Tree Genetics & Genomes (2015) 11: 13
DOI 10.1007/s11295-015-0832-7

ORIGINAL PAPER

Patterns of SNP distribution provide a molecular basis for high genetic diversity and genetic differentiation in *Vitis* species

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Received: 6 December 2013 / Revised: 20 December 2014 / Accepted: 2 January 2015 / Published online: 3 February 2015

Abstract Grape (*Vitis* L.) is one of the most economically valuable fruit crops in the world. The molecular basis for high genetic diversity and genetic differentiation thus conferring broad adaptability in the *Vitis* genus remains elusive. Genetic patterns in *Vitis* were comprehensively characterized by re-sequencing of 6862 nuclear SNPs in this study. Nucleotide diversity (π=0.0073), genetic differentiation (pairwise differences, 0.24) and SNP variation in *Vitis* species were relatively high compared to most woody plant species. Molecular variance among accessions within species (F_{IS}=0.369) and among 30 species (F_{ST}=0.511) suggested strong population structure in *Vitis* (P<0.0001). *Vitis* species are characterized by low frequency (4.7 %) of most minor SNPs and high proportion (>95 %) of minor SNPs to engender high differentiation among species thus high genetic diversity in the whole genus. This confers potentially broad adaptability thus a broad base for natural selection in this genus. Species from three geographical regions were discerned into independent quadrants in the principle coordinate analysis (PCoA) plot and their clustered patterns suggested that their molecular identities represented geographical origins of *Vitis* species. Species extinction will be an irreversible loss for breeding and genetic conservation. Species with low diversity, low heterozygosity, and high agronomical value should be given priority for conservation. Survey of species-specific SNPs will facilitate germplasm conservation and grape breeding programs. This study greatly adds to our knowledge of genetic patterns and molecular bases for ecological habits of *Vitis* species and provides valuable information for germplasm conservation and utilization, and grape breeding.

Keywords Grapes · Nucleotide diversity · Analysis of molecular variance · Genetic relationships · Genetic differentiation · Nei's algorithm

Communicated by D. B. Neale

Electronic supplementary material The online version of this article (doi:10.1007/s11295-015-0832-7) contains supplementary material, which is available to authorized users.

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Introduction

Grape (*Vitis* L.) is one of the most economically important fruit crops in the world (Reisch and Pratt 1996; Alleweldt 1997; El Oualkadi et al. 2011; Myles et al. 2011; Emanuelli et al. 2013). The *Vitis* genus contains approximately 60 species widely distributed across three geographical regions: (1) South Europe and Asia Minor, (2) East Asia, and (3) North and Central America (Reisch and Pratt 1996; Alleweldt 1997; Wan et al. 2008).

Both *Vitis vinifera* ssp. *vinifera* and *Vitis vinifera* ssp. *sylvestris* (refers to '*V. vinifera*' and '*V. sylvestris*' hereafter) originated in South Europe and Asia Minor (Reisch and Pratt 1996). *V. vinifera* grapes are widely cultivated in the world due to their high berry quality, productivity and multiple uses. However, high susceptibility to diseases and abiotic stresses in this species confers enormous losses to global grape production every year (Reisch and Pratt 1996; Alleweldt 1997;

Emanuelli et al. 2013). Thus, extensive ongoing efforts have been put to improve resistance in *V. vinifera* cultivars through exploitation of wild *Vitis* germplasm (Reisch and Pratt 1996; Alleweldt 1997; Emanuelli et al. 2013). East Asia represents a major center of origin of *Vitis* species. China alone has more than 30 wild *Vitis* species (Wan et al. 2007). Chinese *Vitis* species possess good berry quality, high biotic and abiotic stress resistance, and are used for *V. vinifera* improvement (Wan et al. 2007). North and Central America form another major diverse *Vitis* gene pool with more than 30 wild *Vitis* species (Reisch and Pratt 1996).

Several species within the genus, e.g., *Vitis rotundifolia*, *Vitis labrusca*, *Vitis amurensis*, and *Vitis quinquangularis*, are cultivated for various grape purposes for human consumption, but most direct use of wild *Vitis* species has been to cross them with *V. vinifera* to develop cultivars with better biotic and abiotic endurance. Such interspecific crosses produce berries that are superior in quality in comparison to those in wild species that are directly cultivated (Reisch and Pratt 1996; Alleweldt 1997; El Oualkadi et al. 2011).

Wild species represent a unique, invaluable genetic resource for viticulture and grape breeding (Reisch and Pratt 1996; Alleweldt 1997; Martinez et al. 2006). However, the geographic distribution of wild grapevines has been dramatically reduced over the last 150 years (Grassi et al. 2006), particularly in China because of tremendous threats from rapid expansion of human population and industrialization therein (Wan et al. 2008). The extinction of wild species would be an irreversible loss for breeding programs and ecosystems (Grassi et al. 2006; Derero et al. 2011; Hadziabdic et al. 2012).

Characterization of molecular diversity, population structure, and molecular variation throughout the whole genus adds to knowledge of molecular basis for ecological habits of wild species and insures that we efficiently collect and conserve the germplasm *in situ* and choose novel genetic materials in breeding programs (Pavek et al. 2003; Emanuelli et al. 2013).

Nuclear single-nucleotide polymorphisms (SNPs) have been widely used for genetic diversity studies (Zhu et al. 2003; Lijavetzky et al. 2007; Rikkinen and Virtanen 2008; Emanuelli et al. 2013). Assessment of molecular diversity in grapevines has been reported using microsatellites (SSRs) and SNPs for *V. vinfera* and *V. sylvestris* (Grassi et al. 2006; Salmaso et al. 2004; Martinez et al. 2006; Myles et al. 2011; Emanuelli et al. 2013), cp-SSRs for *V. sylvestris* (Arroyo-Garcia et al. 2006; Grassi et al. 2006), and a few cp-SNPs, nuclear SSRs and amplified fragment length polymorphisms (AFLPs) for genetic relationships within the genus of *Vitis* (Ingrouille et al. 2002; Rossetto et al. 2002; Soejima and Wen 2006; Tröndle et al. 2010; El Oualkadi et al. 2011; Péros et al. 2011; Aradhya et al. 2012). Because of extremely low variation in cp-DNA and problematic homoplasy in SSR and AFLP markers, they have inherent limitations for study of population genetics in plants (Zhang and Hewitt 2003; DeWoody et al. 2010).

Every wild species may represent inherently unique genetic features and ecological habits, which can serve as a valuable resource for conservation and utilization (Mace and Purvis 2008; Wan et al. 2008; Pauls et al. 2013). To date, few studies have been performed concerning comprehensive assessment of genetic makeup and patterns across the *Vitis* genus using large numbers of nuclear SNPs (Salmaso et al. 2004; Lijavetzky et al. 2007; Myles et al. 2011; Péros et al. 2011). High morphological diversity has long been recognized in *Vitis* (Salmaso et al. 2004; Lijavetzky et al. 2007; Myles et al. 2011; Péros et al. 2011). High genetic diversity suggests potential broad adaptability of the population (Zhu et al. 2003). However, molecular basis for broad adaptability in *Vitis* is unclear. A number of strategies, e.g., based on genetic diversity, molecular heterozygosity, biodiversity-evolutionary perspective or range of the species habit, have been postulated for germplasm conservation (Mace and Purvis 2008; Pauls et al. 2013). However, these hypotheses have not yet been tested for their applicability to conservation of *Vitis* species. This study, based on empirical nuclear re-sequencing data, aimed to: (1) characterize patterns of genetic diversity and molecular variation in the different levels in *Vitis*, (2) discern genetic relationships and genetic partitioning and differentiation of *Vitis* species, (3) illustrate the molecular basis for high genetic diversity and genetic differentiation that confer broad adaptability in *Vitis*, (4) test the influence of genetic loss of one or two species on the genetic components in *Vitis*, and (5) comprehensively evaluate genetic features in *Vitis* thus to provide strategies for genetic conservation and utilization of this germplasm. This study not only greatly aids our understanding genetic patterns, molecular basis for broad adaptability of *Vitis* species, but also is critical to establishment of a basic molecular reference for germplasm conservation and utilization of *Vitis* species.

Materials and methods

Plant materials and sampling strategy

Plant materials were obtained from three germplasm repositories: (1) the Grape Germplasm Repository at the Northwest A & F University (NAFU), Yangling, Shaanxi Province, China, (2) USDA-ARS, Plant Genetic Resources Unit (PGRU), Geneva, NY, USA, and (3) USDA-ARS, National Clonal Germplasm Repository (NCGR), Davis, CA, USA (Table S1). No material collected from private/commercial vineyards was used to guarantee the trueness of accession of the materials.

Initially, 286 accessions were re-sequenced. Accessions with percentage of more than 2.5 % missing re-sequencing

data and all potential hybrids between species were excluded as to minimize any experimental error. Consequently, a total of 248 accessions from 48 species or varieties (not cultivars for wild species, referred as 'species' hereafter) of *Vitis* (~80 % coverage of the extant species) were used in this study (Table S1, S2). Three large population groups were delineated in the complete set of samples according to physical geographical barriers: South Europe and Asia Minor (Eur.), East Asia, and North and Central America (NC Amer.).

DNA isolation

DNA was isolated from young leaves and apical meristems of accessions using a modified CTAB (cetyltrimethylammonium bromide) protocol (Lodhi et al. 1994) followed by NaCl and ethanol precipitation to remove polysaccharides.

