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A comparison of DNA sequences, SSR and AFLP for systematic study of *Dipelta* (Caprifoliaceae)

Zhan-Lin Liu[1,*], Peng Tian[1], Jian-Fang Li[1], Chunce Guo[1] & Edward Biffin[2]

[1)] *Key Laboratory of Resource Biology and Biotechnology in Western China (Ministry of Education); College of Life Sciences, Northwest University, Xi'an 710069, China (*corresponding author's e-mail: liuzl@nwu.edu.cn)*
[2)] *Australian Centre for Evolutionary Biology and Biodiversity, School of Earth and Environmental Science, The University of Adelaide, Adelaide, South Australia 5005, Australia*

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Dipelta (Caprifoliaceae) is endemic to China, and comprises 3–4 species. Here we used DNA sequences, and SSR and AFLP data to investigate relationships among these taxa. Cluster analysis of all the data showed that *D. elegans*, an endangered species, was early separated from the others. Both SSR and AFLP data indicated that individuals of *D. wenxianensis* and *D. floribunda* were mixed together, suggesting that they were conspecific. STRUCTURE analysis revealed the introgression between *D. floribunda* and *D. yunnanensis*, which might produce new "taxa" like *D. wenxianensis*.

Introduction

The Qinghai–Tibetan Plateau (QTP), the highest and largest plateau in the world, is regarded as a biodiversity hotspot due to its high level of plant diversity and endemism. Of the 12 000 species in this area, approximately 20% are endemic (Wang *et al*. 1993). This endemism is mainly attributed to the heterogeneous habitats of QTP and the complicated evolutionary history affected by geological uplift and the Quaternary climatic oscillation (Xu *et al*. 2010). Hybridization and genetic introgression are common issues for species delimitation in this area and adjacent regions (Hewitt 2004, Wang *et al*. 2011, Xu *et al*. 2010). In this study, we use *Dipelta* as an example to explore the taxa relationships and potential introgression in Gansu, the northeast part of QTP.

Dipelta (Caprifoliaceae) (Donoghue *et al*. 2001), a genus endemic to China, appears to have radiated in the late Eocene/early Oligocene (Manchester & Donoghue 1995) and was supported as a monophyletic group in phylogenetic studies (Donoghue *et al*. 2001, Landrein *et al*. 2012). It comprises three (Yang & Landrein 2011) or four (Wang *et al*. 1994) species, most of which have been widely cultivated as decorative garden plants. *Dipelta floribunda* and *D. yunnanensis*, two wide ranging and sympatric species, are mainly distributed in the north and the south of the QTP and adjacent regions, respectively. They extend their range along the eastern edge of the QTP to central China (Fig. 1). Two rare and endangered taxa, *D. elegans* and *D. wenxianensis*, occur in the overlapping region (Gansu, northeast of the QTP) and only two and one wild population are found, respectively (Fig. 1). Southern Gansu is one of the refugia for Chinese plants that has been identified by several recent studies (Xu *et al*. 2010, Wang *et al*. 2011).

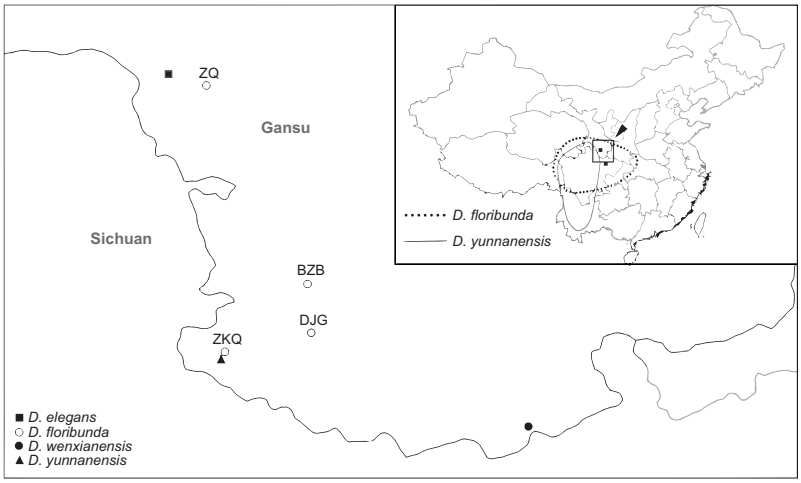

Fig. 1. Study area (indicated by an arrow), sampling locations and geographic ranges of two widely distributed species (*Dipelta yunnanensis* and *D. floribunda*). Only two main populations (filled squares) of *D. elegans* are recorded. ZQ = Zhouqu, BZB = Wudu, ZKQ = Wenxian, DJG = Longnan.

