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Molecular phylogeny of the genus *Vitis* (Vitaceae) based on plastid markers[1]

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- *Premise of the study*: This work represents the first molecular phylogeny of the economically important genus *Vitis*, an important genetic resource for breeding in grapevine, *Vitis vinifera*.
- *Methods*: A molecular phylogeny of *Vitis* using a combined data set of three noncoding regions of the plastid DNA genome was constructed from 47 accessions covering 30 species of *Vitis.* The data for the *trnL-F* marker were combined with previously published data across the Vitaceae.
- *Key results*: The molecular phylogeny demonstrated monophyly of the genus *Vitis*. Based on the combined analysis of three genes, *Vitis* is split into three clades that mirror the continental distribution of these accessions. The diversity is highest in the Asian clade, but the general genetic distances across taxa from different continents are relatively small.
- *Conclusions*: The findings support a relatively recent and intense gene flow between East Asia and North America and the possible impact of hybridization on the evolution of the genus *Vitis.* Taxon identity in important stock collections should be screened carefully because roughly 10% of the accessions analyzed in the present study had been misidentified.

Key words: molecular systematics; phylogeny; *trnL-F*; Vitaceae; *Vitis*; *Vitis vinifera.*

Grapevine, *Vitis vinifera* L. subsp. *vinifera*, is not only among the most ancient crop plants, but it also has exerted considerable impact on the formation of human civilization in the Near East and the Mediterranean areas. According to the Organisation International de la Vigne et du Vin (OIV, 2009) at present, around 8 million hectares worldwide are cultivated with grapevine, yielding more than 200 million liters of wine. Due to the high price per yield ratio, viticulture contributes considerably to the economy of the corresponding regions. The ancestral species, *Vitis vinifera* L. subsp. *sylvestris* (C. C. Gmel.) Hegi represents the only taxon of this genus that naturally occurs in Europe. In contrast, numerous species of *Vitis* are indigenous to North America and East Asia. Although these wild species are only peripherally used for human consumption, they are of great economic importance as a gene pool for resistance breeding and as rootstocks for the highly susceptible *Vitis vinifera* L. subsp. *vinifera*. Many taxa of this genus have been described independently such that some 140 synonyms are used for the presently recognized 65 (USDA, 2010) to 70 species (Soejima and Wen, 2006). The numerous synonyms seem to be one reason that the evolutionary relationships within the genus *Vitis* have not been elucidated to date. However, wild species of *Vitis* are of tremendous importance for grapevine breeding. For instance, wild species of *Vitis* differ qualitatively in their response to pathogens such as false downy mildew (Jürges et al., 2009), and these differences have been used to develop environmentally friendly and sustainable strategies of plant protection such as resistance breeding or specific interference with host–pathogen signaling.

Most *Vitis* species inhabit the temperate regions, usually humid forests, where they grow as lianae on supporting trees (Zohary, 1996). Arid regions form biogeographical barriers for *Vitis*, and these barriers may have confined autochthonous taxa of *Vitis* to the northern hemisphere (which, however, cannot account for the distribution of *Vitis tiliifolia* Humb. & Bonpl. ex Schult. in Central and South America). Typically, *Vitis* species produce sweet berries that are conspicuously colored by anthocyanins such that they are efficiently propagated through birds, often over large distances (Martin et al., 1961). Most wild species of this genus are dioecious (Levadoux, 1956). The nectaries of the female flowers are strongly reduced and have little reward for a potential pollinator and no conspicuous attraction. It is therefore assumed that pollination occurs mainly through wind and, considering the ecological niche typical for *Vitis*, this secondary anemophily is efficient only over relatively short distances (Olmo, 1996).

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This poses the question of how founder populations can be established from individual seeds that have been transported by birds. There is one genetic locus, *Su*, with three alleles, which determine gender in *Vitis*: Su^m (suppressor of staminal development, recessive), Su^F (suppressor of carpellate development, dominant), and Su^+ (hermaphrodite development, recessive to Su^F, but dominant to Su^m). By loss of function of Su^F, even in individual flowers, the ancestral hermaphrodite state is restored (Olmo, 1996). Hermaphrodism is readily achieved by alterations of hormonal balance as shown by conversions of male into hermaphrodite flowers by cytokinins (*V. vinifera* subsp. *sylvestris* Negi and Olmo, 1966; *V. thunbergii* Izuka, 1967). Upon cross fertilization with plants properly expressing the Su^F locus, such convertants can produce dioecious offspring that segregate into male and female plants. Such sexual conversions seem to be essential during pioneering situations, when a population is established from few individuals that have been introduced by birds (Negi and Olmo, 1971).

