was used as one of the key morphological characters in sectional classification of *Vigna* subgenus *Ceratotropis* (Sects. *Angulares*, *Ceratotropis* and *Aconitifoliae*; Table 1) (Tomooka *et al.*, 2002*a*, *b*). The cpDNA phylogeny present here indicates that the germination type evolved independently multiple times (Fig. 3), suggesting the taxonomic limit of the utility of seedling germination. Based on morphological cladistic analysis, the hypogeal germination was suggested as the primitive state in the subgenus *Ceratotropis* (Tateishi, 1985, 1996); however, the present results assume its derived condition and that it evolved in three independent lineages (Fig. 3). Furthermore, seedling traits are evolutionarily conservative, reflecting phylogenetic niche conservatism (Ibarra-Manrìquez *et al.*, 2001). Dispersal of *Sophora tomentosa* in the tropics appears to be correlated with its hypogeal germination (Heenan *et al.*, 2004). In the genus *Manihot*, a phylogenetically based survey of seedling functional morphology is associated with habitat and life form (Pujol *et al.*, 2005), although it is difficult to identify the relationship between habitat and germination type within some families such as Restionaceae (Linder and Caddick, 2001). The phylogeny generated in this study suggests that seedling germination type is probably associated with a shift in habitat conditions since species with epigeal germination are mainly distributed in dry and tropical lowland habitats, while the subhumid and warm temperate regions are mainly occupied by species with hypogeal germination except for *V. stipulaceae*, *V. trinervia* and *V. reflexo-pilosa* (Figs 2I and 3).

Diversification time in the subgenus Ceratotropis

The combined cpDNA phylogenetic tree showed two major geographical groups in the subgenus *Ceratotropis*; the Indian subcontinent group and East Asia–Southeast Asia group, suggesting the process of interspecific diversification to different climatic and ecological habitats, e.g. temperate, subtropical and tropical groups (Fig. 2I). The estimated divergence time (3·62 ± 0·3 Mya) between the Indian subcontinent group and East Asia–Southeast Asia group was less than that of the Tibetan uplift (8 Mya or 15 Mya; Harrison *et al.*, 1992; Spicer *et al.*, 2003), suggesting the diversification within the subgenus *Ceratotropis* may have occurred after the collision of the Indian subcontinent and the Asian plate. The recent diversification of the subgenus *Ceratotropis* was congruent with the findings of Lavin *et al.* (2005) who proposed that the age of the African crown clade including African and Asian *Vigna* (*V. mungo* and *V. umbellata*) was about 5·1 Mya. Moreover, molecular dating indicates late Pliocene diversification of the temperate and subtropical groups (2·0 ± 0·2 Mya) in the subgenus *Ceratotropis*. However, diversification spread through late Pliocene and into the Pleistocene. The north-east Indian passageway (Cordaux *et al.*, 2004), a narrow land bridge between the Indian subcontinent and East Asia which was created by the collision of Indian subcontinent with Asia, might be an important geographic barrier for species migration, together with the formation of high mountain ranges between the two regions. Therefore, formation

and persistence of seasonal climate after the collision of the Indian subcontinent with the Asian plate and long-distance dispersal could have subsequently facilitated the Pliocene–Pleistocene diversification of the subgenus *Ceratotropis* into different eco-geographic groups.

Conclusions

The evidence presented here clearly demonstrates the geography and ecological habitat as important factors in shaping phylogenetic relationships of the subgenus *Ceratotropis*. Indels in the four cpDNA intergenic spacers provide useful information to infer the phylogenetic relationships at the interspecific levels in the subgenus *Ceratotropis*. It is important to note that DNA-based identification in the subgenus *Ceratotropis* would be much more challenging for a future study. Mapping seedling germination used taxonomically in the subgenus *Ceratotropis* into the phylogeny indicates the multiple origins, thus limiting its taxonomic utility, and a reassessment of diagnostic characters in the current classification of subgenus is thus necessary. Ultimately, this study provides a framework for future studies that deal taxonomically with *Vigna* species.

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