The sympatric distribution of all *Dipelta* taxa in Gansu could also be explained by the refugium hypothesis. Although all four species are usually morphologically distinguishable, their genetic diversity is unknown. In addition, *D. wenxianensis* is morphologically intermediate between *D. floribunda* and *D. yunnanensis* (Yang & Landrein 2011). Here we used DNA sequences, nuclear microsatellites (SSR) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*. 1995) markers to investigate the interspecific relationships. Our questions were: (1) Are all four taxa supported as distinct entities? (2) If not, is there any introgression among them?

Material and methods

Plant samples

Leaves were collected from the Gansu province in the northeast of the QTP, where all four *Dipelta* species are sympatrically distributed. Only one population of *D. elegans*, *D. wenxianensis* and *D. yunnanensis* were sampled because of their rarity in Gansu. A total of nine populations were sampled for the systematic study, including two outgroups: *Abelia engleriana* and *Kolkwitzia amabilis* (Table 1 and Fig. 1).

DNA sequencing

Total genomic DNA was isolated from silica-dried leaf tissues using the CTAB method (Doyle & Doyle 1990). Seven chloroplast regions (*rpl*20–*rps*18, *trn*V, *rrn*5–*trn*N, *rbc*L, *po*C2–*rps*2, *psa*B–*prs*14, *trn*L–*trn*F) and one nuclear fragment (Internal transcribed spacer, ITS) were selected for amplification (Table 2). PCR reactions were carried out in a volume of 20 μl containing 50 ng DNA template, 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 1 U Taq DNA polymerase and 1 μM each primer. The reaction conditions were: 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min, ending with a 5-min extension step at 72 °C. PCR products were purified and directly sequenced using the ABI 3730 DNA analyzer. All sequences (GenBank accession No: KC464763-KC464771) were edited and aligned with the BioEdit software (Hall 1999).

Nuclear microsatellites

We developed nuclear microsatellite from *D. floribunda* using the FIASCO (Fast Isolation by AFLP of Sequences Containing Repeats) method (Yang *et al*. 2010). DNA was digested with the *Mse*I restriction enzyme and ligated with *Mse*I AFLP adaptors (5´-TACTCAGGAATCAT-3´, 5´-GACGATGAGTCCTGAG-3´) using T4 DNA ligase. The digestion–ligation mixture was diluted (1:10) and amplified with adaptor specific primers (5´-GATGAGTCCTGAGTAAN-3´) using an initial denaturation of 5 min at 94 °C, followed by 19 cycles of 30 s at 94 °C,

30 s at 53 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. To enrich for SSR, the purified PCR products were hybridized with a 5´-biotinylated probe $(AC)_{15}$ and $(GA)_{15}$ respectively in a 50 μl hybridization solution (6 × SSC with 0.1% SDS) for 10 min at 95 °C, and 30 min at 58 °C. Streptavidin-coated magnetic beads (Promega U.S.) were used to separate and capture DNA fragments hybridized to the probe at 25 °C for 30 min. Target fragments were washed down after 5 min at 95 °C, and amplified with adaptor-specific primers as described above. After purification, the PCR products were ligated into PMD18-T vector, and then transformed into *Escherichia coli* strain TOP10. PCR was carried out using M13 universal primers and $(AC)_{15}$/$(GA)_{15}$ primers to pick out the clones which contain a $(AC)_n$ or $(GA)_n$ motif in a central position of the insert. A total of 82 clones containing simple sequence repeats (SSRs) were detected, of which 56 clones were sequenced using the ABI 3730 Genetic Analyzer (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.). Ten of the 56 sequences were selected for primer design using the Primer Premier 3.0 software (Rozen & Skaletsky 1999).