All *Vitis* species are diploid ($2N = 38$), and many species produce viable, fertile hybrids upon artificial crossing. There is also evidence for interspecies hybrids that occur in natural sympatric populations (Olmo, 1996) although the frequency of such hybridization events seems to be constrained by microgeographic and ecological barriers (Zohary, 1996). Molecular phylogenies based on the entire plastid genome place the Vitaceae into the earliest diverging lineage of rosids (Jansen et al., 2006). Consistent with this early divergence, the fossil records demonstrate that the genus *Vitis* was distributed all over the northern hemisphere, whereby the centers of diversity for this genus have shifted several times between the continents. However, one has to bear in mind that the discrimination between *Vitis* and other Vitaceae is only reliable in fossils where seed remnants could be recovered (Kirchheimer, 1938). Despite this caveat, there is convincing evidence for a rich and differentiated *Vitis* flora in Europe prior to the Pleistocene, whereas the genus had apparently almost gone extinct by the end of the last glaciation about 10 000 yr ago (Kirchheimer, 1938). Thus, the European wild grape, *Vitis vinifera* subsp. *sylvestris*, the ancestor of cultivated grapevine, originates from a very small population that later spread over a relatively large area ranging from Spain and North Africa in the west, over the Central European rivers and beyond the Caucasus in the east. The domestication of the grapevine has been traditionally sited at Transcaucasia, based on archaeological evidence and the largest genetic diversity. However, phylogenetic analysis focusing on the two taxa subsp. *sylvestris* and subsp. *vinifera* and using plastid DNA markers suggests at least two origins for cultivated grapevine, one in the Near East and a second in the Iberian Peninsula (Arroyo-García et al., 2006).

The molecular phylogeny of the Vitaceae has been studied on the family level (*rbcL*: Ingrouille et al., 2002; *trnL-F, atpB-rbcL* and *rps16*: Soejima and Wen, 2006; complete plastid genome: Jansen et al., 2006), and on the subspecies level for *Vitis vinifera* using a set of simple sequence repeat (SSR) markers (Arroya-García et al., 2006). However, a molecular study on the genus level is still lacking for *Vitis*. Based on the economic impact of wild grape species as rootstocks or genetic resources, the phylogenetic relationships between these species are of more than merely academic relevance. We constructed a molecular phylogeny based on plastid markers as a first step to understand the evolution of these important wild relatives of the crop plant *Vitis vinifera* subsp. *vinifera*.

MATERIALS AND METHODS

Plant material—We used fresh leaf material from plants in the living collection of the Botanical Garden of the Karlsruhe Institute of Technology. These accessions were photographically documented, then redetermined using morphological keys and ampelographic descriptors of the Organization Internationale de Vigne et du Vin (OIV, 2010) with the help of Dr. Erika Maul (Julius-Kühn-Institute Institute for Grape Breeding Geilweilerhof), Dr. Jun Wen (Smithsonian Institution, Washington), and Prof. Dr. Claire Arnold (Neuf Châtel, Switzerland). All accessions are maintained as living specimens at the Botanical Garden of the Karlsruhe Institute of Technology. Herbarium vouchers are deposited at the herbarium of the Botanical Garden Heidelberg (HEID) and the Botanical Garden of the Karlsruhe Institute of Technology. From each accession, the fourth and, if necessary due to size, fifth leaf from top was used for DNA extraction. Our study involved 47 accessions from 30 species of the genus *Vitis*. For the first analysis, an accession from the same family, *Rhoicissus rhomboidea* (*Cissus rhombifolia*), was used as the outgroup. When several accessions turned out to be not of the genus *Vitis* but of *Ampelopsis*, these accessions were used as outgroups (Figs. 1, 2). Of the 30 *Vitis* species, 11 species originated from Asia, two from Eurasia (the only species native to Eurasia), including eight cultivars, as well as one unidentified accession from Pakistan, 15 species from North America, and one from Mesoamerica. For voucher information, provenance and synonyms, see Appendix 1. Additional DNA sequence data from the *trnL-F* region were obtained from GenBank (Soejima and Wen 2006; Worberg et al., 2007; Rossetto et al., 2007).

DNA isolation, PCR, purification, and sequencing—For each accession, two to three independent DNA isolates were obtained to check for sequencing errors. Fresh leaf material was shock frozen in liquid nitrogen and homogenized. Genomic and plastid DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hildesheim, Germany) according to the manufacturer's instructions with slight modifications. Quality and quantity of the extracted DNA yield was verified using a Spectrophotometer (NanoDrop, Peqlab, Erlangen, Germany). We amplified four noncoding marker regions of the plastid DNA: *trnL* intron, *trnL-F* IGS, and both parts of the *trnK* intron flanking the *matK* region using the primers specified in Table 1. Nuclear ribosomal internal transcribed spacer (ITS) (Baldwin, 1992) was not suited for *Vitis* because of a second pseudogene. For PCR, we used a proofreading *Taq* polymerase (ExTaq, TaKaRa, Shiga, Japan). PCR was conducted as follows: 96°C for 5 min; 36 cycles consisting of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and synthesis at 72°C for 2 min; final elongation at 72°C for 4 min. The enzyme was added in the "hot-start" mode, i.e., after the initial denaturation to maintain its proofreading function. The amplicons were separated and verified by agarose gel electrophoresis and labeling with CybrSafe (Invitrogen, Karlsruhe, Germany).

