Genetic Structure, Differentiation, and Phylogeny of the Genus *Vitis*: Implications for Genetic Conservation

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Abstract
Examination of phylogenetic patterns within *Vitis* 18 microsatellite markers and AFLPs revealed many affinities differing somewhat from the traditional taxonomic classifications based on morphological criteria. East Asian species from China exhibited significant divergence from the North American group, but some aligned with American species/series. Partitioning of molecular variation suggested a significant amount of total variation (~74%) is accounted for by differences among genotypes within species as compared to among species within series (~17%) and among series within the genus *Vitis* (~9%). Although significant gene flow was evident at all levels of classification there was reasonable differentiation among species and series. The results suggest that *Vitis* represents a complex gene pool within which the interplay of local selection, introgression, drift, and reticulate evolution has played a significant role in shaping genetic structure and phylogeny.

INTRODUCTION
The genus *Vitis* L. (Vitaceae) is typically divided into two subgenera, *Vitis* (2n=6x=38) comprising all but two of the total of about 70 species described in the genus and *Muscadinia* Planch. (2n=6x=40), including two species, *V. rotundifolia* Michx., and *V. popenoei* J.H. Fennel. *Vitis* is a tertiary disjunct with distributions in North America and Eurasia. The biogeographic history of *Vitis* suggests that it occurred widely throughout the Northern Hemisphere during much of the Tertiary (Axelrod, 1966), including the Miocene floras of the western United States (Chaney and Axelrod, 1959), eastern Asia (Chaney and Hu, 1940), and central Europe (Szafer, 1952). *Vitis* is phylogenetically a complex group and much of its taxonomy is solely based on morphological criteria and is riddled with difficulties in establishing the identities of different species due to widespread introgression and clinal variation masking the taxonomic boundaries. The existing classification schemes (Planchon, 1887; Munson, 1909; Bailey, 1934; Comeaux, 1984) do not agree with each other in the circumscription and delineation of species and their relationships (Barrett et al., 1969).

The utility of biochemical and molecular markers in the analyses of species relationships in *Vitis* has not been explored. Transportability of genetic markers across species and their wild relatives allows for efficient evaluation of diversity and relationships within and among gene pools. We have employed population approaches to elucidate the phylogenetic relationships within the genus *Vitis* based on molecular markers.

MATERIALS AND METHODS
Two hundred seventy-three accessions representing 52 species with both Old and New World distributions, including the cultivated *V. vinifera* L. and its putative progenitor, subsp. *sylvestris* (C.C. Gmel.) Hegi, and two species of *Muscadinia*, were analyzed for phylogenetic relationships using polymorphisms at 18 microsatellite loci and 45 AFLP primer-pairs. The standard CTAB method (Doyle and Doyle, 1987) was used for DNA isolation and PCR protocols were according to Dangl et al. (2001) for microsatellites and Aradhya et al. (2004) for AFLPs. Amplified products were resolved...
using capillary electrophoresis on an ABI Prism 3100 genetic analyzer with the data collection software, version 1.2 (PE/Applied Biosystems). The data were further analyzed using Genescan, Version 3.1 and Genotyper, Version 2.5 and data were assembled as microsatellite genotypes as well as in binary format.

The microsatellite and AFLP binary data were combined into a single data matrix to compute a standard distance matrix based on the proportion of shared bands between two accessions for all possible pair-wise combinations. The resultant matrix was subjected to a cluster analysis using the Minimum Evolution (ME) method with the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) for searching an optimum tree. Bootstrap interior branch test (Dopazo, 1994) was used to test the reliability of each interior branch on the tree. Multilocus SSR genotypes were pooled into groups based on the currently recognized series within Vitis (Comeaux, 1984) and analyzed using the Analysis of MOlecular VAriance (AMOVA) as implemented in the software package ARLEQUIN version 3.01 (Excoffier et al., 2005). The total variance was partitioned into variation within and among species and series. The variance components from AMOVA were used to estimate the population subdivisions (Φ statistics) within and among species and series. Contingency χ² analysis was performed to determine the heterogeneity among species and series before performing AMOVA.