Microsatellite PCRs for the population study were conducted in a 10 μl volume containing: 20 ng of genome DNA., 0.6 μM of each primer, 2 × PCR Master Mix 5 μl (Xian Runde Biotechnology Ltd., China) followed the cycling parameters of an initial denaturation at 94 °C for 4 min, 28 cycles at 94 °C, 30 s at 94 °C, 40 s at the annealing temperature (Table 3), 45 s at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were run out on 10% polyacrylamide gel and visualized by silver staining with PUC18 DNA/*Msp*I DNA ladder as the reference.

Genetic parameters (the expected heterozygocity H_e, fixation index F_{ST}) were calculated by GenAlex 6.5 at the population level (Peakall & Smouse 2012). The individual pairwise F_{ST} matrix was used for cluster analysis and hierarchical analysis of molecular variance (AMOVA)

Table 1. *Dipelta* samples and outgroups used in the study.

Species	Location	Lat. (°N)	Long. (°E)	Alt. (m)	Number of individuals			Voucher no.
					Sequencing	SSR	AFLP	
D. wenxianensis	Bikou, Wenxian, Gansu	32.68	105.21	1015	2	29	7	Df0701
D. floribunda	ZKQ, Wenxian, Gansu	32.88	104.40	1700	1	26	3	Df0710
	DJG, Longnan, Gansu	32.93	104.63	1600	1	22	–	Df0705
	BZB, Wudu, Gansu	33.06	104.62	1400	1	10	2	Df0603
	ZQ, Zhouqu, Gansu	33.59	104.35	1800	1	14	2	Df0801
D. yunnanensis	Tielou, Wenxian, Gansu	32.86	104.39	2200	2	19	7	Dy0801
D. elegans	Chagang, Zhouqu, Gansu	33.62	104.25	2200	2	10	7	De0801
Abelia engleriana	Zhouzhi, Shaanxi	33.85	107.82	1400	1	2	1	Ae0703
Kolkwitzia amabilis	Xi'an, Shaanxi	34.25	108.92	400	1	4	1	Ka0601

Table 2. Characteristics of eight DNA fragments of *Dipelta* species and outgroups.

Regions	Aligned length	Constant number	Variable sites	Indel sites	Primer references
*rpl*20–*rps*18	750	685	18	47	Wang *et al.* 1999
*trn*V	562	561	0	1	Wang *et al.* 1999
*rrn*5–*trn*N	586	585	0	1	Suyama *et al.* 2000
*rbc*L	882	877	5	0	Wang *et al.* 1999
*rpo*C2–*rps*2	477	474	1	2	Suyama *et al.* 2000
*psa*B–*rps*14	412	412	–	–	Suyama *et al.* 2000
*trn*L–*trn*F	916	904	11	7	Taberlet *et al.* 1991
ITS	616	571	20	25	Theis *et al.* 2008
Total	5201	5069	55	83	

Note: Amplification failed in *psa*B–*rps*14 region of outgroups. Most variations appear among genera.

Table 3. Characteristics and genetic parameters of six microsatellite loci used in the study. All parameters are calculated at the population level. T_A = annealing temperature (°C); N_A = number of alleles, H_{ue} = unbiased expected heterozygosity, H_o = observed heterozygosity, F_{ST} = fixation index.

Locus	GenBank no.	Primer sequences (5´–3´)	Repeat motif	T_A	N_A	Size (bp)	H_{ue}	H_o	F_{ST}
df01	GU244368	forward: AACCCGGACAAAGTCGCACAAT reverse: CCGCACCCACCAATCGCC	$(TGG)_5(TG)_3(CGTG)_2$	61	12	295–337	0.770	0.310	0.181
df02	GU244369	forward: GTGTGCGGTCTTGGTCC reverse: AAAGTATCAGAGCCCCAT	$A_{10}(TG)_6$	56	9	280–312	0.487	0.296	0.465
df03	GU244370	forward: TTCCTCTTCTGCCGAATACTT reverse: CATCAGACATCCCACCCC	$(CTT)_3(TC)_3(TG)_4$	56	2	90–92	0.041	0.000	0.211
df04	GU244371	forward: ACTTCTTCTCATTCACCCAACT reverse: TATAAAATGAGTTTGGAAGGCTT	$(CT)_3T_2(CT)_4$	56	6	77–97	0.370	0.471	0.544
df05	GU244372	forward: GCAGAATGTTCAGATTTCACAACCC reverse: CGGTTTGAGGGATTTGGCG	$(TTTC)_2(TCC)_2C_7(TC)_4$	56	4	218–230	0.185	0.000	0.747
df06	GU244373	forward: AATGGCGTGATGGTGCAATATGGAGG reverse: ACCTATGGCAGATCCCTGAGCAGTCG	$(TGG)_6T_5$	51	3	305–327	0.082	0.037	0.895
Mean							0.322	0.186	0.507