DNA alignment and analysis—All nucleotide positions were manually verified from the chromatograms, and the single sequences were separately aligned with the program Clustal_X (Larkin et al., 2007). The aligned data sets were manually edited using the program MEGA version 4.0b (Kumar et al., 2008). All four single sequences of each accession were combined, creating a contiguous sequence alignment shown in Appendix S2 (see Supplemental Data with the online version of this article). Indels were coded by a binary matrix appended to the alignment. A minimal evolutionary tree based on neighbor-joining (NJ) was calculated with the program PAUP* version 4.0b10 (Swofford, 2002) based on the nucleotide substitution model of Hasegawa, Kishino, and Yano (HKY) and gamma distribution. Bootstrap analyses were run with 1000 replicates, using tree-bisection-reconnection (TBR) branch swapping, Multrees in effect, and Maxtrees set to 100. Parsimony analyses were run also with PAUP* version 4.0b10. Maximum-parsimony analysis was conducted to reconstruct a strict consensus tree, using a heuristic search strategy with TBR branch swapping and random addition of taxa, collapse option, and Multrees option. The number of replicate searches was 10, and the number of trees held for each step search was 1.

Bayesian analyses were performed with the program MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). Prior and likelihood settings were defined using MrModeltest version 3.7 (Nylander, 2004). Each analysis started with random trees and employed four Markov chain Monte Carlo runs, covering 2 000 000 generations per chain, sampling trees every 1000th generation. This analysis was repeated twice to verify the results. Negative log likelihood values were plotted against number of generations to define the point were the log likelihood values had stabilized. All trees following this burn-in point were used to calculate a consensus tree including posterior clade probabilities.

TABLE 1. Designations, sequences, and literature references for the oligonucleotide primers used to amplify the plastidic marker sequences used in this study.

Name	Primer	Sequence	Reference
trnL intron	5′trnL^{UAA}F (C)	5′-CGA AAT CGG TAG ACG CTA CG-3′	Taberlet et al., 1991
	3′trnL^{UAA}R (D)	5′-GGG GAT AGA GGG ACT TGA AC-3′	Taberlet et al., 1991
trnL-F IGS	3′trnL^{UAA}F (E)	5′-GGT TCA AGT CCC TCT ATC CC-3′	Taberlet et al., 1991
	5′trnF^{GAA}R (F)	5′-ATT TGA ACT GGT GAC ACG AG-3′	Taberlet et al., 1991
trnK-matK intron	3′trnK^{UUU}R (matK1)	5′-AAC TAG TCG GAT GGA GTA G-3′	Samuel et al., 2005
	5′matK F (matK8F)	5′-TCG ACT TTC TTG TGC TAG AAC TTT-3′	Samuel et al., 2005
matK-trnK intron	3′matK R (matK5)	5′-TGT CAT AAC CTG CAT TTT CC-3′	Samuel et al., 2005
	5′trnK^{UUU}F (matK6)	5′-TGG GTT GCT AAC TCA ATG G-3′	Samuel et al., 2005

To compare our data with previous familywide analysis, we used the alignment for *trnL-F* sequence data as presented by Soejima and Wen (2006) and fitted our sequences into this alignment. This previous study comprised a *trnL* intron and *trnL-F* spacer matrix of 108 accessions with *Leea* and *Rhamnus* as outgroups (including also 10 *Vitis* accessions). We added additional four sequences from *Vitis* (Rossetto et al., 2007; Worberg et al., 2007). This matrix was expanded by our newly generated *trnL-F* sequences to examine phylogenetic relationships within the context of the whole family (shown in online Appendix S1). The *trnL-F* region has been used as a suitable marker to reconstruct family and higher-order phylogenetic relationships by Koch et al. (2007) and Borsch et al. (2007).

RESULTS

Alignments and DNA sequence data—The familywide *trnL-F* analysis was based on an alignment of 1343 nt (online Appendix S1) and also contained our *trnL* intron and *trnL-F* intergenic spacer (IGS) sequences (see below). This alignment was adjusted to that presented by Soejima and Wen (2006), containing 707 constant characters, 227 variable but parsimony uninformative characters, and 456 parsimony-informative characters (including also 48 gaps coded separately as a binary matrix).