Identification of polymorphic DNA regions

An in-house pipeline containing the SEAN SNP Prediction package, Phrap (Ewing and Green 1998; Ewing et al. 1998), and Primer 3 (Rozen and Skaletsky 2000) was used to subcluster and survey potential SNPs in *V. vinifera* using ESTs database in 2004 (NCBI Unigene set). This strategy for search of potential SNPs was successfully used in previous studies (Krutovsky and Neale 2005; Branca et al. 2011; Keller et al 2011; Myles et al 2011). PCR primers were designed to bind in conserved areas flanking polymorphic regions (Labate and Baldo 2005; Somers et al. 2003).

Primer design and selection

Primers were designed for gene fragments containing moderate polymorphism and 281 pairs of primers were selected to examine their PCR quality on agarose gels using DNA samples of three species representing genetic background from three major continents, e.g., *V. vinifera* ('Rotberger', DVIT2339) for Eur, *V. rotundifolia* (DVIT1689) for NC Amer, and *V. romanetii* (Jiangxi2) for East Asia. Ninety-six pairs of primers with robust, single band PCR product were selected to examine re-sequencing quality using eight species representing distinct genetic backgrounds (*Vitis cinerea* (PI588575), *Vitis labrusca* (PI588194), *V. amurensis* (Zuoshan1), *V. quinquangularis* (Weinan3), *Vitis romanetii* (Pingli7), *Vitis davidii* (Xuefeng), *Vitis hancockii* (Lingye_F), and *Vitis yeshanensis* (Yanshan_F)). Thirty gene fragments (Table S3, S4) with suitable polymorphisms and only minor sequence length variation in the eight tested accessions were re-sequenced in a total of 248 accessions. These 30 gene fragments were mapped onto 14 of 19 (or 20 for *V. rotundifolia*) genetic linkage groups based on alignment of the entire grapevine genome (Figure S1) and their unigene annotations were listed in Table S3.

PCR and re-sequencing protocol

PCR reactions for primer screening were performed in 25 μl volume consisting of 20 ng DNA, 3 mM $MgCl_2$, 0.2 mM each dNTP, 8.0 pmol primer, 0.25 U GoTaq polymerase, and its commercial buffer (Promega, Madison, WI). PCR for sequencing was in 50 μl volume consisting of 20 ng DNA, 3 mM $MgCl_2$, 0.2 mM dNTPs, 12.0 pmol primer, 0.5 U GoTaq polymerase, and its commercial buffer. The PCR conditions were: 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 42–56.8 °C for 1 min, 72 °C for 2 min, plus 72 °C for 7 min and 4 °C hold. Fifty microliters of PCR products were cleaned using the Edge Biosystems QuickStep™ PCR cleaning kits (www.EdgeBio.com), then concentrated to 12 μl for cycle sequencing reactions. Cycle sequencing reactions were performed in 12 μl volume consisting of 5.0 μl concentrated PCR product DNA, 3.6 μl 2.5× sequencing buffer, and 1.0 μl BigDye Ready Reaction Mix (Applied Biosystems, Foster City, CA). The PCR conditions for cycle sequencing reactions were: 95 °C for 3 min, followed by 50 cycles of 96 °C for 10 s, 58 °C for 4 min, plus 72 °C for 7 min and 4 °C hold. Cycle sequencing reaction products were cleaned using 96-well cartridge (Applied Biosystems, Foster City, CA) and dried in a speed vacuum, then resuspended in 10 μl formamide. The sequencing was performed on ABI-3100×1 sequencer (Applied Biosystems, Foster City, CA) at the USDA-ARS, Plant Genetic Resource Unit (Geneva, NY, USA).

Sequence editing and alignment

The software "Proseq v2.9" was used to visualize and analyze the sequence data (Filatov 2009). Sequencing with the forward primers produced sufficiently reliable sequences for all accessions; however, the reverse strand was also sequenced for all accessions as control. The software "Clustal W" (Thompson et al. 1994) was used to align the sequences. The software "Proseq" was used to visually identify SNPs and manually create a spreadsheet containing all scored SNPs. All sequences were released in NCBI with NCBI accession numbers [Genbank: JX952227-JX960379, EMBL: HF544510-HF544512] (Wan et al. 2013).

Assessment of genetic diversity and genetic differentiations

Nine tests with different levels were designed for estimation of genetic diversity using five statistical indices, including nucleotide diversity (π), gene diversity (θ), numbers of haplotypes, numbers of segregating sites, and mean numbers of pairwise differences between haplotypes and the statistically expected estimators for four indices above except nucleotide diversity (Nei 1987; Tajima 1983, 1989; Schneider et al. 2000). All parameters were estimated using Arlequin software version

3.1 (Nei 1987; Tajima 1983, 1989; Excoffier et al. 2005). Nucleotide diversity is the probability that two randomly chosen homologous nucleotides are different:

$$\pi \frac{\sum_{i=1}^{k} \sum_{j<i} p_i p_j d_{ij}}{L}$$

where d_{ij} is an estimate of the number of mutations having occurred since the divergence of haplotypes i and j, k is the number of haplotypes, p_i is the sample frequency of haplotype i, and L is the number of loci.

Gene diversity (Nei 1987) is the probability that two randomly chosen haplotypes are different:

$$\theta = \frac{n}{n-1}\left(1-\sum_{i=1}^{k} p_i^2\right)$$

where n, i and j, k is the number of haplotypes, p_i is the sample frequency of the haplotype i.

Twelve tests with different levels were designed to manually calculate variation of SNPs based on five estimates including numbers of SNPs per gene fragment or per species (Per. SNPs), percentage of segregating sites (PSS), numbers of SNPs per segregating site (SNPs/SS), numbers of SNPs per haplotype (SNPs/Hap), numbers of haplotypes per sampled accessions (HPA).

Pairwise differences were used for assessment of the extent of genetic differentiation between pairs of 30 *Vitis* species. These were based on Wright's F-statistics (F_{ST}, number of permutations=1,000), P value for F_{ST} values (number of permutations=1,000) and significance of P value (level=0.05) as estimated using Arlequin software Version 3.1 (Excoffier et al. 2005).

Analyses of molecular variance

Ten population structures were designed to perform analyses of molecular variance (AMOVA) (based on 1,023 random permutations for P values) among the three geographic groups, among the 30 species (species containing at least three accessions were used for estimation of molecular variances), among the accessions within the species and among 248 accessions within *Vitis* genus using Arlequin software version 3.1 (Weir 1996; Excoffier et al. 2005; Fitzpatrick 2009). Coefficient of variation (CV=(standard variation/mean) multiplied by 100) (Hendricks and Robey 1936) was used for assessment of extent of molecular variation among accessions, among species or among gene fragments. SAS program (http://www.sas.com/) was used for calculation of correlation coefficients (r) between pairs of molecular indices and statistical significance. Permutations were performed based on the biological assumption of "expectation under random mating" and resampling the data for "pseudo-replication".

Analysis of genetic relationships of species

Nei's algorithms (Nei 1972) were used to estimate genetic distance between pairs of accessions and pairs of species using Arlequin software version 3.1. Principle coordinate analysis (PCoA) was used to illustrate the patterns of the genetic relationships using GenAlEx 6.0 (Peakall and Smouse 2006). Each accession (Table S1) and species was accordingly coded (Table S1 and S2), e.g., as "002N" or "S01A," when the patterns of their relationships were discerned on the PCoA plots.

To test influence of species' loss on genetic components of the whole dataset, one to two species were taken out from the dataset to compare molecular features between two populations: the complete population (comprising 48 species, CP) and the population without one or two species (the substracted population, SP) using software of Arlequin and GenAlEx 6.0. Mantel tests (Mantel 1967; Peakall et al. 2003) were performed to test statistical significance of relatedness between these two populations.

Results

Molecular features of nuclear sequences in *Vitis*

Seven DNA samples (0.09 %) did not amplify for the gene fragment '7393'. In total, 7,433 amplicons were successfully re-sequenced and aligned. The length of these 30 gene fragments ranged from 251 to 690 bp, averaging 413.9±116.5 bp (mean±s.d., n=30, the statistical values in this context are all presented as mean±s.d., except as noted). In total, ~12.4 Kb for each accession and ~3.07 million nucleotides for all 248 accessions were re-sequenced. A total of 6,862 SNPs, 2,752 segregating sites, and 4,248 haplotypes were found among 248 accessions of *Vitis* (Table 1).