implemented in the Arlequin 3.5 software (Excoffier & Lischer 2010). Population structure of the *Dipelta* species was assessed with a model-based Bayesian clustering method implemented in the STRUCTURE software (Pritchard *et al*. 2000). STRUCTURE was run using the "admixture model" and with correlated allele frequencies, with a burn-in period of 10 000, followed by 1 000 000 iterations. Under the assumption that individuals belonged to an unknown number of *K* genetically distinct clusters, we carried out set priors of *K* from 2 to 7 to estimate the average posterior probability values for *K* from 10 runs each. The most likely value of *K* was determined by an *ad hoc* statistic ΔK (Evanno *et al*. 2005). The results of STRUCTURE were visualized with CLUMPP (Jakobbson & Rosenberg 2007) and DISTRUCT (Rosenberg 2004).

AFLP

AFLP was performed with an AFLP kit (EcoRI/MseI, Beijing Dingguo Changsheng Biotechnology Co. Ltd.) following the manual. Eight pairs of selective primer combinations (E-AAC/M-CAG, E-AAC/M-CAT, E-AAG/M-CAA, E-AAG/M-CAC, E-AAG/M-CAG, E-AAG/M-CAT, E-ACA/M-CAG, E-ACA/M-CTC) were chosen for selective amplification of all samples with FAM labeled *Mse*I primers. PCR products were separated by ABI 377 sequencer (Applied Biosystems, USA).

Raw AFLP data were collected by GeneScan 3.7 (Applied Biosystems, USA) and the presence/absence data were used to calculate genetic similarities for all possible pairwise comparisons of individuals. A UPGMA (Unweighted Pair Group Method using Arithmetic Averages) tree based on average similarity was constructed using FAMD ver. 1.25 (Schlüter & Harris 2006) with 1000 bootstrap replicates. Population structure was analyzed by software Arlequin and STRUCTURE (with no admixture model) as discussed above.

Results

Aligned length of eight DNA fragments varied from 412 bp for *psa*B–*rps*14 to 916 bp for

*trn*L–*trn*F with a total of 5201 bp for the concatenated alignment. There were 138 variable sites including 55 nucleotide substitution and 83 insertion-deletion (indels) sites, with the ITS and *rpl*20–*rps*18 regions showing the greatest level of variability (Table 2). With the exception of ITS, however, all sequences within *Dipelta* were identical. Relative to the other *Dipelta* taxa, the ITS region of *D. elegans* had unique traits, two substitutions and one insertion of 24 bp length, which were identical to the two outgroups.

Of the ten nuclear microsatellite loci tested, six had high cross-species transferability in *Dipelta* with the exception of locus df02 in *D. elegans*, which also failed to amplify in *Abelia engleriana*. Other loci were excluded for their low resolution (Table 3). At population level, the number of alleles per locus ranged from three (df06) to twelve (df01) with an average of six. The unbiased expected (H_{ue}) and observed heterozygosities (H_o), as well as fixation indices (F_{ST}) varied at different loci (H_{ue} = 0.041–0.770, H_o = 0–0.471, F_{ST} = 0.181–0.895). In contrast to independence of *D. elegans*, individuals of *D. wenxianensis* in the UPGMA tree did not belong to any species-specific lineage. Members of *D. wenxianensis* and *D. floribunda* were always mixed. The same issue existed between *D. yunnanensis* and population ZQ of *D. floribunda* (Fig. 2a). They could also be identified by STRUCTURE when all *Dipelta* individuals were assigned into two clusters for ΔK analysis (K = 2; Fig. 3a). We also calculated K = 3, and found differentiation among populations of *D. floribunda*: population DJG was different from ZKQ (Fig. 3b). AMOVA showed that 20.4% of variation was partitioned among taxa (Table 4).

