Genetic diversity and its conservation implications of *Vitex rotundifolia* (Lamiaceae) populations in East Asia

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27 **Abstract**

28 *Vitex rotundifolia* is an important coastal and medicinal plant, and is recorded in the List of the

29 Important Wild Plants for Conservation in China and Japan. However, an effective conservation

30 strategy is lacking. In the present study, the genetic diversity and population structure were

31 analyzed using phylogeographical methods based on the *trnH-psbA* and *trnG-trnS* intergenic

32 spacers of the chloroplast DNA (cpDNA) sequences from 157 individuals from 25 sampling sites

33 for *V. rotundifolia* and *V. trifolia* plus the internal transcribed spacer (ITS) of the nuclear

34 ribosomal DNA (nrDNA) sequences of 177 individuals from 27 sampling sites. The results

35 showed that *V. rotundifolia* and *V. trifolia* had eight cpDNA and two nrDNA haplotypes,

36 respectively, and the *V. rotundifolia* has a low level of genetic diversity (haplotype diversity (h_d),

37 $_{cp}$ = 0.360, $h_{d,\ nr}$= 0.440), a more pronounced genetic differentiation among populations

38 (population differentiation at the species level (G_{ST}) = 0.201, population differentiation at the

39 allele level (N_{ST}) = 0.462), and an insignificantly different phylogeographical structure (N_{ST} >

40 G_{ST}, P > 0.05). In addition, haplotype network analyses indicated that *V. rotundifolia* and *V.*

41 *trifolia* have distinct haplotypes. Divergence dating based on BEAST software analyses showed

42 that most cpDNA clades diverged in the late Pleistocene era. Demographic analysis indicated

43 that *V. rotundifolia* underwent a rapid demographic expansion. Some scientific strategies are

44 suggested for resource conservation of *V. rotundifolia* based on its genetic diversity and

45 population structure.

47 **Introduction**

48 *Vitex rotundifolia*, which often grows on beaches and sand dunes, is a widely distributed

49 shoreline shrub of the family Lamiaceae, (Cantino, 1992; Harley, 2004; Cousins et al., 2010). *V.*

50 *rotundifolia* plays an important ecological role in stabilizing sand dunes in coastal areas

51 (Gresham & Neal, 2004; Kim, 2005). In addition, the fruit of *V. rotundifolia*, known as

52 Manjingzi, is a herbal medicine commonly used in China and Japan to prevent and treat colds,

53 headache, and migraine (Sung et al., 1996). However, the increased use of the plant as a medical

54 resource, coastal overexploitation, and environmental destruction, have accelerated the

55 degradation of *V. rotundifolia* populations, decreased the intraspecific variation, severely

56 destroyed its natural habitats, and even threatened the survival of the species. Given the

57 vulnerability of *V. rotundifolia*, it has been recorded in the List of the Important Wild Plants for

58 Conservation in China and Japan (http://www. cites.org. cn/article/show.php?itemid=589;

59 Ohtsuki et al., 2014). Therefore, it is necessary to provide an effective conservation strategy for

60 this species.

61   Based on a comprehensive allozyme study of *V. rotundifolia* Korean populations, Yeeh et al.

62 (1996) reported that the levels of genetic variation and differentiation within populations are

63 considerably lower, but are higher among populations. By contrast, levels of genotypic diversity

64 within and among populations were moderate. They indicated that clonal reproduction might act

65 as an enhancer of genetic drift by reducing the effective size of local *V. rotundifolia* populations.

66 Ohtsuki et al. (2014) developed ten microsatellite makers from *V. rotundifolia* Japanese

67 populations; however, no further data were presented. Hu et al. (2008) investigated the genetic

68 variation of *V. rotundifolia* at more extensive sampling areas in Chinese populations using inter

69 simple sequence repeat (ISSR) markers. Their fine-scale spatial autocorrelation analysis showed

70 a clear within-population structure, with gene clusters of approximately 20 m, which could act as

71 a guide for sampling strategies. They also reported the overall genetic diversity (GD) of *V.*

72 *rotundifolia* on China was moderate (GD = 0.190) and the genotypic diversity was greater than

73 the average values for a clonal plant, indicating its significant reproduction through seedlings.

74   Pharmacological research discovered that the genetic variation pattern was closely associated

75   with the chemical constituents in the fruit of *V. rotundifolia* (Hu et al., 2007). Currently, our

76   understanding of the genetic variation background of this species is poor, especially concerning

77   the population genetic structure and phylogeography. In addition, we lack specific advice for the

78   conservation of *V. rotundifolia*.

79   *V. trifolia* is another important medicinal plant and is the closest relative of *V. rotundifolia. V.*

80   *trifolia* is only distributed in a very narrow area in Southwest of China and Taiwan province, and

81   the independent species taxonomic status between *V. trifolia* and *V. rotundifolia* has long been

82   disputed (Munir, 1987; Kok, 2007, 2008; Barger et al., 2012; Chang, Kim, & Chang, 2014;

83   Franck et al., 2016). The fruit of *V. trifolia* have also been recorded in the Pharmacopoeia of

84   China as Manjingzi (Editorial Committee of Chinese Pharmacopoeia, 2015) for medical use.

85   Therefore, the addition of *V. trifolia* in this study could help to analyze the evolutionary history

86   of *V. rotundifolia* and to better understand their genetic differentiation.

87   The analyses of population genetic structures, genetic variations across populations, and the

88   geographical distributions of the species at risk of extinction will help to develop appropriate

89   conservation decisions and sustainable utilization strategies (Millar & Libby, 1991).

90   Phylogeographical methods are often used to assess the variability of molecular markers within a

91   taxon across both time and space, to deduce the historical processes that may have been

92   responsible for the contemporary geographical distributions of individuals and population

93   genetic structures, and help to prioritize areas of high value for conservation (Avise & John,

94   2000; Franck et al., 2016; Kumar & Kumar, 2018). Chloroplast DNA (cpDNA), which is

95   maternally inherited in most angiosperm plants, is commonly considered as a single non-

96   recombinant unit of inheritance and often is used to investigate the phylogeographical processes

97   associated with seed dispersal, such as range expansion (Clegg et al., 1994; Petit et al., 1997).

98   Nuclear ribosomal DNA (nrDNA), which exhibits different modes of inheritance, can also be

99   used to determine a species' genetic structure. The combination of highly conserved and variable

100   regions allows us to make phylogenetic inferences across a wide range of evolutionary

101 timescales and to unravel the genetic variation and demographic history of a plant species

102 (Weitemier et al., 2015).

103   In the present study, we analyzed the cpDNA and internal transcribed spacer (ITS) haplotype

104 sequences from 23 sampling sites covering most distribution ranges of *V. rotundifolia* in East

105 Asia; and four sampling sites of *V. trifolia*, to explore the genetic variation pattern, population

106 structure, and the evolutionary history of *V. rotundifolia*, with the aim of proposing effective

107 strategies for its resource conservation.

108

109 **1. Materials and Methods**

110 **1.1 Population sampling**

111 A total of 157 individuals of *V. rotundifolia* and *V. trifolia* from 25 sampling sites for cpDNA

112 and 177 individuals of *V. rotundifolia* and *V. trifolia* from 27 sampling sites for ITS were

113 sampled from distribution areas in East Asia (Table 1 and Fig.1). Leaf samples were collected

114 from individuals separated by more than 20 m to avoid collecting the same clones (genotypes).

115 The leaf samples were dried in silica gel and then stored in a freezer (−20 °C) for use. A sample

116 of *V. negundo* from Shanghai was also collected as the outgroup. Voucher specimens were

117 deposited in the herbarium of the Second Military Medical University (Shanghai, China).