Molecular genetic diversity in *Vitis* species

Nucleotide diversity and gene diversity among 248 accessions were 0.0150 and 0.927 ("T1" in Table 2), respectively. On average, these two estimates for accessions within species were 0.0073 and 0.699 ("T3" in Table 2), respectively. Numbers of segregating sites, numbers of haplotypes, and mean numbers of pairwise differences among 248 accessions were 91.7, 141.6, and 6.10 ("T1" in Table 2), respectively. These three parameters for accessions within species were

Table 1 Molecular features of the 30 gene fragments among 248 accessions of *Vitis*

Fragments	TL (bp) [a]	NSS(No.) [b]	NH(No.) [c]	SNPs(No.) [d]	SS/TL (%) [e]	SNP/SS (No.) [f]	SNP/ Hapl(No.) [g]	Two SNPs (%) [h]	Three SNPs (%) [i]	Two SNPs and three SNPs (%) [j]	HPA [k]
241	407	87	127	230	21.4	2.6	1.8	56.3	29.9	86.2	0.5
590	294	63	126	151	21.4	2.4	1.2	71.4	22.2	93.7	0.5
689	305	22	42	57	7.2	2.6	1.4	40.9	59.1	100.0	0.2
765	445	141	230	397	31.7	2.8	1.7	44.0	35.5	79.4	0.9
1165	525	90	109	220	17.1	2.4	2.0	61.1	34.4	95.6	0.4
1313	506	121	170	294	23.9	2.4	1.7	70.3	33.9	104.1	0.7
1314	630	103	127	261	16.4	2.5	2.1	59.2	31.1	90.3	0.5
1529	330	78	76	181	23.6	2.3	2.4	75.6	21.8	97.4	0.3
1973	536	58	115	144	10.8	2.5	1.3	53.5	46.6	100.0	0.5
2129	478	83	141	211	17.4	2.5	1.5	51.8	44.6	96.4	0.6
2415	646	154	243	386	23.8	2.5	1.6	58.4	35.7	94.2	1.0
3026	501	126	191	307	25.2	2.4	1.6	65.9	29.4	95.2	0.8
3221	358	172	258	426	48.0	2.5	1.7	63.4	29.1	92.4	1.0
3389	376	69	88	167	18.4	2.4	1.9	66.7	29.0	95.7	0.4
4581	294	49	52	115	16.7	2.4	2.2	67.4	32.7	100.0	0.2
5069	531	118	93	264	22.2	2.2	2.8	79.7	18.6	98.3	0.4
5693	366	73	153	188	20.0	2.6	1.2	53.4	39.7	93.2	0.6
6054	277	47	106	118	17.0	2.5	1.1	61.7	31.9	93.6	0.4
6670	402	33	44	84	8.2	2.6	1.9	57.6	30.3	87.9	0.2
7022	303	75	119	186	24.8	2.5	1.6	54.7	44.0	98.7	0.5
7029	452	84	149	223	18.6	2.7	1.5	53.6	25.0	78.6	0.6
7230	386	55	97	146	14.3	2.7	1.5	49.1	40.0	89.1	0.4
7312	314	42	125	95	13.4	2.3	0.8	78.6	21.4	100.0	0.5
7351	251	57	94	138	22.7	2.4	1.5	59.7	31.6	91.2	0.4
7362	690	236	322	579	34.2	2.5	1.8	62.3	30.5	92.8	1.3
7386	291	47	74	121	16.2	2.6	1.6	57.5	36.2	93.6	0.3
7393[a]	409	132	163	329	32.3	2.5	2.0	60.6	31.1	91.7	0.7
7413	451	113	207	291	25.1	2.6	1.4	54.9	36.3	91.2	0.8
7434	324	104	176	249	32.1	2.4	1.4	69.2	24.0	93.3	0.7
7447	338	120	231	304	35.5	2.5	1.3	58.3	35.8	94.2	0.9
Total	12,416	2752	4248	6862	–	–	–	–	–	–	–
Average	413.9	91.7	141.6	228.7	23.0	2.5	1.7	60.6	33.0	93.6	0.6
s.d.	116.5	46.3	67.4	116.1	8.7	0.1	0.4	9.2	8.4	5.6	0.3

[a] Total length; [b] Numbers of segregating sites; [c] Numbers of haplotypes; [d] Numbers of SNPs; [e] Percentage of segregating sites; [f] Numbers of SNPs per segregating site; [g] Numbers of SNPs per haplotype; [g] Percentage of segregating sites with which one site produced two SNPs; [h] Percentage of segregating sites with which one site produced two SNPs; [i] Percentage of segregating sites with which one site produced three SNPs; [j] Percentage of segregating sites with which one site produced two or three SNPs; [k] Numbers of haplotypes per accession

7.9, 6.1, and 2.85 ("T3" in Table 2), respectively. Genetic diversity was the highest in NC Amer among the three geographic regions ("T4–T9" in Table 2), indicating sampling size (number of total accessions or species) is an important contributor to genetic diversity.

For 248 accessions, 67 % of correlation coefficients were significant between pairs of five molecular indices above (Table S32). For 30 species, all correlation coefficients were significant (Table S32). For accessions within species, 94 % of correlation coefficients were significant (Table S33). This suggests that these five indices had similar trends of variation in *Vitis*.

Genetic differentiations between *Vitis* species

The F_{ST} values in pairwise differences for estimation of genetic differentiation between *Vitis* species was mostly in the range of 0.10–0.40 (Fig. 1). On average (n_1=30 gene fragments, $n2$=29 pairs of comparisons), the F_{ST} value of a species with the remaining 29 species was 0.24 (Fig. 1). In all,

Table 2 Genetic diversity and their comparisons based on statistical random permutations in *Vitis* (mean±s.d.) (detailed information in Table S5–S31)

Tests	ND	GD	NSS (no.)	NH (no.)	MPD (no.)
T1	0.0150±0.0093	0.927±0.074[a] (73 %)[b]	91.7±46.3 (100 %)	141.6±67.4 (100 %)	6.10±3.75 (90 %)
T2	0.0136±0.0092	0.929±0.077 (70 %)	100.0±86.1 (100 %)	136.6±66.1 (100 %)	5.48±3.68 (97 %)
T3	0.0073±0.0020	0.699±0.093 (20±16 %)	7.9±3.1 (97±4 %)	6.1±2.4 (78±15 %)	2.85±0.79 (97±13 %)
T4	0.0089±0.0081	0.729±0.180 (10 %)	12.2±5.7 (100±0 %)	11.1±6.0 (97 %)	3.52±3.25 (100 %)
T5	0.0078±0.0020	0.698±0.104 (17±19 %)	8.3±1.1 (98±2 %)	6.8±0.0 (83±19 %)	3.11±0.84 (100±0 %)
T6	0.0110±0.0084	0.877±0.102 (47 %)	39.4±7.7 (98±9 %)	46.0±19.3 (97 %)	4.35±3.04 (97 %)
T7	0.0058±0.0011	0.642±0.081 (12±9 %)	5.6±2.0 (97±4 %)	5.0±1.7 (85±8 %)	2.23±0.44 (93±22 %)
T8	0.0156±0.0107	0.914±0.104 (70 %)	63.3±6.3 (100 %)	93.7±44.5 (93 %)	6.25±4.26 (100 %)
T9	0.0080±0.0019	0.731±0.088 (25±18 %)	9.2±3.1 (97±4.8 %)	6.7±2.7 (73±17 %)	3.17±0.76 (98±2 %)

ND nucleotide diversity, *GD* gene diversity, *NSS* numbers of segregating sites, *NH* numbers of haplotypes, *MPD* mean numbers of pairwise differences

T1, average genetic diversity among 248 accessions within the genus (n=30 gene fragments)

T2, average genetic diversity among 243 accessions within the subgenus of *Euvitis* (n=30 gene fragments)

T3, average genetic diversity within species (n=30 species)

T4, average genetic diversity among 21 accessions within Eur (n=30 gene fragments)

T5, average genetic diversity of two species in Eur (n=2 species)

T6, average genetic diversity among 79 accessions in Asia (n=30 gene fragments)

T7, average genetic diversity of ten species in Asia (n=10 species)

T8, average genetic diversity among 148 accessions in NC Amer (n=30 gene fragments)

T9, average genetic diversity of 18 species in NC Amer (n=18 species)

[a] The observed values, [b] Percentage of gene fragments or species presenting the observed values (refers to Table S30) greater or equal to their statistical estimators based on random permutations (refers to Table S31), numbers of permutations=3000 based on random mating

86.8 % of 13,021 pairwise comparisons had significant P values (P<0.05). Thus, the 30 *Vitis* species were highly genetically differentiated from each other.

Analysis of molecular variance (AMOVA)

Variance among 248 accessions was 0.684 (F_{IT} in "Str1" of Table 3), suggesting a strong population structure across the entire genus (Excoffier et al. 2005; Peakall and Smouse 2006; Straub and Doyle 2009). On average, variance among accessions within species was 0.369 (F_{IS} in "Str2" of Table 3), suggesting population structure within most *Vitis* species is also strong (Excoffier et al. 2005; Peakall and Smouse 2006; Straub and Doyle 2009). Variance among 30 species was 0.511 (F_{ST} in 'Str3' of Table 3), indicating high genetic differentiation among species. For three geographic regions, estimated F_{ST} representing variance among *Euvitis* species was in this order: Asia > NC Am > Eur (F_{ST} in "Str7,", "Str8," and "Str10" of Table 3), suggesting the strongest structure within Asia. However, estimated F_{IS} representing variance among accessions within *Euvitis* species was in this order: NC Am > Asia > Eur (F_{IS} in "Str7," "Str8," and "Str10" of Table 3), indicating that NC Am species had the strongest population structure among three regions.