In the AFLP data set, eight primer-pair combinations of AFLP produced in total 1451 bands, of which 1419 were polymorphic. Phylogenetic tree analyses supported *D. elegans* as an independent and basal taxon sister to the remaining *Dipelta* species. *Dipelta wenxianensis* and *D. floribunda* were not distinguished from each other. *Dipelta yunnanensis* and one individual of *D. floribunda* (population ZQ) formed a distinct lineage, suggesting a possible genetic introgression between them (Fig. 2b). This was also supported by STRUCTURE when the favorite K was equal to three (Fig. 3c). AMOVA indicated that 28.5% of total variation was partitioned among the species (Table 4).

Discussion

Dipelta wenxianensis has been proposed as a new species (Wang *et al*. 1994). It can be distinguished from *D. floribunda* by its pilose style, from *D. yunnanensis* by its peltate bracts, and from *D. elegans* by its divided calyces. In this study, multiple molecular markers were used to explore the systematic relationship among *Dipelta* taxa. All data, particularly the SSR and AFLP analyses, found that individuals of *D. wenxianensis* and *D. floribunda* could not be separated from each other, supporting the notion that *D. wenxianensis* should be included in *D. floribunda* rather than recognized as an independent species. *Dipelta wenxianensis* shows intermediate traits between *D. floribunda* and *D. yunnanensis* (Yang & Landrein 2011). STRUCTURE analyses of the AFLP and SSR data indicated that *D. wenxianensis* populations have two almost equal genetic components from *D. floribunda* and *D. yunnanensis*, respectively (Fig. 3). Therefore, both morphological and genetic data imply that *D. wenxianensis* might be a hybrid of the former species.

In fact, introgression between the two widely distributed species is possibly more common in the sympatric regions than expected (Yang & Landrein 2011). For example, in the populations ZKQ and ZQ of *D. floribunda*, we observed that the shapes of accrescent bracts of fruit diversified from peltate (*D. floribunda*-like) to reniform (*D. yunnanensis*-like), although their calyces were divided (*D. floribunda*-like). Both SSR and AFLP data indicated the genetic admixture of the two species in these populations (Fig. 3). In particular, high differentiation was detected in population ZQ. Some individuals carried genetic components identical to *D. yunnanensis* while others had those of *D. floribunda* (Figs. 2b and 3). In the area studied, the anthesis of *D. floribunda* is from March to May while *D. yunnanensis* flowers from the end of April to May. The overlapping anthesis makes hybridization possible although interspecific pollination has not been tested. In contrast, *D. elegans* might not be involved because it flowers later (in June). Its