118 **1.2 Sequencing of cpDNA and nrDNA**

119 The noncoding regions DNA sequences of cpDNA *trnG* (encoding tRNA glycine)-*trnS*

120 (encoding tRNA serine) and *trnH* (encoding tRNA Histidine)-*psbA* (encoding photosystem II

121 protein D1) and ITS intergenic spacer region DNA sequences were examined to analyze the

122 genetic variation in *V. rotundifolia* and *V. trifolia* from different geographical locations

123 (Hamilton, 1999; Shaw et al., 2007; Zhang et al., 2014). Total DNA was extracted using a Hi-

124 DNA secure Plant Kit (Tiangen Biotech (Beijing) Co., Ltd, Beijing, China). PCR amplification

125 was conducted in a total reaction volume of 50 μl, containing 10–20 ng total DNA, with 1 μl of

126 each primer, 21 μl of ddH$_2$O, and 25 μl of 2 × Trans Taq High Fidelity (HiFi) PCR SuperMix

127 (Transgen Biotech, Beijing, China). Double-stranded DNA was amplified after 3-min incubation

128   at 94 °C; followed by 30 cycles at 94 °C for 30 s, 54/56 °C for 30 s, and 72 °C for 30 s; with a

129   final extension at 72 °C for 15 min. To examine the geographical distribution of cpDNA and

130   nrDNA in *V. rotundifolia* and *V. trifolia*, we amplified two noncoding regions of cpDNA *trnG-*

131   *trnS* and *trnH-psbA* and ITS intergenic spacer regions using primers that successfully amplified

132   the expected DNA fragments and exhibited some variations in our preliminary experiments

133   (Hamilton, 1999; Shaw et al., 2007; Zhang et al., 2014). Primers used for PCR amplification

134   were also used as sequencing primers. The PCR products were assessed using electrophoresis

135   through 1.0% agarose gels and then used as templates for direct sequencing. Sequencing was

136   conducted from both ends. The cpDNA and ITS sequences data of *V. rotundifolia* and *V. trifolia*

137   samples have been submitted to the GenBank database, and the accession numbers for the *trnG-*

138   *trnS*, *trnH-psbA*, and ITS sequences are MG822129–MG822287, MG822468–MG822626, and

139   MG822288–MG822467, respectively.

140   **1.3 Phylogenetic analyses**

141   Geneious Pro V4.8.5 (http://www.geneious.com/) was used to check and align the DNA

142   sequences. Bayesian analysis (BI) was performed on MrBayes3.1.2 to construct the phylogenetic

143   tree of haplotypes based on the combined cpDNA data (Ronquist et al., 2012). T92+G was

144   selected as the model for the cpDNA dataset using Modeltest version 3.06 (Posada & Crandall,

145   1998) and the Akaike Information Criterion (AIC) was used to calculate the parameters and

146   assumptions for the sequence partitions. Two runs of four simultaneous Monte Carlo Markov

147   Chains (MCMC) analyses were performed for 10,000,000 generations, with sampling done every

148   100 generations. Log-likelihood values were examined for stationarity to determine the burn-in

149   value, which was 5,000 trees in this case. TCS 1.21 (Clement, Posada & Crandall, 2000) was

150   applied to build a genotype network based on the 90% parsimony criteria with the option of

151   treating gaps as a fifth base.

152   **1.4 Molecular dating**

153   The program BEAST version 1.5.3 (Drummond et al., 2012) was used to estimate the divergence

154   times of the major lineages to the most recent common ancestor (TMRCA). Considering there

155   are no fossil records and no specific substitution rates to calibrate the molecular clocks, the

156   published nucleotide substitution rates were used to estimate the divergence time between two

157   species, and a rate of about 1.2–1.7×10^{-9} substitutions per neutral site per year (s/s/y) was used

158   to obtain absolute values of TMRCA (Eyrewalker, 2000).

159   **1.5 Population genetic diversity and genetic differentiation**

160   The program DnaSP v5.10 was used to calculate the haplotype diversity (h_d) and nucleotide

161   diversity (π_d) based on the cpDNA and nrDNA sequences of *V. rotundifolia* and *V. trifolia*

162   (Librado & Rozas, 2009). To prevent an insufficient sampling affect, we re-analyzed the

163   nucleotide diversity of *V. rotundifolia* excluding poorly sampled populations.

164   The analyses of molecular variance (AMOVA) in ARLEQUIN 3.5 were performed to

165   calculate the genetic variation between *V. rotundifolia* and *V. trifolia* groups, among populations

166   within groups, and within populations, using a significance test based on 1000 permutations

167   (Excoffier & Lischer, 2010). The program PermutCpSSR_1.2.1 (Pons & Petit, 1996) was used to

168   calculate the within-population diversity (h_S), total diversity (h_T), geographical total haplotype

169   diversity (V_T), geographical average haplotype diversity (V_S), the level of population

170   differentiation at the species level (G_{ST}), and an estimate of population subdivisions for

171   phylogenetically ordered alleles (N_{ST}). G_{ST} and N_{ST} are often used to assess the geographical

172   structure affecting population differentiation.

173   **1.6 Demographic history**

174   Tajima's D test and Fu's Fs test in the ARLEQUIN 3.5 software, with 1000 permutations, were

175   applied to detect historical demographic range expansions of *V. rotundifolia* and *V. trifolia*

176   (Excoffier & Lischer, 2010). The significance of the D value is associated with bottlenecks,

177   selective effects, population expansion, or heterogeneity of mutation rates (Tanaka et al., 2011).

178   In addition, 'mismatch distributions' analysis was performed using the DnaSP v5.10 program to

179   detect any recent demographic expansions of *V. rotundifolia* or *V. trifolia* (Librado & Rozas,

180   2009).

181

182   **Results**

183   **2.1 Genetic diversity based on cpDNA and ITS sequences**

184   Based on the 157 samples of *V. rotundifolia* and *V. trifolia* individuals, the nucleotide sequence

185   lengths were 848 bp and 271 bp for the *trnG-trnS* and *trnH-psbA* regions, respectively. The

186   combined cpDNA sequence length after multiple alignments was 1119 bp. In total, six

187   polymorphic sites and eight haplotypes were detected, and the aligned sequence variations are

188   summarized in Table 2. The number of haplotypes, haplotype diversity (h_d), and the nucleotide

189   diversity (π_d) within each population are presented in Table 1. Finally, three haplotypes were

190   obtained from 17 populations, plus four sites with a single sample of *V. rotundifolia*, and five

191   haplotypes were obtained from four sampling sites of *V. trifolia* (Fig. 2). The value of haplotype

192   diversity ($h_{d,\ cp}$) ranged from 0 to 0.639, and the nucleotide diversity ($\pi_{d,\ cp}$) from 0 to 0.00065 in

193   the 21 sampling sites for *V. rotundifolia*. At the species level, $h_{d,\ cp}$ and $\pi_{d,\ cp}$ were 0.360 and

194   0.00010, respectively. When excluding six poorly sampled populations, $h_{d,\ cp}$ and $\pi_{d,\ cp}$ slightly

195   changed to 0.363 and 0.00010, respectively. Within the four *V. trifolia* sampling sites, $h_{d,\ cp}$

196   ranged from 0 to 0.639 and $\pi_{d,\ cp}$ from 0 to 0.00065, and at the species level, $h_{d,\ cp}$ was 0.812 and

197   $\pi_{d,\ cp}$ was 0.00039. These results indicated that both *V. rotundifolia* and *V. trifolia* have relatively

198   low genetic diversity. The highest haplotype diversity occurred in population WN for *V.*

199   *rotundifolia* and 3_YN for *V. trifolia*, indicating that Wanning of Hainan Province and

200   Xishuangbanna of Yunnan Province may be the centers of biological diversity of *V. rotundifolia*

201   and *V. trifolia*, respectively.

202   The length of the ITS sequence was 334 bp for 177 individuals, but only two haplotypes and

203   one polymorphic site were found in *V. rotundifolia* and *V. trifolia* (Fig. 3, Table 3). In 23

204   sampling sites for *V. rotundifolia*, the total haplotype diversity ($h_{d,\ nr}$) was 0.440, the range of

205   haplotype diversities within population was from 0–0.667; the nucleotide diversity ($\pi_{d,\ nr}$) was

206   0.00000; and the range of nucleotide diversities within population was 0–0.00200. When we

207   excluded six poorly sampled populations, the ITS total $h_{d,\ nr}$ and $\pi_{d,\ nr}$ slightly changed to 0.443

208   and 0.00000, respectively. Within the four *V. trifolia* sampling sites, the total values of $h_{d,\ nr}$ and

209   $\pi_{d, nr}$ were 0.516 and 0.00000, respectively. These results indicated that based on the ITS nuclear

210   sequence, *V. rotundifolia* and *V. trifolia* both have a low genetic diversity.

211   Furthermore, PermutCpSSR_1.2.1 analysis based on cpDNA sequences deduced that the h_T,

212   V_T, h_S, and V_S values for *V. rotundifolia* were 0.281, 0.285, 0.225, and 0.285, respectively, and

213   those for the *V. trifolia* populations were 0.869, 0.921, 0.690, and 0.553, respectively, indicating

214   that *V. rotundifolia* populations also have low genetic diversity, and that the genetic diversity of

215   the *V. trifolia* populations was much higher than that of *V. rotundifolia* populations. The

216   parameters of h_T, V_T, h_S, and V_S based on ITS sequence could not be calculated because only two

217   haplotypes were found in *V. rotundifolia* and *V. trifolia*.