Variance components, i.e., percentage of variance, varied among structures in some cases, e.g., variances among accessions within species (e.g., 'Str 2 and Str 3' of Table 3). The reason for this is that variances for each component were the co-variances of the entire structure, and this may result in relatively different values for the same estimates among different structures (Schneider et al. 2000; Excoffier et al. 2005). However, the associated F statistics and percentage (%) of 30 gene fragments presenting significant P values for the same component were close among the different structures (e.g., in Str 2 and Str

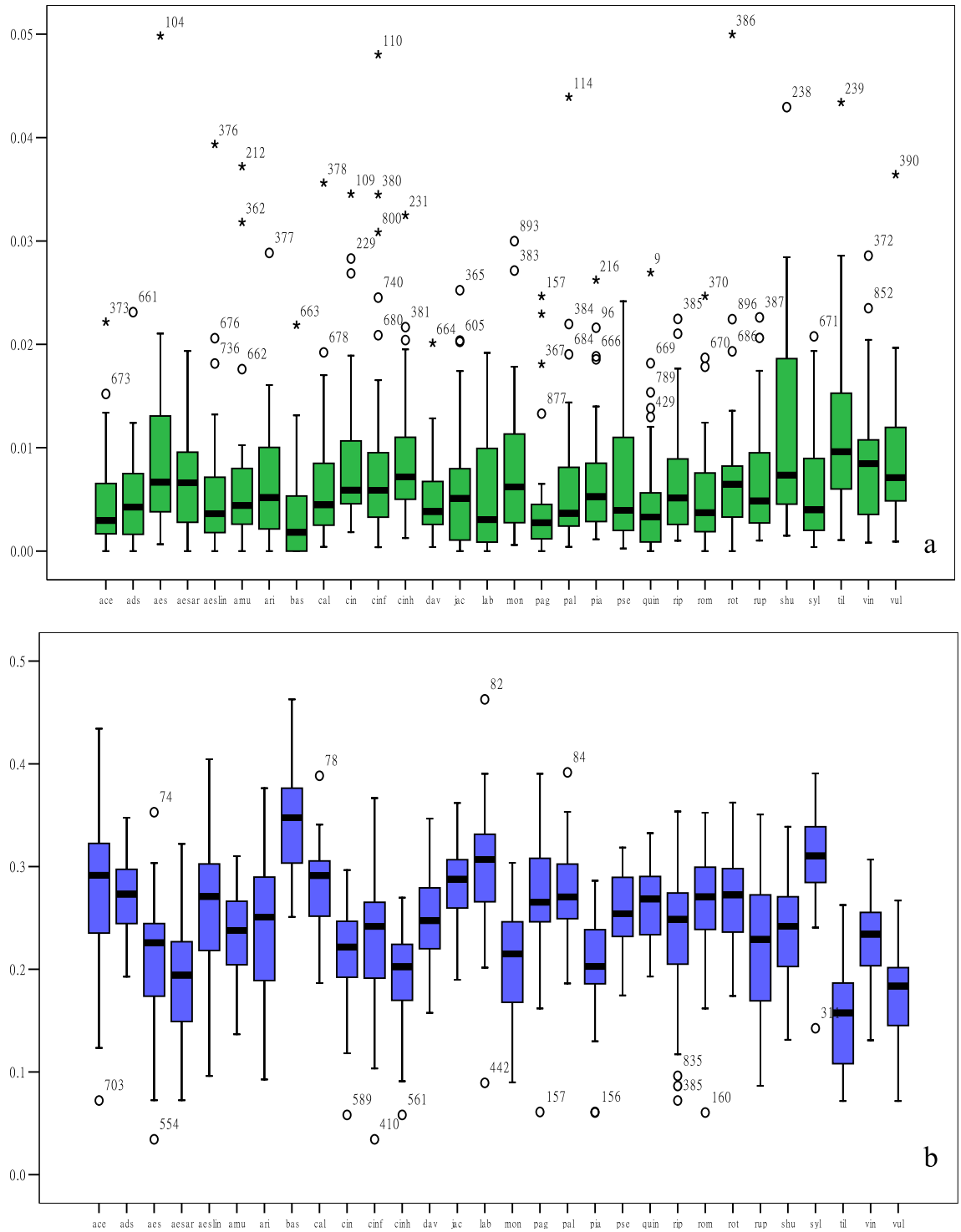


Fig. 1 Box plots of nucleotide diversity within and genetic differentiations between 30 *Vitis* species. **a** nucleotide diversity (π) of 30 *Vitis* species estimated from 30 gene fragments. Nucleotide diversity shows extensive variation among genes and among species in *Vitis*. **b** Average genetic differentiation of a *Vitis* species with the remaining 29 species (average value from 29 pairs of estimations)

3, Table 3), indicating that these results were consistent. Molecular variances were significant in all of the ten designed structures (Table 3). Because genotypes or species in *Vitis* are highly genetically differentiated from one another as analyzed above, when assessing species differentiation using a metric designed for populations, one would expect significant differentiation (Table 3).

Table 3 Analysis of molecular variance in the *Vitis* based on the ten designed structures with three or four hierarchies (mean, s.d. n=30 gene fragments) (detailed information in Table S34–S43)

Structures		Percentage of variance (%)				F values for				Percentage (%) of fragments presenting significant P values for			
		Va	Vb	Vc	Vd	F_{ST} or F_{CT}	F_{SC}	F_{IS}	F_{IT}	F_{ST} or F_{CT}	F_{SC}	F_{IS}	F_{IT}
Str 1	Mean	17.9	50.6	31.6	–	0.1787	–	0.6212	0.6845	97	–	97	97
	S.D.	9.3	25.9	26.8	–	0.0930	–	0.2861	0.2675	–	–	–	–
Str 2	Mean	12.8	41.2	14.9	31.16	0.1276	0.4654	0.3691	0.6884	73	100	97	97
	S.D.	11.3	18.2	18.5	27.13	0.1125	0.1542	0.3091	0.2713	–	–	–	–
Str 3	Mean	51.1	16.0	32.9	–	0.5110	–	0.3691	0.6708	100	–	97	97
	S.D.	14.6	19.2	27.7	–	0.1458	–	0.3091	0.2772	–	–	–	–
Str 4	Mean	19.5	45.6	34.9	–	0.1952	–	0.5758	0.6512	100	–	97	97
	S.D.	9.1	24.5	26.7	–	0.0914	–	0.2840	0.2670	–	–	–	–
Str 5	Mean	16.0	33.8	16.0	34.61	0.1565	0.4012	0.3656	0.6540	70	100	97	97
	S.D.	9.9	11.1	18.4	27.10	0.1008	0.1151	0.3127	0.2710	–	–	–	–
Str 6	Mean	45.9	17.3	36.8	–	0.4593	–	0.3656	0.6318	100	–	97	97
	S.D.	11.8	19.1	27.6	–	0.1181	–	0.3127	0.2764	–	–	–	–
Str 7	Mean	22.2	19.8	58.0	–	0.2223	–	0.2779	0.4198	60		50	60
	S.D.	19.8	34.3	40.0	–	0.1978	–	0.4295	0.3998	–	–	–	–
Str 8	Mean	44.0	14.6	41.5	–	0.4398	–	0.3148	0.5855	100		67	97
	S.D.	14.8	22.3	31.8	–	0.1481	–	0.3579	0.3179	–	–	–	–
Str 9	Mean	47.0	18.1	34.9	–	0.4703	–	0.3789	0.6512	100	–	97	97
	S.D.	15.7	19.4	27.2	–	0.1570	–	0.2937	0.2720	–	–	–	–
Str 10	Mean	38.9	20.8	40.4	–	0.3889	–	0.3745	0.5963	100	–	97	97
	S.D.	11.2	19.4	26.8	–	0.1123	–	0.2950	0.2675	–	–	–	–

In the three hierarchical structures, F_{ST}, F values for Va, F_{IS}, F values for Vb, F_{IT}, F values for Vc; in the four hierarchical structures, F_{CT}, F values for Va, F_{SC}, F values for Vb, F_{IS}, F values for Vc, F_{IT}, F values for Vd

Str 1, three geographic groups as 'populations' and sampling accessions as 'individuals', Va, variance among three geographical groups (*d.f.*=2), Vb, variance among sampling accessions within the geographic groups (*d.f.*=245, but *d.f.*=238 in the fragment '7393'), Vc, variance among 248 accessions (*d.f.*=248, but *d.f.*=241 in '7393')

Str 2, three geographic groups as 'groups' and 30 species as 'populations', and sampling accessions as 'individuals', Va, variance among three geographical groups (*d.f.*=2), Vb, variance among species within the geographic group (*d.f.*=27, but *d.f.*=26 in '7393'), Vc, variance among accessions within species (*d.f.*=189, but *d.f.*=184 in '7393'), Vd, variance among 222 sampling accessions (*d.f.*=219, but *d.f.*=213 in '7393')

Str 3, the 30 species as 'populations' and sampling accessions as 'individuals', Va, variance among 30 species (*d.f.*=29, but *d.f.*=28 in the fragment '7393'), Vb, variance among sampling accessions within species (*d.f.*=189, but *d.f.*=184 in '7393'), Vc, variance among 222 accessions (*d.f.*=219, but *d.f.*=213 in '7393')

Str 4, three geographic groups as 'populations' and 243 sampling accessions (without 5 accessions from *Vitis rotundifolia*) as 'individuals', Va, variance among three geographical groups (*d.f.*=2), Vb, variance among sampling accessions within the geographic groups (*d.f.*=240, but *d.f.*=238 in '7393'), Vc, variance among 248 accessions (*d.f.*=243, but *d.f.*=241 in '7393')

Str 5, three geographic groups as 'groups' and 29 species as 'populations', and 243 sampling accessions (without 5 accessions from *Vitis rotundifolia*) as 'individuals', Va, variance among three geographical groups (*d.f.*=2), Vb, variance among species within the geographic groups (*d.f.*=26), Vc, variance among accessions within species (*d.f.*=184), Vd, variance among 222 sampling accessions (*d.f.*=213)

Str 6, the 29 species as 'populations' and sampling accessions as 'individuals', Va, variance among 29 species (*d.f.*=28), Vb, variance among sampling accessions within species (*d.f.*=185, but *d.f.*=184 in '7393'), Vc, variance among 222 accessions (*d.f.*=214, but *d.f.*=213 in '7393')

Str 7, the two species in Eur as 'populations' and 21 sampling accessions as 'individuals', Va, variance among 2 species (*d.f.* =1), Vb, variance among sampling accessions within species (*d.f.*=19), Vc, variance among 21 accessions (*d.f.* =21)

Str 8, the 10 species in Asia as 'populations' and 79 sampling accessions as 'individuals', Va, variance among 10 species (*d.f.*=9), Vb, variance among sampling accessions within species (*d.f.*=56), Vc, variance among 79 accessions (*d.f.* =66)