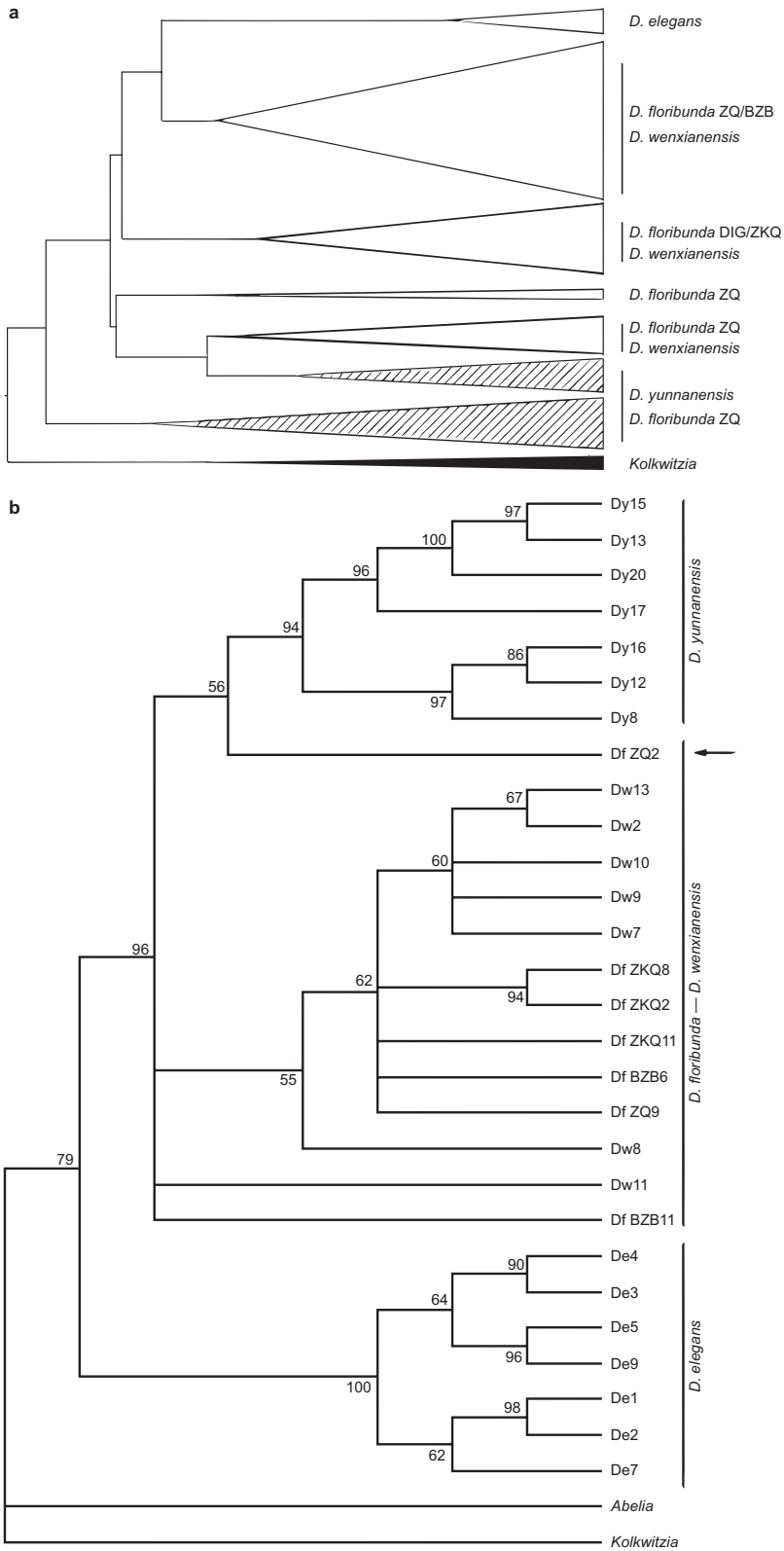

Fig. 2. (**a**) Dendrogram of individuals of SSR and (**b**) AFLP with bootstrap values on the branches. The arrow indicates a potential hybrid individual from ZQ. For locality codes *see* Table 1.