218   AMOVA analysis based on the cpDNA sequences revealed that a total of 79.19% of the

219   variation was between the *V. rotundifolia* and *V. trifolia* populations, and 80.36% of the total

220   cpDNA variation existed within *V. rotundifolia* populations. In addition, 78.25% of the

221   variations based on cpDNA were found between *V. rotundifolia* and *V. trifolia* populations (P <

222   0.01; Table 3), indicating that there was a significant genetic differentiation between these two

223   species. Based on ITS sequences from combined *V. rotundifolia* and *V. trifolia* populations,

224   AMOVA analysis showed that 6.29% of the variation existed between the species, 6.70% of the

225   variation was caused by differences between the populations within the species, and 87.02% of

226   the variation existed within the populations (Table 4).

227   **2.2 Population structure and phylogeographical analysis**

228   The phylogeographical analysis based on the cpDNA sequences found that the N_{ST} value was

229   significantly greater than the corresponding G_{ST} value (0.751 *vs.* 0.436, P < 0.05), showing a

230   noticeable phylogeographical structure across 25 *V. rotundifolia* and *V. trifolia* populations, and

231   the eight haplotypes displayed a clear geographical pattern. However, the N_{ST} value was higher

232   than its corresponding G_{ST} value (0.462 *vs.* 0.201, P > 0.05) in 21 *V. rotundifolia* populations,

233   indicating that there was an unclear cpDNA phylogeographical structure. As shown in Fig 4,

234   statistical parsimony analysis found a single haplotype network based on cpDNA samples of *V.

235   rotundifolia* and *V. trifolia* populations. Furthermore, the TCS 1.21 network analysis of cpDNA

236   haplotypes revealed the relationship of the interior (ancestral) and the tip (derived) haplotypes.

237   Haplotype H4 was inferred as the ancestral haplotype, as determined by outgroup weight based

238   on haplotype positions in the network. H1, H7, and, H8 were shared only by *V. rotundifolia*. H2–

239   H6 were shared only by *V. trifolia*. H1 was the most frequent and widely distributed haplotype in

240   17 of the 21 populations of *V. rotundifolia*.

241   The phylogenetic relationships between the eight cpDNA haplotypes were assessed under

242   Bayesian inferences drawn using *V. negundo* as an outgroup. The result showed that the

243   phylogenetic tree of the eight cpDNA haplotypes had a comb-like structure (Fig. 5). This result

244   may have been caused by insufficient information sites, or by the rapid expansion of *V.*

245   *rotundifolia* and *V. trifolia*. We could not perform the haplotype network and phylogenetic

246   analyses based on the ITS sequences because there were only two haplotypes.

247   **2.3 Divergence times and demographic history**

248   BEAST, a program for Bayesian analysis, was used to estimate the divergence times. The result

249   showed that the effective sample size (ESS) value ranged from 919 to 1070 for all the nodes, the

250   divergence time between *V. rotundifolia* and *V. trifolia* was 0.23 mya (0.096–0.396, 95% highest

251   posterior density (HPD)) (Fig. 6). Hence, the divergence between *V. rotundifolia* and *V. trifolia*

252   appeared during the late Pleistocene era.

253   Based on the cpDNA data, mismatch distribution analysis was performed to test whether the

254   current expansion occurred in *V. rotundifolia* populations and *V. trifolia* populations separately.

255   The results found that *V. rotundifolia* and *V. trifolia* populations each had a relatively smooth

256   unimodal distribution, which was consistent with distribution under an exponential growth rate

257   (Fig. 7), indicating that the two species had a rapid demographic expansion. In addition, among

258   the 21 *V. rotundifolia* populations, Tajima's *D* and Fu's test gave apparently positive results, as

259   evidenced by the value of *D* and *F*s (D = −0.3997, *F*s = 0.2787; both *P* > 0.05), indicating that *V.*

260   *rotundifolia* has not passed through a recent demographic expansion. In the four *V. trifolia*

261   population, Tajima's *D* (*D* = 1.8690, *P* > 0.05) and Fu's *F*s (*F*s = 0.95759, *P* > 0.05) tests also

262   showed no demographic expansion (Table 5). This result is inconsistent with that derived from

263   the mismatch distribution plots. This discrepancy might be caused the insufficient number of

264   variable sites provided by only two pairs of cpDNA regions (*trnG-trnS* and *psbA-trnH*).

265

266   **3. Discussion**

267   **3.1 A large-scale distribution and low genetic diversity of *V. rotundifolia***

268   *V. rotundifolia* is native to seashores from eastern India to Hawaii and from Japan to Australia,

269   and can also be found in Brazil, Mauritius, Bangladesh, and Sri Lanka (Moldenke, 1977; Munir,

270   1987). In China, *V. rotundifolia* grows on the beach, seaside, and lakeside, mainly along the

271   eastern coastal region (Wang et al. 2013). Based on our fieldwork, we investigated 14 sampling

272   sites throughout the coastal areas and three sampling sites at lakesides in inland of China. The

273   whole distribution areas of *V. rotundifolia* in China were covered and also extended to Korea and

274   Japan, including sites with few samples. The latitude and longitude of these locations range from

275   18°44′4.68 to 39°51′26.24″ and from 9°28′37.83″to 140°0′46.99″, with the sampling distance

276   crossing almost 3000 km. However, the present study found that *V. rotundifolia* has extremely

277   low levels of genetic diversity ($h_{d,\ cp}$ = 0.360, $\pi_{d,\ cp}$ = 0.00010; $h_{d,\ nr}$ = 0.440, $\pi_{d,\ n\ r}$= 0.00000)

278   compared with most other plants (Yan, 2007; Chen et al., 2008; Chen et al., 2012; Xing et al.,

279   2017). Such low level of nucleotide diversity has also been found in the coastal plant *Rhizophora*

280   (Rhizophoraceae), with nucleotide diversities for *trnG-trnS* and *trnH-rpl2* being 0.04 ± 0.02 and

281   0.03 ± 0.02, respectively, and π_{nr} = 0.02 ± 0.01 (Lo, Duke & Sun, 2014). However, for *V.*

282   *rotundifolia*, using allozyme markers (Yeeh et al. 1996) and ISSR markers (Hu et al. 2008),

283   moderate genetic diversity was reported, which was slightly higher than that observed in the

284   present study. This discrepancy might be attributed to differences molecular markers used. In the

285   present study, even when excluding poorly sampled populations, the secondary analysis also

286   indicated that both the chloroplast and nuclear sequences have extremely low nucleotide

287   diversity in *V. rotundifolia.*

288   The large-scale distribution of *V. rotundifolia* with a low level of genetic diversity may be

289   attributed to three reasons: (1) Long-distance dispersal (LDD) by sea-drifted fruit: Long-distance

290   dispersal can result in the homogeneous distribution of DNA haplotypes at the spatial scale

291   (Miryeganeh, 2013). Our results revealed that 17 of the 21 *V. rotundifolia* sampling sites,

292   including the most distant populations covered by the geographical distribution range of

293   sampling, such as XL (Xinglong of Hainan province) and JP (Japan), shared the common

294   haplotype H1. This wide distribution of haplotypes may be caused by long-distance dispersal of

295   this plant's fruit. The *V. rotundifolia* fruit is covered with a thick hydrophobic coating that can

296   resist water penetration, rendering it able to float in the ocean, drift onto the beach, and disperse

297   over long distance via sea drift (Munir, 1987; Cousins et al., 2010). Therefore, this rapid long-

298   distance dispersal ability of *V. rotundifolia* fruit may be responsible for the low genetic diversity

299   of this plant. (2) The similar ecological environment: Life history traits and environmental

300   factors affect the genetic diversity and structure of species (Loveless & Hamrick, 1984; Nybom,

301   2004). *V. rotundifolia* usually grows on beaches, sand dunes, and rocky shorelines at low

302   elevations and can tolerate the highly salt, drought, full sun, and sandy or well-drained soils

303   (http://www2.hawaii.edu/~eherring/hawnprop/vit-rotu.htm). These similar ecological niches

304   could limit genetic differences and lead to a low genetic diversity of *V. rotundifolia*. (3) The

305   asexual reproductive mode: This species proliferates by sexual reproduction, as well as clonal

306   propagation through the elongation of rhizomes and root systems

307   (http://hgic.clemson.edu/factsheets /HGIC2315.htm; Gresham & Neal, 2004). Clonal

308   reproduction usually leads to the loss of genetic diversity within populations (Cook, 1983).