Str 9, the 18 species in NC Amer as 'populations' and 148 sampling accessions as 'individuals', Va, variance among 18 species (*d.f.*=17, but *d.f.*=16 in the fragment '7393'), Vb, variance among sampling accessions within species (*d.f.*=114, *d.f.*=109 in '7393'), Vc, variance among 148 accessions (*d.f.*= 132, but *d.f.*=126 in '7393')

Str 10, the 17 species (without *Vitis rotundiforlia*) in NC Amer as 'populations' and 148 sampling accessions as 'individuals', Va, variance among 17 species (*d.f.*=16), Vb, variance among sampling accessions within species (*d.f.*=110, but *d.f.*=109 in '7393'), Vc, variance among 143 accessions (*d.f.*= 127, but *d.f.*=126 in '7393')

Features of segregating sites and SNPs in *Vitis*

Numbers of SNPs per segregating site were similar among all different level tests (Table 4). For 248 accessions, on average, 22.0±8.7 % of polymorphic sites (TL1 in Table 4, Table 1), i.e., one segregating site was found in every 3 to 8 bp among 248 accessions of *Vitis*. This value is relatively high compared to most plant species (Schneider et al. 2000; Tenaillon et al. 2001; Somers et al. 2003; Zhu et al. 2003; Krutovsky and Neale 2005; Heuertz et al. 2006; Pyhäjärvi et al. 2007; Chen et al. 2010; Keller et al. 2011; Wachowiak et al. 2011; Ismail et al. 2012; Emanuelli et al. 2013; Xin et al. 2013). However, after a detailed analysis, we found that 6862 SNPs were produced by 2,752 segregating sites, indicating that there was on average 2.5±0.1 SNPs per segregating site. Percentage of segregating sites with which one site produced two SNPs and three SNPs, on average, accounted for 61 and 33 %, respectively (Table 1). Almost all allelic SNPs were distributed unevenly among segregating sites throughout the 248 accessions. On average, 95 % of 248 accessions shared an allelic SNP (referred as the genus major SNP). The remaining few accessions possessed other SNPs (referred as genus minor SNPs) (Table S48). Most genus minor SNPs were found within just one or a few species. Therefore, certain SNPs found as minors within the genus were identified as the majors within a species. Some of these SNPs showed no segregation within one or a few species, indicating that they may be unique to one or a few species. On average, 67.8 % of segregating sites in the genes produced SNPs with low frequency of 0.4–2.0 % (Table S45). In addition, the total frequency of the genus minor SNPs was 4.7 % (Table S44). Thus, numerous "subtle" variants were found in the *Vitis* gene pool.

Table 4 Characterization of segregating sites and SNPs in *Vitis* (mean± s.d.) (detailed information in Table S44–S50)

Tested levels	Per. SNPs	PSS (%)	SNPs/SS (No.)	SNPs/Hap (No.)	HPA
TL1	228.7±116.1	22.0±8.7	2.5±0.1	1.7±0.4	0.6±0.3
TL2	17.6±7.0	1.9±0.8	2.1±0.2	2.7±0.8	0.9±0.2
TL3	17.6±10.7	1.9±1.1	2.1±0.1	2.7±1.2	0.9±0.3
TL4	30.1±28.7	3.2±1.7	2.3±1.1	2.6±1.8	0.5±0.3
TL5	18.7±14.2	2.0±1.4	2.3±0.2	2.5±1.1	0.7±0.3
TL6	18.7±2.0	2.0±0.3	2.3±0.1	2.5±0.3	0.7±0.3
TL7	100.6±59.8	10.2±3.3	2.5±1.2	2.2±1.4	0.6±0.2
TL8	12.4±6.8	1.3±1.0	2.0±0.2	2.2±0.9	0.8±0.3
TL9	12.4±4.6	1.3±0.6	2.0±0.3	2.2±0.4	0.8±0.2
TL10	174.1±103.8	16.4±4.4	2.8±1.7	2.1±1.7	0.6±0.3
TL11	20.4±13.5	2.2±1.3	2.1±0.1	3.0±1.6	1.0±0.3
TL12	20.3±7.0	2.2±0.8	2.1±0.2	3.0±0.9	1.0±0.2

Per. SNPs numbers of SNPs per gene fragments, *PSS* percentage of segregating sites (%), *SNPs/SS* numbers of SNPs per segregating site, *SNPs/Hap* numbers of SNPs per haplotype, *HPA* numbers of haplotypes per accession

TL1, among 248 accessions (n=30 gene fragments)

TL2, among accessions within species (n=30 gene fragments)

TL3, among 30 species within gene fragment (n=30 species)

TL4, among 21 accessions in Eur (n=30 gene fragments)

TL5, among 30 gene fragments within species in Eur (n=30 gene fragments)

TL6, among 2 species within gene fragment in Eur (n=2 species)

TL7, among 79 accessions within Asia (n=30 gene fragments)

TL8, among 30 gene fragments within species in Asia (n=30 gene fragments)

TL9, among 10 species within gene in Asia (n=10 species)

TL10, among 148 accessions in NC Amer (n=30 gene fragments)

TL11, among 30 gene fragments within species in NC Amer (n=30 gene fragments)

TL12, among 18 species within gene in NC Amer (n=18 species)

SNPs/Hap and HPA within species were much higher than those among 248 accessions. However, percentage of segregating sites among accessions within species was greatly lower than that among 248 accessions (Table 4). Frequency of segregating sites among accessions within species, i.e., one segregating site in every 45 to 70 bp (TL2, Table 4), was higher than most reported plant species (Schneider et al. 2000; Tenaillon et al. 2001; Somers et al. 2003; Zhu et al. 2003).

Five indices, including Per. SNPs, PSS, SNPs/SS, SNPs/Hap, and HPA, were the highest in the NC Amer among three geographic groups (Table 4). With the exception of SNPs/Hap, the remaining four indices among accessions were the lowest in Eur, while five indices among accessions within species were the lowest in Asia among the three geographic groups (Table 4, Table S49 and S50).

Extent of molecular variation in *Vitis*

Twenty genetic structures comprising ten molecular indices (Table 5) were designed to evaluate the genetic variation (CV) in *Vitis*. CV in *Vitis* was characterized by: (1) CV among 248 accessions (SL1, Table 5) was much lower than that among 30 species (SL4, Table 5); (2) CV of nucleotide diversity (variation) was generally the highest among ten molecular indices, followed by mean numbers of pairwise differences and numbers of SNPs per gene fragment or per species; (3) CV of numbers of SNPs per segregating site and gene diversity were the lowest two indices among the ten molecular indices (Table 5, Table S51-S64), indicating that *Vitis* species exhibit a relatively wide extent of nucleotide variation while they maintain a relatively narrow range of gene variation (Fig. 1); (4) CV among gene fragments within species (SL2, Table 4, Table S52) was relatively the highest among the twenty tests, indicating that molecular variation ranged extensively among genes; (5) CV of numbers of haplotypes per sampled accession were within a relatively narrow range (mostly around 47 %) among the twelve tests, i.e., every two sampled accessions produced one haplotype; (6) CV among

Table 5 Coefficients of variation (CV) in the ten molecular indices of *Vitis* based on 20 genetic structures (mean ± s.d.; detailed information in Table S51-S64)

Sub-levels	ND	GD	NSS	NH	MPD	Per. SNPs	PSS	SNP/SS	SNP/Hapl	HPA
SL1	53.3±4.3	1.1±2.5	50.4	47.6	49.5±3.7	50.8	39.7	4.9	25.0	47.6
SL2	96.7±17.9	35.1±12.3	89.6±21.5	47.5±10.0	97.0±18.6	61.9±21.5	54.5±20.1	6.2±16.2	44.8±30.1	47.7±9.9
SL3	71.8±6.0	16.6±5.4	–	–	63.8±5.3	–	–	–	–	–
SL4	69.6±28.6	33.3±15.1	68.0±19.4	52.8±6.1	70.1±29.3	67.6±18.7	68.1±19.4	24.2±12.2	61.2±34.2	45.8±9.6
SL5	71.8±10.0	16.6±6.4	–	–	63.8±8.9	–	–	–	–	–
SL6	67.0±16.3	8.6±7.0	46.3	53.9	60.3±14.7	95.2	54.4	48.0	70.4	53.9
SL7	58.4±6.5	2.4±2.1	19.4	42.1	52.8±5.8	59.4	32.0	46.7	61.2	42.1
SL8	55.2±5.3	1.1±1.4	10.0	47.5	49.9±4.9	59.6	26.7	63.0	84.6	47.5
SL9	93.9±32.8	34.3±11.6	87.4±31.9	49.2±18.9	93.8±33.3	84.7±31.1	81.1±28.4	20.8±9.1	69.9±28.1	49.2±18.9
SL10	108.2±18.0	41.9±13.2	95.1±20.5	53.4±10.9	103.5±18.8	93.2±20.2	92.7±17.7	30.4±17.5	80.5±22.2	53.4±10.9
SL11	92.4±17.7	29.4±12.3	78.9±18.5	44.6±10.6	89.9±19.1	78.4±17.3	74.3±16.7	14.6±13.1	76.4±38.4	45.1±10.5
SL12	75.0±7.1	16.6±6.6	–	–	61.9±16.6	–	–	–	–	–
SL13	73.3±8.4	17.8±6.2	–	–	65.1±7.2	–	–	–	–	–
SL14	70.1±5.1	14.7±5.4	–	–	62.5±4.6	–	–	–	–	–
SL15	38.8±30.4	23.0±22.4	28.3±27.2	22.9±18.6	38.7±30.3	27.5±25.8	28.3±27.2	9.6±9.3	23.3±23.0	48.9±25.4
SL16	73.2±30.1	40.4±19.3	74.8±26.8	52.6±13.5	73.6±30.9	75.5±25.7	82.7±24.7	30.6±19.5	58.5±28.6	47.6±15.3
SL17	58.8±29.7	28.9±18.0	58.4±17.8	51.7±7.9	59.0±29.8	58.6±17.3	58.6±17.8	19.5±14.1	54.6±32.1	41.1±11.9
SL18	72.6±18.9	14.9±11.1	–	–	64.9±16.9	–	–	–	–	–
SL19	73.3±14.1	17.9±6.8	–	–	65.1±12.4	–	–	–	–	–
SL20	70.9±10.2	16.2±7.6	–	–	62.9±9.1	–	–	–	–	–