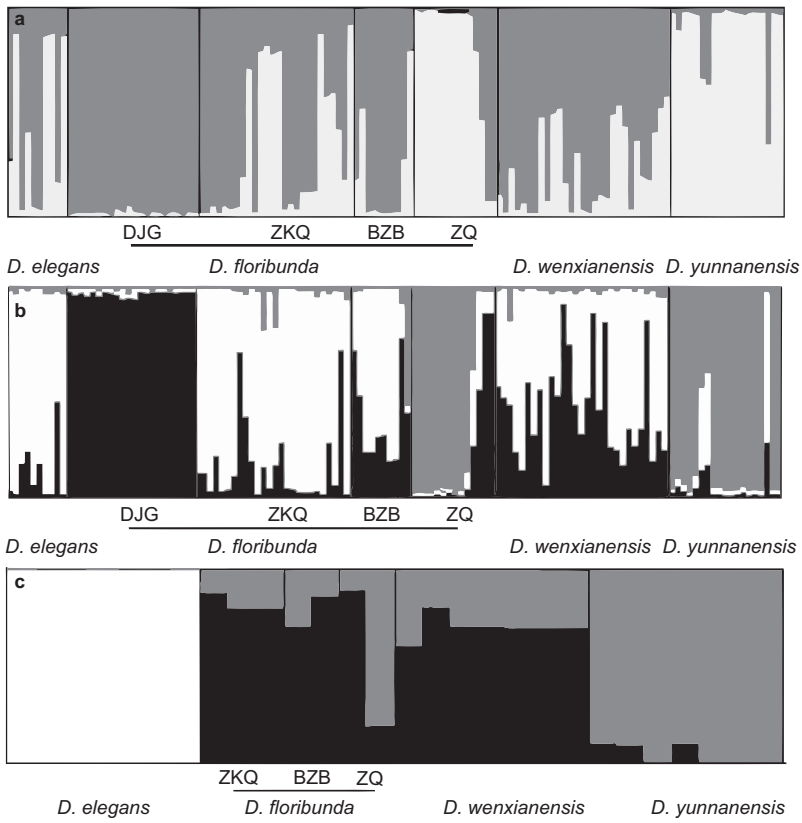

Fig. 3. Individual assignment of *Dipelta* based on the STRUCTURE analysis of SSR [(**a**) $K = 2$ and (**b**) $K = 3$] and AFLP [(**c**) $K = 3$].

mixed components in STRUCTURE (Fig. 2a) could possibly result from the failed amplification of the df02 locus.

According to biogeographic analysis, *D. floribunda* was proposed as the basal taxon in the genus (Wu *et al*. 2003). However, our data supported *D. elegans* as sister to the remaining *Dipelta* species. In the phylogenetic analyses of the AFLP data (Fig. 2b), this position is strongly supported. Furthermore, ITS sequences of *D. elegans* have a 24 bp insertion, found also in the outgroups, which is absent from other *Dipelta* species. The SSR results did not completely conflict with the hypothesis. Both *D. elegans* and *Abelia engleriana* failed to amplify the df02 locus, which might reflect a close relationship between them. Unlike the hypothesized hybrid origin of *D. wenxianensis*, *D. elegans* is a possible relic in the North QTP, which has been proposed as a glacial refugium in recent studies, e.g. for *Cupressus chengiana* (Xu *et al*. 2010) and *Metagentiana striata* (Donoghue *et al*. 2001).

Table 4. Analysis of molecular variance (AMOVA) of SSR and AFLP. *Dipelta wenxianensis* and *D. floribunda* were considered as conspecific.

Markers	Source of variation	df	Sum of squares	Variance components	Percentage of variation	*F*-statistics
SSR	Among species	2	124.638	1.422	20.4	$F_{CT} = 0.204$
	Among populations	4	140.175	1.589	22.8	$F_{SC} = 0.286$
	Within populations	123	487.725	3.965	56.8	$F_{ST} = 0.432$
AFLP	Among species	2	1368.214	55.983	28.5	$F_{CT} = 0.285$
	Among populations	5	483.095	9.967	5.1	$F_{SC} = 0.071$
	Within populations	29	2863.976	130.181	66.4	$F_{ST} = 0.336$

The molecular data used here provide different levels of resolution among *Dipelta*. Low resolution from the chloroplast data could be attributed to their relatively low evolutionary rates (Wolfe *et al*. 1987) and/or the narrow sampling. In some taxa (e.g. *Pinus torreyana*), even rapidly evolving loci (such as chloroplast microsatellites) can show a low level of variation (Wolfe *et al*. 1987). Low evolutionary rates could be compounded by relatively recent divergence. Although *Dipelta*-like fossils are recorded in England in late Eocene (Manchester & Donoghue 1995), extant *Dipelta* is found only in China today and may have radiated much more recently (ca. 6–9 Mya for Linnaeaceae = Linnaeoideae of Caprifoliaceae) (Bell & Donoghue 2005). There has not been enough time for species-specific mutations to accumulate. In contrast to the poor resolution from the DNA sequences, AFLP offered clear resolution of interspecific relationships, while the SSR data provided less resolution due to relatively fewer loci. Although these markers are criticized for phylogenetic reconstruction due to their potential homoplasy, they still are informative at species or even genus level (Ochieng *et al*. 2007, Stagel *et al*. 2008). In our study, AMOVA performed on both data sets found similar differentiation among species (0.20 *vs*. 0.28). Recently, highly variable chloroplast markers were found for evaluating phylogeny at low taxonomic level (Dong *et al*. 2012). In future work, we will use these markers, together with new interspecific transferable microsatellites, to investigate the genetic introgression among populations across the range of *Dipelta* species.

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