309   **3.2 Population structure and demographic history**

310   Based on the cpDNA data, the results showed that all populations of *V. rotundifolia* and *V.*

311   *trifolia* had a significant phylogeographical structure (G_{ST} = 0.436, N_{ST} = 0.751; N_{ST} > G_{ST}, P <

312   0.05). The current distribution of haplotypes may be caused by climate oscillation during the

313   Quaternary period, which resulted in further large-scale migration of most plants and animals,

314   and the subsequent accumulation of genetic variations and specific phylogeographical structures

315   (Hewitt, 2000, 2004). *V. rotundifolia* populations showed an insignificant phylogeographical

316   structure. (G_{ST} = 0.201, N_{ST} = 0.462; N_{ST} > G_{ST}, P > 0.05). Furthermore, AMOVA analysis

317 showed low genetic differentiation among *V. rotundifolia* populations, indicating that rapid

318 spread by sea currents may have played a key role. The unclearly phylogeographical structure

319 and the low genetic differentiation in *V. rotundifolia* populations may relate to the rapid long-

320 distance dispersal by sea drifts. This is not consistent with the report by Hu et al (2008), in which

321 ISSR analysis showed a relatively high genetic differentiation (Gst = 0.587) among populations

322 of *V. rotundifolia*; the authors stated that such characteristics of *V. rotundifolia* are likely

323 attributed to its sexual/asexual reproduction and limited gene flow. These contradictory results

324 may have been caused by the different gene markers used (ISSR *vs*. cpDNA).

325   Estimated divergence times indicated that the genetic divergence between *V. rotundifolia* and

326 *V. trifolia* occurred about 0.23 mya (0.096–0.396, 95% HPD), indicating a relatively short

327 divergence time, corresponding to the late Pleistocene era, before the onset of the last glacial

328 maximum (LGM). Following this divergence, long-distance dispersal may have been a major

329 historical process for *V. rotundifolia*, because they are present throughout the Pacific, including

330 the coastal areas of the continents and many islands. Additionally, we detected a recent

331 population expansion of *V. rotundifolia* based on the apparent mismatch distribution, showing

332 that *V. rotundifolia* had undergone a long-distance dispersal expansion in the short term.

333   The ancestral haplotypes are located in a central position within the phylogeographical

334 network (Crandall & Templeton, 1993). Haplotype H4 is located in the center of the haplotype

335 network, and is the closest haplotype to H9 (outgroup), and H1 has evolved from H4. Therefore,

336 we suggest that H4 is the ancestral haplotype, which was detected in two populations (3_WC and

337 3_YN) in *V. trifolia*. Populations 3_WC and 3_YN are from the southern part of China and have

338 higher haplotype diversities in all populations. Moreover, the results of the TCS network analysis

339 also showed that the haplotypes shared by *V. rotundifolia* were deduced from those of *V. trifolia*.

340 Thus, we speculated that Yunnan and Wenchang regions might be two origin areas of *V.*

341 *rotundifolia*. During the Quaternary period, *V. rotundifolia* expanded to eastern China rapidly.

342   The analysis of cpDNA data in this study showed that the *V. rotundifolia* and *V. trifolia*

343 populations have no shared haplotypes. In addition, analysis of molecular variance (AMOVA)

344   showed a significant difference in genetic differentiation between *V. rotundifolia* and *V. trifolia*

345   ($F_{ST, cp}$ = 0.79195). These findings supported the view that *V. rotundifolia* and *V. trifolia* are two

346   separate species.

347   **3.3 Conservation strategy**

348   It is essential to study the morphological variation, genetic diversity, and population structure to

349   provide basic information on plant conservation (Feng, Wang & Gong, 2014; Fan et al., 2017).

350   Climate change, the rapid growth of the human population, and economic development all

351   contribute to the deterioration of *V. rotundifolia's* habitat, reducing the number of individuals in

352   some populations. Therefore, *V. rotundifolia* resource protection is urgently required. The

353   management priority is to conserve the population of the species that has the greatest diversity.

354   Our results showed that the WN, XL, and QD populations have the highest haplotype diversity

355   and nucleotide diversity in *V. rotundifolia*; therefore, conservation priority should be considered

356   for these populations. Hu et al. (2008) proposed that the Xinjian population of *V. rotundifolia*

357   from Jiangxi province of China had a considerably high genotypic diversity index and should be

358   prioritized for protection measures.

359   Furthermore, population size is an important factor threatening the existence of species, and for

360   many wildlife species, conservation should prioritize the smallest populations. Our field surveys

361   found that most extant *V. rotundifolia* populations in China face a serious threat of extinction

362   because of the limited number of individuals. Therefore, initial conservation measures for *V.*

363   *rotundifolia* should be considered to increase the population size and genetic diversity. Among

364   the extant populations, there are fewer than 10 individuals in the DL, LYG, and XL populations.

365   Hence, we need to increase the population size for *in situ* conservation with high priority in these

366   populations. In addition, habitat protection is also an important concern in *V. rotundifolia*

367   conservation because of habitat damage by increased human activities and over-exploitation of

368   the plant as a medical resource. Therefore, it is necessary to establish local nature reserves,

369   especially for those small-sized and seriously disturbed populations. *Ex situ* conservation

370   strategies, including germplasm collection, culture in botanical gardens (Kunming, Beijing), or

371   reintroductions at appropriate areas with similar habitats, are also effective measures to preserve

372   the genetic resources of *V. rotundifolia*. When the *ex situ* conservation is put in practice, samples

373   should be collected from as many individuals as possible, because a large portion of the genetic

374   diversity exists within, rather than among, populations. Sample collection from the WN, XL, and

375   QD populations is recommended. Our experience, together with the results from previous

376   studies, suggested that individuals in a population should be collected at > 20 m spatial intervals

377   to avoid collecting individuals with identical or similar genotypes (Hu et al., 2008).

378

379   **Conclusions**

380   In summary, the present studies revealed the intraspecific genetic variation pattern of *V.*

381   *rotundifolia* and *V. trifolia*, and identified genetic divergence that occurred during the late

382   Pleistocene era. Our results showed that *V. rotundifolia* underwent a rapid long-distance

383   dispersal expansion with low genetic differentiation among populations with an unclear

384   phylogeographical structure. In addition, it has a low level of genetic diversity with a large-scale

385   distribution. In conservation terms, the populations containing high genetic diversity (WN, XL,

386   and QD) should be protected. A balanced and dynamic conservation strategy for *V. rotundifolia*

387   has been proposed.

388

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392

393

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Figure 1

The sample locations of *V. rotundifolia* and *V. trifolia* were used in this study.