ND nucleotide diversity, *GD* gene diversity, *NSS* numbers of segregating sites, *NH* numbers of haplotypes, *MPD* mean numbers of pariwise differences, *Per. SNPs* numbers of SNPs per gene contis, *PSS* percentage of segregating sites, *SNP/SS* numbers of SNPs per segregating site, *SNP/Hapl* numbers of SNPs per haplotype, *HPA* numbers of haplotypes per accession

SL1, among 248 accessions (n=30 fragments)

SL2, among 30 gene fragments within the species (n=30 species)

SL3, among accessions within the species (n=30 species)

SL4, among 30 species (n=30 fragments)

SL5, among accessions within the species and within the gene fragment (n=30 fragments)

SL6, among 21 accessions within Eur (n=30 fragments)

SL7, among 79 accessions within Asia (n=30 fragments)

SL8, among 148 accessions within NC Amer (n=30 fragments)

SL9, among gene fragments within the species in Eur (n=2 species)

SL10, among gene fragments within the species in Asia (n=10 species)

SL11, among gene fragments within the species in NC Amer (n=18 species)

SL12, among accessions within the species in Eur (n=2 species)

SL13, among accessions within the species in Asia (n=10 species)

SL14, among accessions within the species in NC Amer (n=18 species)

SL15, among species within the gene fragment in Eur (n=30 fragments)

SL16, among species within the gene fragment in Asia (n=30 fragments)

SL17, among eighteen species within the gene fragment in NC Amer (n=30 fragments)

SL18, among accessions within the species and within gene fragment in Eur (n=30 fragments)

SL19, among accessions within the species and within gene fragment in Asia (n=30 fragments)

SL20, among accessions within the species and within gene fragment in NC Amer (n=30 fragments)

accessions within the geographic region was the highest in Eur and the lowest in Asia; however, CV among gene fragments or among species was the highest in Asia and the lowest in NC Amer in most cases among the three geographic groups (SL6-SL20 in Table 4, Table S56-S64). Thus, the geographic region comprising relatively high genetic diversity (T4–T9 in Table 2), i.e., NC Amer, may not present a wide extent of molecular variation (SL6-SL20 in Table 4).

The genetic patterns among *Vitis* species

The patterns of genetic relationships of accessions and species in *Vitis* were characterized by: (1) two sub-genera, *Muscadinia* and *Euvitis*, were extensively differentiated from each other (Fig. 2a, c), (2) two species from Eur were discerned close to each other, but they both fell to different quadrants from American species in the PCoA plot (Fig. 2b, d), (3) Asian species were mainly discerned into two groups (Fig. 2b, d), one represented by *Vitis adstricta* (S01A), *V. hancockii* (S34A), *Vitis pseudoreticulata* (S08A), *V. quinquangularis* (S09A), *Vitis bellula* (S31A), *Vitis ficifolia* (S32A), and *Vitis bashanica* (S03A), the other represented by *Vitis shenxiensis* (S37A), *V. romanetii* (10A), *V. davidii* (S04A), *Vitis piasezkii* (S06A), *Vitis qinlingensis* (S36A), *V. piasezkii* var. *pagnuccii* (S07A), *Vitis betulifolia* (S47A), *V. amurensis* (S02A), *Vitis yenshanensis* (S38A), *Vitis liubanensis* (S35A), *V. davidii var. cyanocarpa* (S48A) and *Vitis jacquemontii* (S05A), (4) American species within *Euvistis* were mainly assigned into four groups (Fig. 2c), consisting of (1) *Vitis arizonica* (S17N), *Vitis girdiana* (S43N), *Vitis acerifolia* (S13N), *Vitis riparia* (S25N), *Vitis blancoii* (S41N), *Vitis bloodworthiana* (S23N), *Vitis monticola* (S23N), and *Vitis rupestris* (S27N), (2) *Vitis tiliifolia* (29N), *Vitis nesbittiana* (S44N) and *Vitis shuttleworthii* (S28N), (3) *Vitis aestivalis* var. *aestivalis* (39N), *Vitis cinerea* var. *helleri* (S21N), *Vitis cinerea* (S19N) and *Vitis biformis* (S40N), and (4) *Vitis cinerea* var. *floridana* (S20N), *V. aestivalis* (S14N), *V. aestivalis* var. *argentifolia* (S15N), *V. aestivalis* var. *lincecumii*(S16N), and *Vitis labrusca* (S22N).

Effects of genetic loss on genetic component of genus

We found that haplotype frequency and genetic differentiation between CP and SP were non-significant ($P=1.00\pm0.00$) for all the tests (450 tests for one species loss (=(30 gene fragments×30 species)/2) and 6,750 tests for two species losses (=(30 gene fragments×30 species×29 species)/2)). However, mean numbers of pairwise differences were extremely significant between two populations for all tests ($P=0.00\pm0.00$), suggesting each species represents unique genotype and its extinction would result in an irreversible loss of genetic variation from the gene pool of the genus.

Discussion

Comprehensive interpretation of genetic relationships of *Vitis* species

A precise resolution of genetic relationships is a prerequisite for genetic analyses, germplasm conservation and breeding programs (Grassi et al. 2006; Soejima and Wen 2006; Tröndle et al. 2010). In this study, all samples were obtained from governmental collections and any potential hybrids within the wild species germplasm were removed from analyses. Thus, all species are assumed to represent their natural identities. This ensured accuracy in discernment of genetic relationships among *Vitis* species in this study.

Although the principle of PCoA was proposed 50 years ago (Torgerson 1958), this method and its powerful attributes have not until relatively recently been recognized in the study of population genetics (Peakall and Smouse 2006). This method, unlike the tree construction methods, does not assume a hierarchical genetic structure in the population. The hierarchical assumption is rational at higher taxonomic levels. But it is not always true at the population level. Another disadvantage of tree building methods is that results may be very distinct among algorithms, among different numbers of permutation tests and among hierarchical genetic structures when the number of samples is vast for analyses (Peakall and Smouse 2006). In addition, strong evolutionary signals (historic hybridization and splitting, e.g., in *Vitis* (Wan et al. 2013) may lead to unwieldy trees that are difficult to interpret. PCoA uses a strategy in which the essential signals in the data were quantified and used for estimation, thus minimizing influence from conflicting signals of patterns of genetic relationships among operational taxonomic units (OTUs) (Peakall and Smouse 2006). Another advantage of PCoA is that patterns of genetic relationships and extent of differentiation among accessions or species in the PCoA plot (e.g., in Fig. 2) can be visually identified (Peakall and Smouse 2006).

The patterns between the accession-by-accession and species-by-species graphs were very similar for the whole genus (e.g., in Fig. 2a, c) and for the subgenus of *Euvitis* (e.g., in Fig. 2b, d), considering that coordinate orientations may vary among permutations of the different tests (Peakall and Smouse 2006). If number of accessions per species was the same for all species and molecular variation among accessions within the species was very close among species, the patterns between the accession-by-accession and species-by-species graphs would be very similar. All of these evidences suggested that the patterns in the PCoA plots in this study can reflect the intrinsic genetic relationships of the species in *Vitis*.

However, some differences were found between patterns between the graphs of the accession-by-accession and species-by-species (Fig. 2a and c, b and d). For example, 21 accessions from Eur overlapped with accessions from Asia in the accession-by-accession plot (Fig. 2b). However, two species from Eur were distinct from Asian species (Fig. 2d). The reason for this is that PCoA calculates the vector of each variable based on the whole matrix dataset, thus the results may vary among permutations (Peakall and Smouse 2006). The accession-by-accession analysis was a good reference with which to examine if any accession was unexpectedly assigned and for comparisons with the species-by-species results, thus

to decide with confidence whether the accessions and species were consistently discerned.

The overall patterns in the PCoA plots (Fig. 2) reflected features of geographic distribution of species. The Asian species mainly fell to different quadrants from American species, indicating that genetic materials have inherited with unique molecular features to represent their identity in each of the three geographic regions since continental isolation. Our study suggested that two species from Eur were genetically closer to Asian species than American species (Fig. 2), consistently with previous studies (TrÖndle et al. 2010; Zecca et al. 2012). Fourteen accessions of *V. sylvestris* had relatively centralized distribution, but seven accessions of *V. vinifera* had relatively scattered distribution (Fig, 3a). This observation is also confirmed by the recent report (Emanuelli et al. 2013). This may indicate that high molecular variation has been maintained in the cultivated species by human selection and asexual propagation during domestication (Myles et al. 2011; Emanuelli et al. 2013).

Except for two varieties, *V. aestivalis var. argentifolia* and *V. aestivalis var. lincecumii*, that were closely related to each other, all remaining varieties were differentiated from each other and from their original species (Fig. 2d). Thus, classification for sub-species or varieties should be carefully treated, because genotypes in *Vitis* were genetically highly differentiated from each other. High differentiation among accessions within species can be frequently observed (Fig. 2b, SL3 in Table 5).