RESULTS

Evolutionary history of the genus Vitis inferred from the ME tree (Fig. 1) revealed several affinities within the New World group roughly corresponding to the Series recognized on morphological criteria. As expected the two subgenera exhibited a major divergence with Muscadinia forming a well-supported distinct basal sister group (C1) to subgenus Vitis (C2). Vitis formed three major clusters with two representing the New World species (C2.1 and C2.2) and one exclusively containing the Old World species (C2.3). Two well-supported subclusters are evident within the Old World group; one (C2.3.1) with the cultivated Eurasian grape V. vinifera subsp. vinifera and its wild progenitor, subsp. sylvestris along with a West Asia species, V. jacquemontii R. Parker as a sister group, and another entirely composed of the East Asian species from China (C2.3.2). The two major clusters within the New World group exhibited many affinities at species level and some even representing varieties.

Series Labruscae Planch., appeared to show differentiation between V. labrusca L. and its sister species V. mutangensis Buckley and V. shuttleworthii House, which formed a close and well-supported cluster within C2.1. Similarly, members of Series Precoces Munson, V. riparia Michx. and V. rupestris Scheele occupied cluster C2.1, while a closely associate species, V. acerifolia Raf., was placed in cluster C2.2. Except for V. cinerea (Engelm.) Engl. ex Millard var. cinerea and V. tilifolia Humb. & Bonpl. ex Schult., which occupied cluster C2.1, all other members of Series Cinerascenes Planch., V. cinerea var. berlandieri Planch., V. cinerea var. floridana Munson, V. biflora Rose, and V. peninsularis M.E Jones are scattered within cluster C2.2. In the case of Series Aestivales Planch., except for V. aestivalis Michx. var. aestivalis which stayed in cluster C2.1, all other members, mostly varieties within V. aestivalis Michx., V. aestivalis Michx. var. lincecumii (Buckley) Munson, V. aestivalis var. glauca (Munson) L.H. Bailey, V. aestivalis var. smalliana (L.H. Bailey) Comeaux, and V. nesbittiana Comeaux occupied cluster C2.2. All the members of the series Occidentales Munson, V. californica Benth., V. girdiana Munson, V. treleasei Munson ex L.H. Bailey, V. monticola Buckley, and V. bloodworthiana Comeaux are found clustered together in C2.2. Members of the series Vulpinae Comeaux, V. vulpina L. and V. palmata Vahl are found within cluster C2.1.

Among the Chinese species, V. ficifolia Bunge, V. pseudoreticulata W.T. Wang, and V. pisaezkii Maxim. are well differentiated and the remaining species do not show definite affinities within and between them. Vitis thunbergii Siebold & Zucc., native to Southeast Asia, and variants of V. pseudoreticulata and V. amurensis Rupe. and three unknown species, probably from East Asia showed affinity with the New World species by clustering together within C2.2.
Hierarchical partitioning of molecular variation (AMOVA) suggested that a significant amount of the total variation (~74%) is accounted for by inherent differences among genotypes within species, while smaller portions of molecular variation were partitioned for genetic differences among species within series (~17%) and among series within the genus (~9%) (Table 1). Fixation indices $\Phi_{ST}$, $\Phi_{SC}$, and $\Phi_{CT}$, which reflect the correlations between uniting gametes sampled randomly from different series, from different species within series and from within species were 0.2561, 0.1847, and 0.0876 and estimation of gene flow based on F-statistics were 0.7262, 1.1032, and 2.6200, respectively.