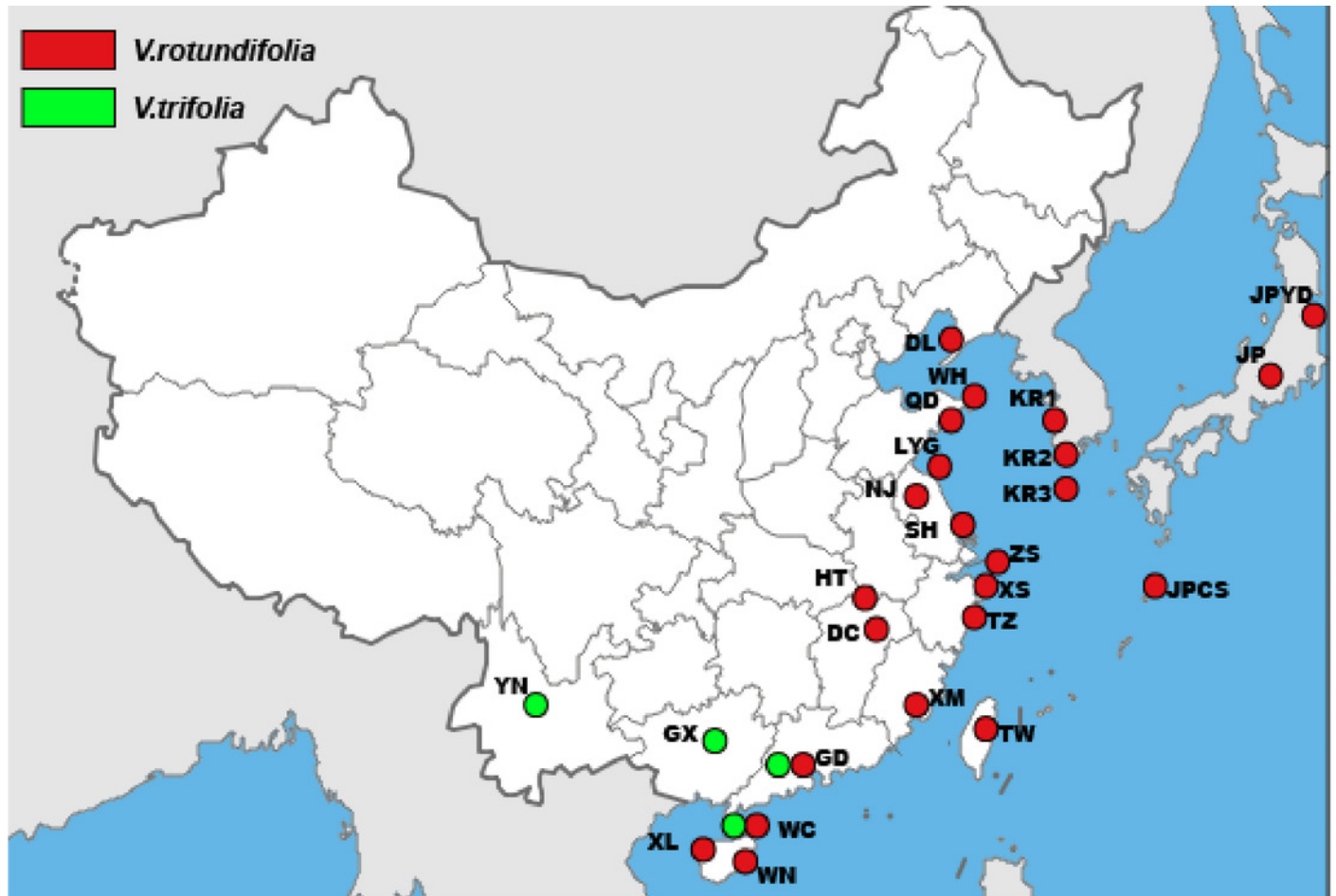



Figure 2

Geographic distribution of the cpDNA haplotypes in *V. rotundifolia* and *V. trifolia*.

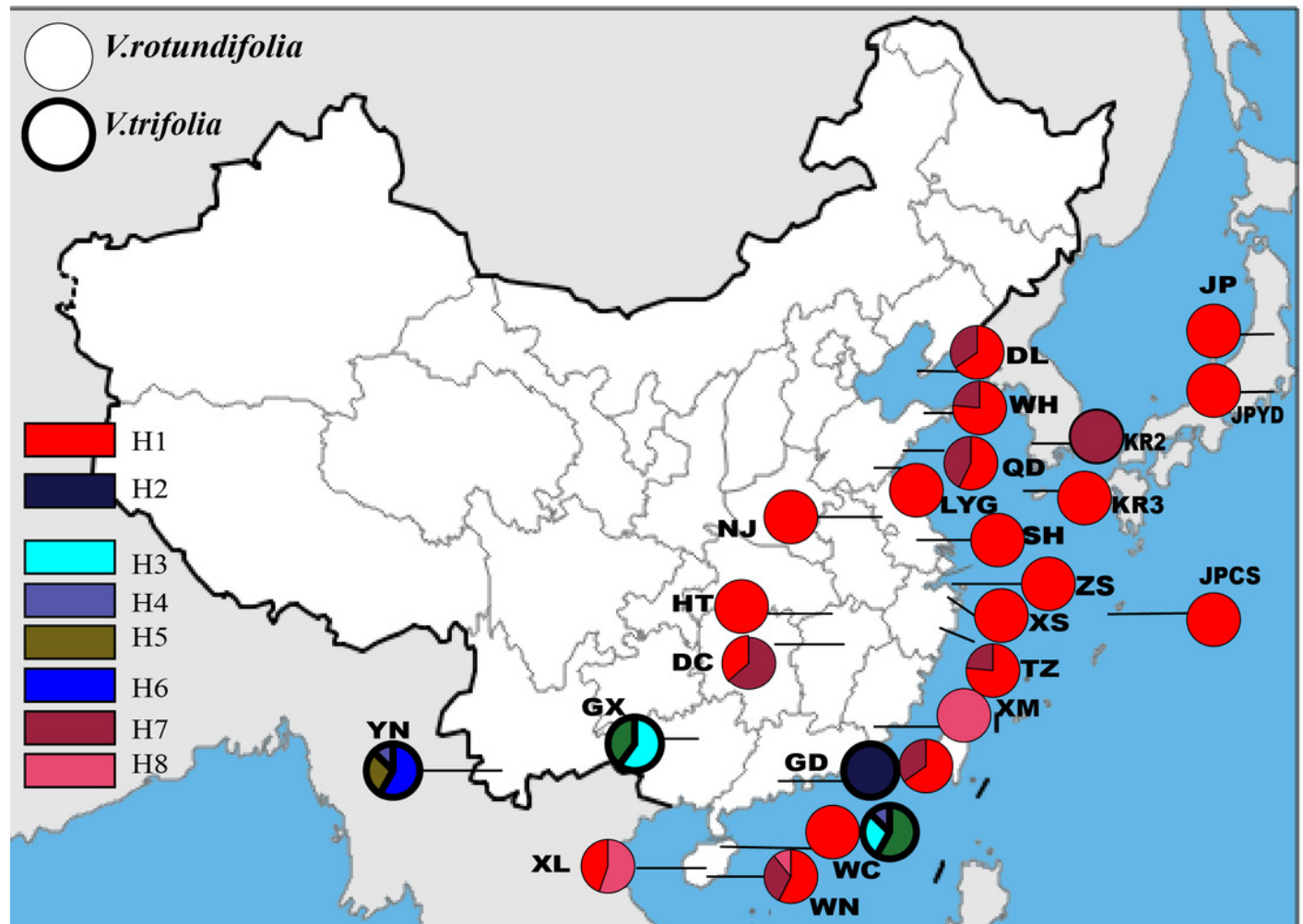



Figure 3

Geographic distribution of the nrDNA haplotypes in *V. rotundifolia* and *V. trifolia.*