The overall patterns in the PCoA plots (Fig. 2) representing genetic relationship between accessions and between species in this study are very similar to those in our previous study estimated by other algorithms (Wan et al. 2013).

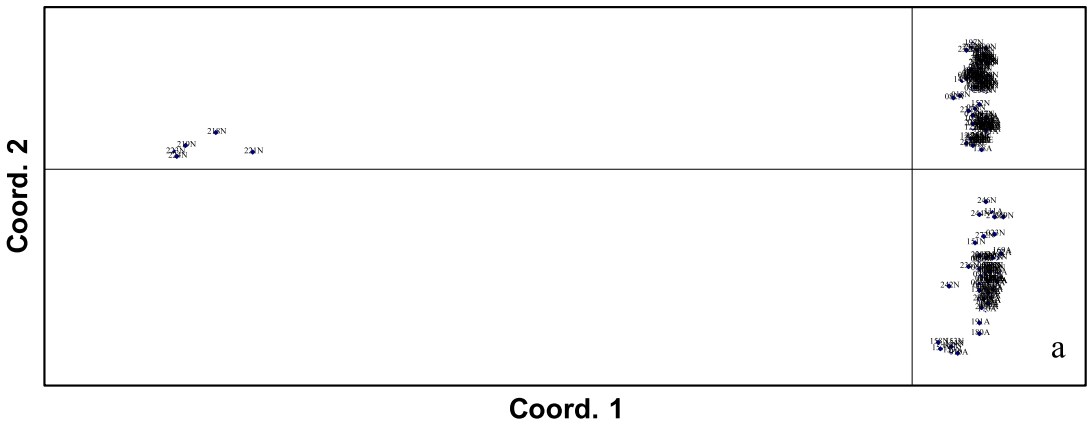
Comprehensive evaluation of genetic diversity in *Vitis*

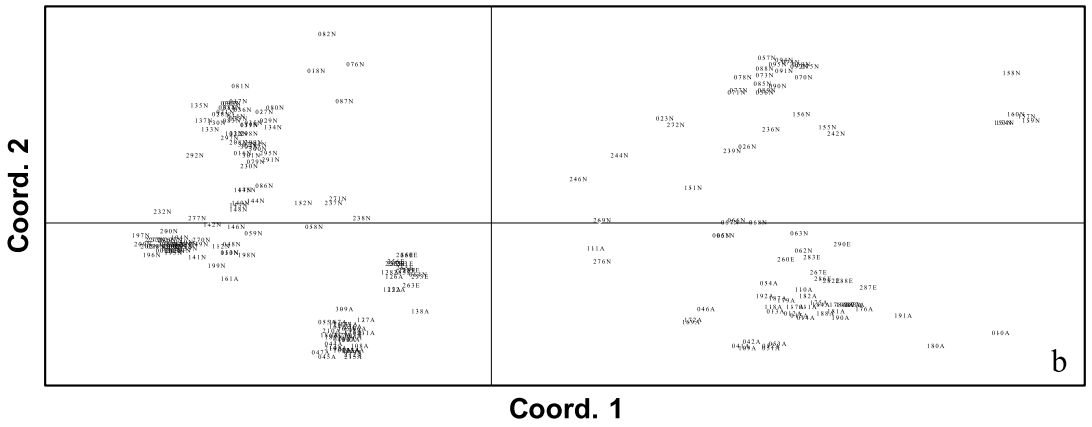
Molecular polymorphism varied among genes and among regions within the gene in this study (Table S20). This may result from distinct conserved extent among genes and among regions within the gene (Lande 1988; Mace and Purvis 2008). Thus, choice of molecular markers is critical to assessment of genetic patterns of species. For example, nucleotide diversity in *Picea abies* estimated in one study (Heuertz et al. 2006) was twofold relative to a different study (Chen et al. 2010). This discrepancy may result from marker choice and sampling bias. An increase of number of accessions (≥3 accessions) per species may have little influence on assessment of molecular variance within species if there has been appropriate sampling. However, number of species (≥6 species) is very important for a precise assessment of genetic features within higher-level population structures (Fitzpatrick 2009). Linkage disequilibrium estimates suggested that the 30 gene fragments used in this study were genetically independent overall. We selected 30 gene fragments comprising moderate polymorphisms for evaluation of genetic diversity in *Vitis*, assuming that estimation based on these 30 gene fragments could represent the molecular make-up of a random sampling of a coding region.

Fig. 2 The molecular genetic structure in *Vitis* based on Nei's algorithms and PCoA methodology. **a** An accession-by-accession plot for the 248 accessions of the *Vitis* genus, the up-left five accessions were *V. rotundiforlia*, and the remaining 243 accessions on the right were from *Euvitis*; **b** an accession-by-accession plot for the 243 accessions of the *Euvitis* sub-genus, these accessions were mainly clustered into seven major groups; **c** a species-by-species plot for the 48 species of the *Vitis* genus, the left one species was *V. rotundiforlia*, and the remaining 47 species were from *Euvitis*; **d** a species-by-species plot for the 47 species of the *Euvitis* sub-genus. Comparisons between the accession-by-accession plot and the species-by-species plot support the conclusion that genetic patterns of the accessions and species were consistently and correctly discerned. Another advantage of the PCoA method is that extent of genetic differentiation among accessions or species can be easily visually identified

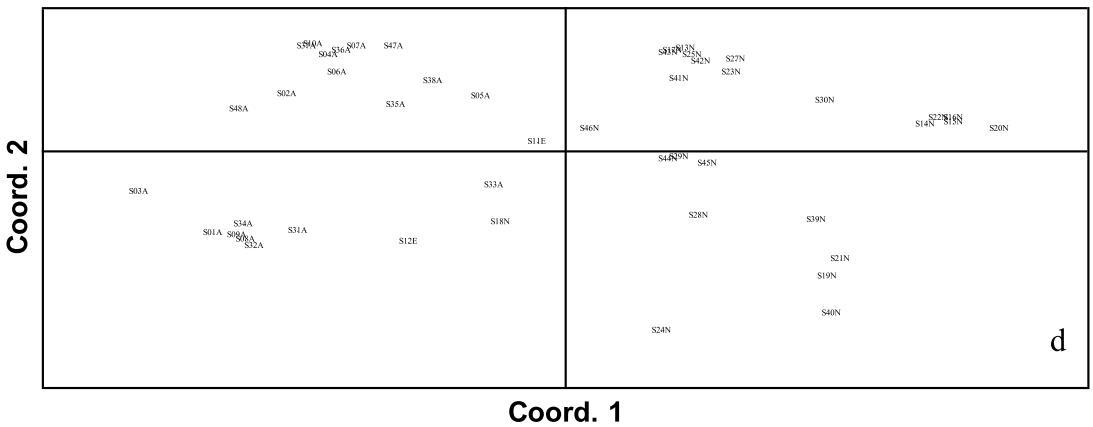
There are numerous molecular indices (e.g., five basic indices in this study) for assessment of genetic diversity. Their values were significantly related to each other based on correlation analysis between pairs of five indices ($P<0.05$), indicating that these molecular indices may clearly discern patterns of genetic diversity in *Vitis* species. Nucleotide diversity of the two sub-species *V. vinifera* and *V. sylvestris* estimated in this study was higher than a previous report (Riahia et al. 2013). However, gene diversity of these two sub-species was lower than Riahia et al. (2013), suggesting estimation of gene diversity is affected by sample number (number of accessions per species). Previous studies suggested use of nucleotide diversity for estimation of genetic diversity of species (Liu and Burke 2006; Song et al. 2009; Chen et al. 2010; Branca et al. 2011; Riahia et al. 2013). However, our estimates of gene diversity and heterozygosity were based on the same formula in Arlequin (Excoffier et al. 2005). Thus, estimates of gene diversity represent extent of heterozygosity of *Vitis* species in this study. Compared to reports of other species based on nuclear sequencing (Table S65), nucleotide diversity of 80 % of *Vitis* species was relatively high. Numbers of segregating sites, numbers of haplotypes, and mean numbers of pairwise differences were equal to or higher than their expected estimators in *Vitis* in most cases (Table 2, Table S30-S32). Genetic diversity in *V. bashanica*, *V. piasezkii var. pagnuccii*, *V. acerifolia*, *V. adstricta*, *V. davidii*, *V. quinquangularis* and *V. labrusca* was low compared to the relative high diversity in *V. shuttleworthii*, *V. tiliifolia*, *V. cinerea var. floridana*, *V. cinerea var. helleri* and *V. vinifera* (Table S30).

The species presenting high genetic diversity may not contribute proportionally to the genetic diversity of the genus. For example, *V. rotundifolia* had moderate genetic diversity and *V. tiliifolia* possessed relatively high genetic diversity compared to the remaining species in our study (Table S30). But contribution of *V. rotundifolia* to the genetic diversity of the genus was much higher than that of *V. tiliifolia* (Table S66 and


Coord. 2
Coord. 1
a


Coord. 2
Coord. 1
b


Coord. 2
Coord. 1
c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d

S67). The reason for this was that a species possessing high genetic diversity may not be highly genetically differentiated from other species (Rao and Hodgkin 2002).