**DISCUSSION**

**Biogeography**

*Vitis* is a Tertiary disjunct with modern distribution restricted to two discontinuous geographic areas, Eurasia and North America (Axelrod, 1966; Chaney and Hu, 1940). Fossil members of *Vitis* date back to the Early Eocene and the Miocene Epoch and have been discovered in many parts of China, Europe, Japan, Greenland, Alaska and other parts of North America (Kirchheimer, 1939; Galet, 2000), suggesting early diversification and widespread distribution of the genus across the Northern Hemisphere during the Paleocene (Graham, 2007), including North America (Chaney and Axelrod, 1959), eastern Asia (Chaney and Hu, 1940) and central Europe (Szafer, 1952). Climatic fluctuations and geographic changes during the Late Tertiary and Quaternary periods resulted in range reduction and widespread extinction of *Vitis* in the northern latitudes pushing the distribution limit southward, leaving members of the genus isolated in North America and Eurasia. Further southward migration in Eurasia coupled with the Pleistocene glaciations effectively eliminated many species, leaving the Balkans and Caucasus as refugia (Axelrod, 1966; Graham, 1972).

The modern distribution and the biogeographic history of *Vitis* indicate that the extant species represent relatively recent evolutionary diversification, probably occurring during the Quaternary period in isolation in North America and Eurasia (Mullins et al., 1992). According to Negrul (1936) evolution and domestication of the cultivated grape, *V. vinifera* subsp. *vinifera* from its wild ancestor, subsp. *sylvestris* probably happened sometime during the Early Holocene in the Transcaucasian region between the Black Sea and Caspian Sea, where wild grape and spontaneous transitional forms of *V. vinifera* coexist in nature. However, fossil records (Turner, 1968) and archaeological evidence (Renfrew, 1973) suggest that the wild grape was distributed throughout Pleistocene Europe and probably became extinct locally in northern Europe during glaciations.

**Genetic Relationships within *Vitis***

The evolutionary history inferred through molecular marker analysis confirmed two well-supported major clades corresponding to the two subgenera, *Muscadinia* and *Vitis*, which can be easily distinguished on morphological, anatomical, and cytological differences (Mullins et al., 1992) and breeding barriers that restrict gene flow between them. The monophyly of the genus *Vitis* is well-supported. The placement of subgenus *Muscadinia* sister to subgenus *Vitis*, together with the presence of a fossil species, *V. ludwigii* closely resembling the seed characteristics of *Muscadinia* and *V. teutonica* similar to modern *Vitis* in the Tertiary sediments of Northern Europe (Kirchheimer, 1939) suggest early divergence dating back to the Tertiary period. The finding of *Muscadinia*-like fossils in northwestern Europe also perhaps suggest that it once was widely distributed in northeastern America connecting to Europe across a land bridge, but perhaps became extinct during the Late Tertiary and Quaternary glaciations leaving behind a present day distribution restricted to southeastern United States and Mexico.

The study also supports several relationships within and between the Old World and New World species of the subgenus *Vitis*, but it also indicated many problems concerning taxonomic delimitation and placement of some species within and between
clades as encountered in earlier studies (Moore and Giannasi, 1987, 1994; Comeaux et al., 1987). Within the subgenus \textit{Vitis}, the Old World species have clearly diverged from the New World group indicating that these two groups have evolved in isolation. Although the long history of ecological isolation resulted in significant differentiation, it did not bring about any significant breeding barriers. Interspecific hybrids within and between these groups are fully fertile and produce normally segregating progenies. The phylogenetic resolution within and between the Old or New World groups is poor and series as described in the current classification systems are not supported. However, the identity of many species at species and even variety level is evident, suggesting the existence of taxonomically and genetically definable units with some exceptions. Earlier studies on the taxonomic delimitation of species and phylogenetic relationships within North American \textit{Vitis} using foliar flavonoids have reported a complex evolutionary scenario involving parallel flavonoid evolution within each series thus masking subgeneric and interserial distinctions (Moore and Giannasi, 1987, 1994). However, Comeaux et al. (1987), studying the native grapes of North Carolina, concluded that phenological and ecological differences between species promote reproductive isolation between species within subgenus \textit{Vitis}.