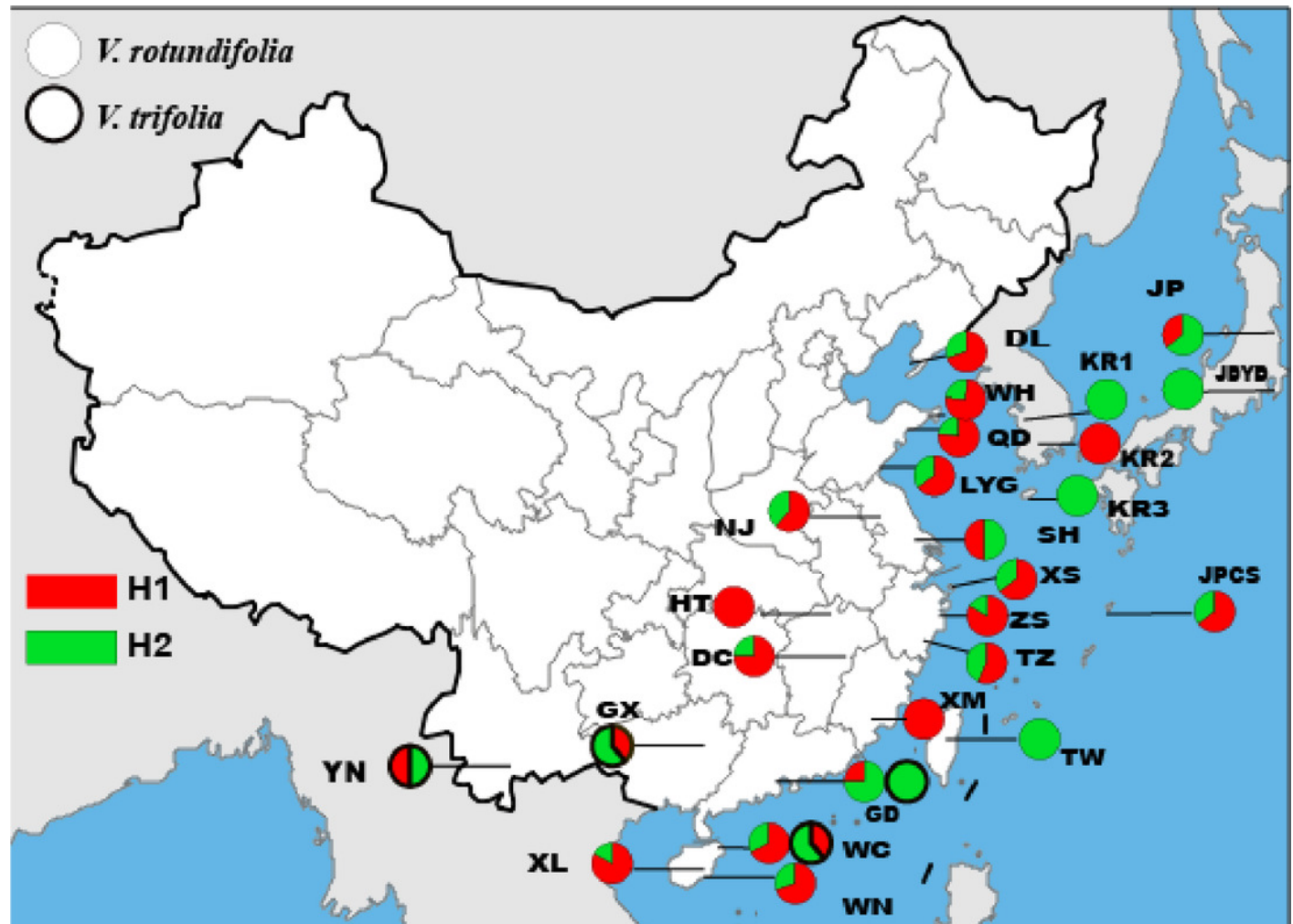



Figure 4

Minimum spanning network of eight cpDNA haplotypes in *V. rotundifolia* and *V. trifolia*.

The network was rooted at the *V. negundo*. Circle sizes are proportional to the number of samples per haplotypes. Hollow dots indicate the number of mutational steps.

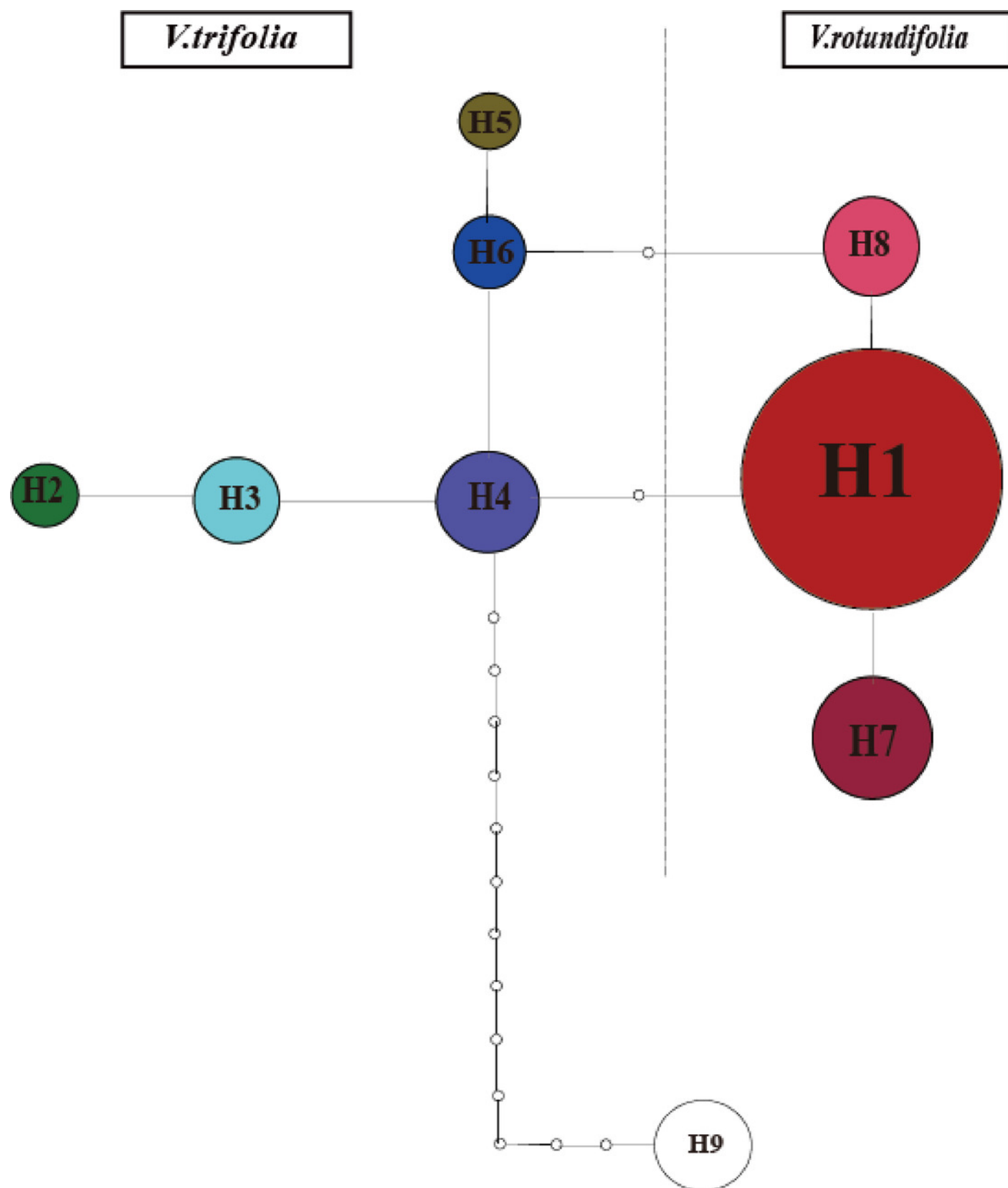



Figure 5

Bayesian inference (BI) phylograms of 9 haplotypes based on chloroplast DNA (cpDNA) sequences.

The *V. negundo* (haplotype 9) was used as outgroup. Posterior probabilities (PP>0.50) are labelled above the nodes.

Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.

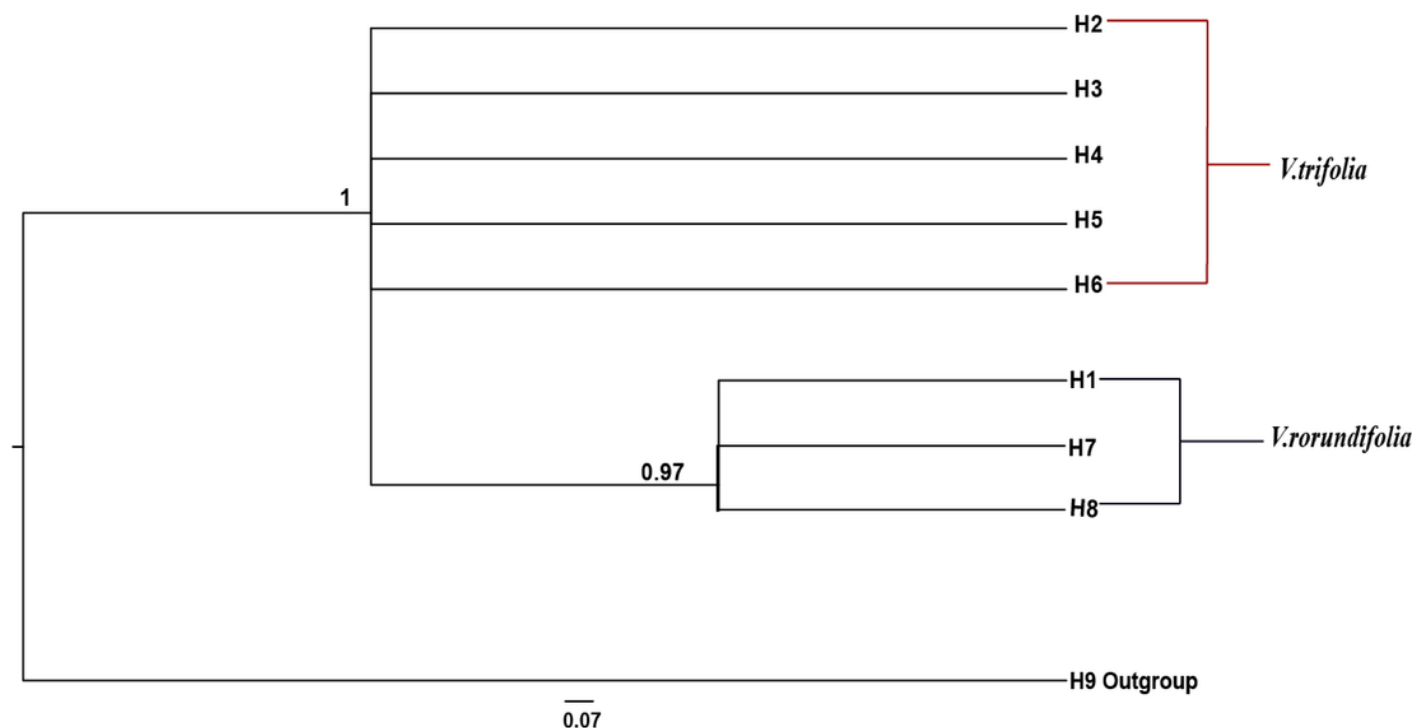



Figure 6

BEAST-derived chronograms of *V. rotundifolia* and *V. trifolia*. based on cpDNA sequences with *V. negundo* used as outgroup.