A molecular basis contributing to potentials broad adaptability in *Vitis*

Our data demonstrated that nucleotide diversity, numbers of segregating sites, and mean numbers of pairwise differences were relatively high in most *Vitis* species. However, most SNPs were at very low frequencies among 248 accessions (Table S48). Percentage of segregating sites in *Vitis* was relatively high, i.e., one segregating site was found in every 3 to 8 bp among 248 accessions within the genus and one in every 45 to 70 bp among accessions within most of species. Thus, *Vitis* species may be inherited with genetic characteristics of maintenance of 'subtle' nucleotide variation, and a high percentage of segregating sites, accumulating these subtle variations as to engender high genetic diversity in most species and high genetic differentiation among species. This molecular basis confers potential broad adaptability of *Vitis* species. Broad geographical distribution in this genus and extensive distinct ecological heterogeneity among species (Reisch and Pratt 1996; Alleweldt 1997) requires high genetic differentiation among species and high genetic diversity in the genus to provide potential broad ecological adaptability of this genus (Mayr 1997; Yokoyama 2002; Orr 2005; Nowak 2006).

Comprehensive interpretation of genetic diversity and genetic differentiation in *Vitis* species

Nucleotide diversity within a species represents genetic variability of the species (Excoffier et al. 2005). The wild *Vitis* species of the subgenus are out-crossing. This habit (natural hybridization) in *Vitis* may facilitate genetic exchange among species, thus increase their genetic diversity, which in turn may improve their fitness and confer their potential for increased adaptability (López-Pujol et al. 2012). High nucleotide diversity (Table S9) and high CV value of nucleotide diversity (Table S53) in most *Vitis* species suggested high genetic variability in these species. Our previous study confirmed broad hybridization among species in the evolutionary history of *Vitis* (Wan et al. 2013). In this case, the phylogenetic species concept may be unsuitable as suggested by López-Pujol et al. (2012). Our phylogenetic study demonstrated that the consensus phylogenetic tree in *Vitis* was impossible to obtain from numerous phylogenetic algorithms (Wan et al. 2013).

Extensive genetic drift may lead to a decline of genetic variability on a large scale, e.g., decrease of genetic differentiation among species (Kekkonen et al. 2011). However, both nucleotide diversity (Table S9) within and genetic differentiation (Fig. 1) among most of the *Vitis* species were high compared to other plant species (Table S65). *Vitis* species inherently showed characteristics of low frequency of most SNPs (Table S48) and a high percentage of segregating sites (Table 4), which together conferred high nucleotide diversity within species. High SNP variation derived from a high percentage of segregating sites at different loci among species can result in high genetic differentiation among *Vitis* species.

Strategies for germplasm conservation and improvement

Our study showed that every accession represents a unique genotype (Fig. 2b) due to highly genetic differentiation among accessions within species. This provides broad potential for improvement of the *V. vinifera* crop using novel germplasm. Given high morphological variation among species and high genetic diversity within *Vitis*, it is important to collect all species within a broad array of ecological, morphological and genetic ranges. However, an exhaustive collection is not feasible because germplasm collection and conservation are very costly for woody plants (Alleweldt 1997; Pavek et al. 2003). Previous studies demonstrated that biotic and abiotic resistance varies among species as well as among accessions within species in *Vitis* (Wan et al. 2007). Thus, certain accessions should be given priority for germplasm collection and conservation (Pavek et al. 2003).

High genetic diversity in taxa does not imply a low threat to the survival of species. Previous studies reported high genetic diversity in certain highly endangered species, e.g., in whorled sunflower (*Helianthus verticillatus*), legume species (*Amorpha georgiana*), giant panda (*Ailuropoda melanoleuca*) and Australian corroboree frogs (*Pseudophryne corroboree* and *Pseudophryne pengilleyi*) (Ellis et al. 2006; Morgan et al. 2008; Straub and Doyle 2009; Zhao et al. 2013). The reason for this phenomenon may be that most molecular studies tend to estimate genetic diversity within the *status quo* of the species rather than adopting a more dynamic approach to reflect changing trends within an evolutionary perspective (Mace and Purvis 2008; Pauls et al. 2013). However, ecological habit is often an important factor in influencing the survival of a species (Mace and Purvis 2008). A narrow and isolated geographical distribution may impose risk of extinction due to restricted gene flow resulting in low genetic heterozygosity thus low adaptability to changing environmental conditions (Ellis et al. 2006). Reed and Frankham (2003) reported a significant positive correlation between heterozygosity (gene diversity) and fitness in a meta-analysis of 34 plant and animal species. Lande (1988) argued that demographic factors may have more impact on population persistence than genetic factors. A number of studies proved that small-range species were more threatened by changing environments (Hering et al. 2009;

Morueta-Holme et al. 2010; Pauls et al. 2013). Thus, both molecular factors (particularly potential of variability) and ecological habits of species should be comprehensively considered when planning germplasm conservation (Ellis et al. 2006; Mace and Purvis 2008; Pauls et al. 2013). Although the ecological diversity and geographical distribution of the flora of the whole genus of *Vitis* are broad, natural distribution of most species is limited within a mono or a few ecological regions (Reisch and Pratt 1996; Alleweldt 1997; Pavek et al. 2003; Wan et al. 2008). Considering their low genetic diversity (Table S9), low heterozygosity (Table S10), and narrow geographical distribution, these species, *V. bashanica*, *V. piasezkii* var. *pagnuccii*, *V. acerifolia*, *V. adstricta*, *V. davidii*, *V. quinquangularis*, should be given priority for collection and conservation. In addition to consideration of species-wide levels of diversity, extent of the partitioning of genetic variation and genetic differentiation between species is also important when designing a strategy for germplasm conservation, especially if not all species can be protected (Ellis et al. 2006). The species relatively highly distinct from other species in the PCoA plot, e.g., *V. flexuosa*, *V. davidii var. cyanocarpa*, *Vitis palmate*, *Vitis vulpine* and *Vitis treleasei* (Fig. 2d), may represent special genetic identities, thus these species should be highlighted for conservation. Although most species possess relatively high genetic diversity, collection and conservation of these species cannot be delayed because of increasing threats from human activity and climate changes (Pavek et al. 2003; Wan et al. 2008; Pauls et al. 2013). Collection and conservation of species from China should be emphasized because increase of human population and rapid expansion of industrialization present tremendous threats to the survival of these Chinese wild flora (Wan et al. 2008).

Grapevine is a perennial woody plant and thus it requires a relatively high cost for biodiversity conservation compared to herbaceous species (Pavek et al. 2003). Thus, a "core collection" strategy was postulated several decades ago for conservation of woody crops in order to conserve land and reduce costs (Pavek et al. 2003). However, no practical and feasible criteria and agreements have been decided on for a core collection of *Vitis* species due to extensive morphological diversity and high levels of interspecific hybridization in most species (Reisch and Pratt 1996; Alleweldt 1997). A number of species-specific SNPs were discovered and the patterns of relationships were clearly presented in this study. These results will provide basic tools for taxonomy, and germplasm conservation and development. Natural hybridization has been shown to commonly occur among species in *Vitis* (Wan et al. 2013), species unique SNPs detected in this study can be useful to classify accessions or discover whether accessions are "hybrid" or derived from several species. These SNPs can also be useful to help assemble a sample suitable for a "core collection" as a potential reference for germplasm collection, conservation, and breeding programs.

Conclusion

Although the grapevine is an economically important crop and use of wild species for improvement of the cultivated species is widely applied throughout the world (Reisch and Pratt 1996; Alleweldt 1997; Wan et al. 2008), comprehensive evaluation of genetic features in this genus using a large number of SNPs has not yet been conducted. This is first study using a large number of (6862) SNPs and a number of methods and estimates including estimation of genetic diversity, AMOVA, CV, and PCoA to comprehensively characterize genetic features among the hierarchical levels of *Vitis* species. Our data indicated that genetic diversity in most *Vitis* species is relatively high based on a comparison with that reported for other woody species. A molecular basis for high genetic diversity and genetic differentiation that together confer potential adaptability in *Vitis* was postulated in this study. Patterns in the PCoA plot represent genetic relationships, extent of differentiation of species and molecular identities for their origins from three geographic regions. Based on a comprehensive consideration of genetic features and ecological habits of *Vitis* species, the species with relatively low genetic diversity, low heterozygosity, and narrow geographic distribution should be given high priority for conservation. Given the ease of interspecific hybridization among most *Vitis* species and importance of novel agronomic traits for germplasm development (Reisch and Pratt 1996; Alleweldt 1997; Wan et al. 2008), the accessions with high agronomical values including resistance to multiple stresses and representing the genetic uniqueness inherent to the species should also be highlighted for conservation. Determination of species-specific SNPs in this study facilitates our identification of a "core collection" and detection of parentage in the grape breeding program. The strategy of vegetative propagation in this crop will facilitate the wide applicability of these markers in germplasm conservation and grape breeding programs. This study not only greatly adds to our knowledge of genetic features and molecular bases for ecological habits of *Vitis* species, but also is critical to conservation strategies and molecular tools for grape breeding programs.

Acknowledgments We are grateful to Dr. Joanne A. Labate for making valuable comments and suggestions about this study, to Angela M. Baldo for designing primers for resequencing, to Dr. Peter Cousins (E. &. J. Gallo Winery) for providing expert advice on grape identification and classification, and to Dr. Gan-Yuan Zhong for valuable discussion. USDA provided equal contribution to this study. This work was supported by the National Yangling Bio-Tech Breeding Centre Project (2001-01), China Scholarship Council Project (22861057), the Young Scientist Foundation

of NWAFU (QN2009-013), the 2012-Fund for Returnees Scientists from Foreign Study in Shaanxi Province (A289021201) and the USDA-ARS Plant Genetic Resources Unit CRIS Project 1910-21000-020-00D. USDA is an equal opportunity provider and employer.

Data archiving statement All sequences were released in NCBI with NCBI accession numbers: [Genbank: JX952227-JX960379, EMBL: HF544510-HF544512]

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