Molecular Variation and Gene Flow within and among Species and Series

The significant amount of total variation within species as compared to between species within series and between series within the subgenus suggests considerable gene flow across all taxonomic levels. This observation is further substantiated by the lack of breeding barriers among species with overlapping ecological distributions in the Eastern North America, and perhaps in East Asia, leading to extensive gene flow overriding the factors responsible for genetic differentiation in their natural ranges. Overall, the dynamic interplay of evolutionary forces combined with the impacts of anthropogenic causes interfering with the natural evolutionary processes play a complex role in shaping the phylogenetic structure within \textit{Vitis}. Unlike other plant groups, subgenus \textit{Vitis} is unique in the sense that species both from the Old and New World freely interbreed without any genetic barriers, circulating the genetic diversity within and between species constituting a complex primary gene pool.

Our study permits for some generalizations regarding genetic identity and relationships within and between species and series:

• Monophyly of the genus is established and the two subgenera, \textit{Vitis} and \textit{Muscadinia} are fully resolved into monophyletic lineages.

• Molecular analysis has revealed affinities reasonably supporting the taxonomic delimitations at species and variety levels, but support of series is not evident among the New World \textit{Vitis}.

• The two well-supported clades within New World \textit{Vitis} indicate the existence of two distinct genetic groups, which may deserve some taxonomic reconsideration and the subclades within the major clades may suggest significant substructuring.

• Within series \textit{Labruscae}, \textit{V. labrusca} shows significant differentiation from its closely associate species, \textit{V. mustangensis} and \textit{V. shuttleworthii}.

• Within series \textit{Preoces}, \textit{V. acerifolia} exhibits significant divergence from its two sister species, \textit{V. riparia} and \textit{V. rupestris}.

• Members of series \textit{Cinerascentes} show low to moderate levels of differentiation with \textit{V. cinerea} var. \textit{cinerea} showing significant divergence and closer affinity with members of series \textit{Labruscae}.

• Members of series \textit{Occidentales} exhibit low to moderate differentiation except for \textit{V. bloodworthiana}, which shows sister relationship with the \textit{Cinerascentes-Aestivales} group.

• Members of series \textit{Aestivales}, show affinity with most members of series \textit{Cinerascentes} and these two series need fundamental reclassification.

• The genetic and taxonomic identity of members of series \textit{Vulpinae}, \textit{V. palmata} and \textit{V. vulpina} needs further investigation with larger sampling.
In general, the East Asian *Vitis* are genetically distinct from the North American species, but a few individuals of *V. amurensis*, *V. thunbergii*, and *V. pseudoreticulata* did show affinity with the North American species. Overall, the genetic relationships within East Asia species need re-examination with larger sampling.

Ecological and phenological isolation mechanisms may play a role in reproductive isolation among *Vitis* species, and the results suggest significant gene flow within and between species and series.

Natural selection and drift, combined with gene flow within and between species and series seem to have played a significant role in shaping phylogenetic structure within the genus *Vitis*.

**Literature Cited**


Munson, T.V. 1909. Foundations of American grape culture. T.V. Munson and Son,
Denison, Texas. 252 p.

Tables

Table 1. Hierarchical partitioning of molecular variation.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
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<td>Among groups</td>
<td>13</td>
<td>258.933</td>
<td>0.22922**</td>
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<td>Among populations within groups</td>
<td>46</td>
<td>294.104</td>
<td>0.44116**</td>
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<td>Within populations</td>
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<td>1219.224</td>
<td>1.94764**</td>
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<tr>
<td><strong>Total</strong></td>
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<td>1772.261</td>
<td>2.61803</td>
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</tr>
</tbody>
</table>

Fixation Indices (see text for explanation)

$\Phi_{ST} = 0.25607^{**}$ \hspace{1cm} $Nm = 0.7262$
$\Phi_{SC} = 0.18468^{**}$ \hspace{1cm} $Nm = 1.1032$
$\Phi_{CT} = 0.08756^{**}$ \hspace{1cm} $Nm = 2.6200$

$Nm = \text{Number of migrants per generation}$

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Fig. 1. A condensed minimum evolution tree based on bootstrap interior branch test showing branches that are supported at the cutoff confidence probability value ≥ 95.