Posterior probabilities (PP>0.50) are labelled below the nodes. The bars on each node indicate 95% highest posterior densities (HPDs) of time estimates (years ago).

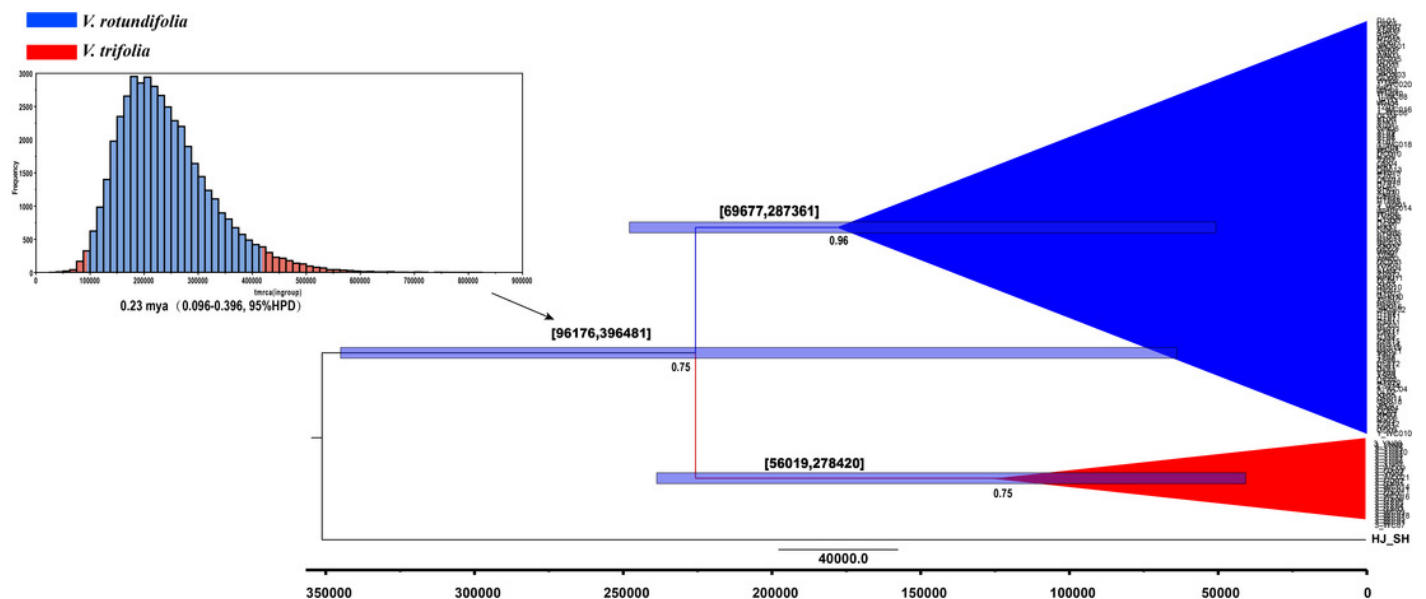
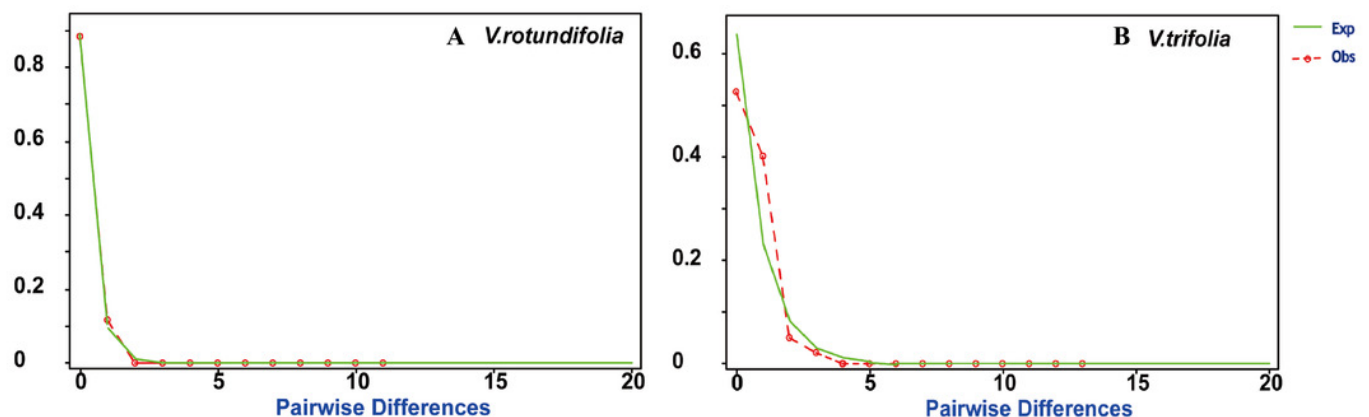



Figure 7

Mismatch distribution plots for *V. rotundifolia* and *V. trifolia* haplotypes.

Observed (Obs) frequency of pairwise differences versus expected (Exp) under exponential population growth model for major cpDNA sequences, (A) *V. rotundifolia.* (B) *V. trifolia.*

Localities and numbers of samples surveyed across the natural range of *V. rotundifolia* and *V. trifolia* and the numbers of haplotypes of cpDNA and nrDNA observed in the populations surveyed.

n_s, the number of samples analysed; n_h, the number of haplotypes obsevered; h, haplotype diversity; π, nucleotide diversity.

1 **Localities and numbers of samples surveyed across the natural range of *V. rotundifolia* and *V. trifolia* and the numbers of**

2 **haplotypes of cpDNA and nrDNA observed in the populations surveyed.**

Population		Latitude (N)	Longitude (E)	Altitude (m)	$n_{s,\ cp}/$ $n_{s,\ nr}$	$n_{h,\ cp}/$ $n_{h,\ nr}$	$h_{cp}/$ h_{nr}	$\pi_{cp}/$ π_{nr}
Vitex. rotundifolia								
DL	Dalian, Liaoning, China	39°10′11.62″	22°04′40.26″	28	10/11	2/2	0.467/0.356	0.00042/ 0.00106
WH	Weihai, Shandong, China	37°31′60.75″	22°02′.25.55″	6	9/8	2/2	0.389/0.429	0.00035/ 0.00128
QD	Qingdao, Shandong, China	36°24′78.35″	20°55′48.07″	5	9/10	2/2	0.500/0.467	0.00045/ 0.00140
LYG	Lianyungang, Jiangsu, China	34°45′74.87″	9°28′37.83″	1	4/7	1/2	0.000/0.571	0.00000/ 0.00171
NJ	Nanjing, Jiangsu, China	32°03′20.78″	118°49′19.85″	25	5/5	1/2	0.000/0.600	0.00000/ 0.00180
DC	Duchang, Jiangxi, China	29°26′06.09″	116°06′42.05″	76	11/12	2/2	0.436/0.409	0.00039/ 0.00123
HT	Houtian, Jiangxi, China	28°26′20.26″	115°48′00.26″	39	12/12	1/1	0.000/0.000	0.00000/ 0.00000
ZS	Zhoushan, Zhejiang, China	29°52′56.42″	22°24′.02.03″	0	9/10	1/2	0.000/0.200	0.00000/ 0.00060
XS	Xiangshan, Zhejiang, China	29°25′86.14″	21°57′76.63″	3	2/3	1/2	0.000/0.667	0.00000/ 0.00200
TZ	Taizhou, Zhejiang, China	28°41′46.83″	21°46′54.73″	10	9/12	2/2	0.389/0.530	0.00035/

		”						0.00159
SH	Shanghai, China	31°13′55.03″	121°28′10.00″	13.31	4/4	1/2	0.000/0.667	0.00000/0.00200
XM	Xiamen, Fujian, China	24°28′56.93″	118°05′4.03″	2	1/1	1/1	0.000/0.000	0.00000/0.00000
GD	Gangzhou, Guangdong, China	23°11′21.66″	113°21′31.41″	22	11/10	2/2	0.467/0.467	0.00042/0.00140
1_WC	Wenchang, Hainnan, China	19°32′42.56″	110°47′51.87″	10	8/10	1/2	0.000/0.467	0.00000/0.00140
WN	Wanning, Hainnan, China	18°47′46.89″	110°23′27.86″	13	9/11	3/2	0.639/0.436	0.00065/0.00131
XL	Xinglong, Hainnan, China	18°44′4.68″	110°11′52.91″	26	10/10	2/2	0.533/0.200	0.00048/0.00060
TW	Pingdong, Taiwan, China	21°59′23.29″	120°44′21.12	5	0/2	0/1	0/0.000	0/0.00000
JP	Akita, Japan	39°51′26.24″	140°0′46.99″	6.5	1/3	1/1	0.000/0.667	0.00000/0.00200
JPYD	Lzu, shizuoka, Japan	34°55'55.36″	139°07'32.80″	162	1/1	1/1	0.000/0.000	0.00000/0.00000
KR1	BoryeongJangan-Beach, Korea	36°12′49.9″	126°32′09.3″	16	0/1	0/1	0/0.000	0/0.00000
KR2	Jeollanam-do Hampyeong HampyeongPort, Korea	35°09′30.4″	126°22′35.9″	12	1/1	1/1	0.000/0.000	0.00000/0.00000
KR3	Jeju-si, Iho-beach, Korea	32°29′54.2″	126°27′14.7″	3.8	2/2	1/1	0.000/0.000	0.00000/

				n_s	n_h		h	π
								0.00000
JPCS	**Nago, Okinawa, Japan**	26°35′29.57″	127°58′38.34″	5.8	3/3	1/2	0.000/0.000	0.00000/ 0.00200
Species level							0.360/ 0.440	0.00010/ 0.00000
Vitex. trifolia								
3_GD	**Gangzhou, GuangDong, China**	23°11′21.66″	113°21′31.41″	22	1/1	1/1	0.000/0.000	0.00000/ 0.00000
3_YN	**Xishuangbanna, YunNan, China**	22°0′38.81″	100°47′49.74″	555	9/8	3/2	0.639/0.571	0.00065/ 0.00171
3_WC	**Wenchang, Hainnan, China**	19°32′42.56″	110°47′51.87″	10	10/11	3/2	0.511/0.545	0.00102/ 0.00163
3_GX	**Longgang, Guangxi, China**	22°28′15.35″	106°57′25.94″	239	7/8	2/2	0.571/0.536	0.00051/ 0.00160
Species level							0.812/0.516	0.00039/ 0.00000

4 n_s, the number of samples analysed; n_h, the number of haplotypes obsevered; h, haplotype diversity; π, nucleotide diversity.

Variable nucleotide sites and length polymorphisms of cpDNA (*trnH-psbA* and *trnG-trnS*) sequences in the *V. rotundifolia* and *V. trifolia*, identifying 9 haplotypes (Hap1-Hap9).

All sequences are compared to the reference Hap1. "-" in sequences denote absence.

1 **Variable nucleotide sites and length polymorphisms of cpDNA (*trnH-psbA* and *trnG-trnS*) sequences in the *V. rotundifolia* and**

2 ***V. trifolia*, identifying 9 haplotypes (Hap1-Hap9).**

Population	Haplotype	cpDNA																			
		407	672	728	782	789	790	797	802	828	848	867	882	883	891	892	893	996	1008	1041	1074
1_WC, DC, DL, GD, HT, JPCS, ZS, JPYD, KR3, LYG, NJ, QD, SH, TZ, WH, WN, XL, XS	Hap1	A	-	G	-	T	C	T	-	-	C	T	-	-	T	T	T	G	G	G.	C
3_GD, 3_GX, 3_WC	Hap2	.	-	.	.	G	A	.	.	.	.	.	T	A	.	.	.	.	.	.	-
3_GX, 3_WC	Hap3	.	-	.	.	G	A	.	.	.	.	.	T	A	.	.	.	.	.	.	.
3_WC, 3_YN	Hap4	.	-	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3_YN	Hap5	C	-	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	-
3_YN	Hap6	C	-	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DC, DL, GD, KR2, QD, TZ, WH, WN	Hap7	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
WN, XL, XM	Hap8	C	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Outgroup	Hap9	.	A	A	T	G	A	A	T	G	A	A	.	.	A	C	A	T	A	C	.

4 All sequences are compared to the reference Hap1. "-" in sequences denote absence.

Variable nucleotide sites and length polymorphisms of nrDNA sequences in the *V. rotundifolia* and *V. trifolia*, identifying 3 haplotypes (Hap1-Hap3).

All sequences are compared to the reference Hap1. "-" in sequences denote absence.

1 Variable nucleotide sites and length polymorphisms of nrDNA sequences in the *V. rotundifolia* and *V. trifolia*, identifying 3

2 haplotypes (Hap1-Hap3).

Population	Haplotype	cpDNA					
		70	72	217	264	274	276
1_WC, 3_YN, 3_GX, 3_WC, DC, DL, GD, HT, JP, JPCS, KR2, LYG, NJ, QD, SH, TZ, ZS, WH, WN, XL, XM, XS, ZS	Hap1	G	C	C	-	G	C
1_WC, 3_YN, 3_GX, 3_WC, 3_GD, DC, DL, GD, HT, JP, JPCS, KR2, LYG, NJ, QD, SH, TZ, ZS, WH, WN, XL, XM, XS, ZS, KR1, KR3	Hap2	.	.	.	C	.	.
Outgroup	Hap3	A	T	T	C	A	T

4 All sequences are compared to the reference Hap1. "-" in sequences denote absence.

Analyses of molecular variance (AMOVA) based on the cpDNA and nrDNA sequences.

Significance was tested by 1000 random permutations. $***$, $P < 0.0001$.

1 **Analyses of molecular variance (AMOVA) based on the cpDNA and nrDNA sequences.**

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices
cpDNA (all populations)					
Among populations	1	53.821	1.19665***	79.19	F_{ST}=0.79195
Within populations	155	48.727	0.31437***	20.81	
cpDNA(species)					
Among species	1	53.821	1.17509***	78.25	F_{CT}= 0.78248
Among populations within species	23	22.242	0.12601***	8.39	F_{ST}=0.86639
Within populations	132	26.485	0.20064***	13.36	F_{SC}=0.38576
cpDNA (*V. rotundifolia*)					
Among populations	20	7.649	0.03763***	19.64	F_{ST}=0.19640
Within populations	109	16.782	0.15396***	80.36	
cpDNA (*V. trifolia*)					
Among populations	3	14.593	0.72257***	63.14	F_{ST}=0.63137
Within populations	23	9.703	0.42188***	36.86	
ITS (all populations)					
Among populations	1	1.075	0.01802	7.39	F_{ST}=0.07392
Within populations	175	39.501	0.22572	92.61	
ITS (species)					
Among species	1	1.075	0.01524	6.29	F_{CT}=0.06286
Among populations within species	25	7.854	0.01624	6.70	F_{ST}=0.12983

Within populations	150	31.647	0.21098	87.02	F_{SC}=0.07146
ITS (*V. rotundifolia*)					
Among populations	22	7.492	0.02231	10.09	F_{ST}=0.10091
Within populations	126	25.045	0.19877	89.91	
ITS (*V. trifolia*)					
Among populations	3	0.362	-0.02429	-9.69	F_{ST}=-0.09685
Within populations	24	6.602	0.27509	109.69	

2 Significance was tested by 1000 random permutations. ∗∗∗, $P <$0.0001

Parameters of mismatch distribution Analysis of *V. rotundifolia* and *V. trifolia* based on cpDNA sequences.

1   **Parameters of mismatch distribution Analysis of *V. rotundifolia* and *V. trifolia* based on**

2   **cpDNA sequences**

	Tajima's D	P	Fu's Fs	P	SSD	P	H_{RAG}	P
V. trifolia	1.8690	0.8820	0.9575	0.7200	0.0051	0.5000	0.0350	0.7400
V. rotundifolia	-0.3997	0.2830	0.2787	0.4830	0.0049	0.0000	0.1936	0.4800