In this study, we amplified three regions of the plastid *trnL* intron, the *trnL-F* IGS, and the *trnK* intron to construct a phylogenetic tree for the genus *Vitis*. The aligned contiguous sequence data matrix was 1265 nt in length, containing 77 single nucleotide polymorphisms (SNPs), 40 of which were parsimony informative (PI). Aligning required 27 gaps, representing indels 1 to 63 nt long (Table 2).

The *trnL* intron region was 494 nt long, included 12 SNPs (four of them PI) and three indels. The *trnL-F* IGS was 411 nt long, included 12 SNPs (six of them PI) and 10 indels. The *trnK* intron, assembled from two partial sequences, was in total 1260 (424 and 836) nt long, included 53 (22 and 31) SNPs (30 [13 and 17] of them PI) and 14 (10 and 4) indels. Table 2 provides an overview; the fully assembled *trnL-F* sequence alignment is shown in online Appendix S1.

Phylogenetic reconstructions—The strict consensus tree from the parsimony analysis based on the familywide *trnL-F* data set is shown in Fig. 1. Total tree length was 1205 steps, including character state changes from 48 gaps coded as binary 0/1 characters (see online Appendix S1). The consistency index (CI) was 0.748, the retention index (RI) was 0.900. Parsimony analysis, neighbor-joining analysis, and the Bayesian approach all provided very similar tree topologies (results not shown). We show the consensus tree from the parsimony analysis (Fig. 1) for direct comparisons with the parsimony analysis presented by Soejima and Wen (2006). In the first familywide analysis presented by Soejima and Wen (2006), they identified six main clades, and all of these were also highly supported in our analysis. They were termed C1 (comprising taxa of the genera *Ampelocissus*, *Pterisanthes*, *Nothocissus*), C2 (comprising taxa of the genus *Vitis*), A (with taxa of the genera *Ampelopsis*, *Rhoicissus*, and three accessions termed *Ampelocissus martinii 7410*, *Cissus striata 7355*, *and Cyphostemma bainesii*), D (with taxa of the genus *Cissus*), and B (with taxa of the genera *Parthenocissus* and *Yua*), and E (with taxa of the genera *Tetrastigma, Cayratia*, and *Cyphostemma*). All the *Vitis* accessions from the present study clustered into clade C2 with the other *Vitis* accessions from Soejima and Wen (2006).

Several accessions that had been determined as *Vitis* taxa were found during this study to be misidentified and based on their morphology were found to be members of the genus *Ampelopsis*. These were VBry (*Vitis bryonica* = *Ampelopsis* cf. *glandulosa*), VDav (*Vitis davidii* = *A. japonica*), VBld (*Vitis berlandieri* = *A. bodinieri*), and Vjap (*V. japonica* = *A.* cf. *japonica*). All of these *Ampelopsis* taxa as well as *Rhoicissus rhomboidea* (= *Cissus rhombifolia*) clustered into clade A together with the other *Ampelopsis* and *Rhoicissus* accessions.

In Fig. 2, the Bayesian inference tree for the *trnL* intron, *trnL-F* IGS, and *trnK* intron sequence data are shown, including the 30 accessions analyzed during the present study, applying the GTR+G model of sequence evolution and calculated from a burn-in of 200 000 generations onward, including posterior clade probabilities (PCP). This tree is congruent with clades C2 and A in Fig. 1, but discriminates plastid DNA relationships within the genus *Vitis* at higher resolution, such that three clades emerge, highly supported by PCPs of around 0.9:

Clade I contains most *V. vinifera* subsp. *sylvestris* haplotypes (Europe), most *V. vinifera* subsp. *vinifera* cultivar haplotypes (Europe), but also the North American haplotypes *V. labrusca* "K", *V. vulpina*, and *V. arizonica*. Genetic distances are very low. Most *V. vinifera* subsp. *sylvestris* accessions, *V. labrusca* "K", and *V. vulpina* show the same haplotype. Cultivar haplotypes differ by maximal 2 SNPs/indels from the *sylvestris* haplotypes.

Clade II contains most Asian haplotypes (*V. flexuosa*, *V. amurenis*, *V. betulifolia*, *V. coignetiae*, *V. jaquemontii*, *V. quinquangularis*, *V. ficifolia*, and *V. thunbergii*), as well as haplotypes of *V. cinerea* and *V. palmata* (both North American).

TABLE 2. Informational content of the sequence alignments used to construct the phylogeny of the genus *Vitis*.

Intron	Total characters[a]	Variable sites	Indels
trnL intron	494	12	3
trnL-F IGS	411	12	10
trnK-matK intron	424	22	10
matK-trnK intron	836	31	4
Assembled sequence	2165	77	27

[a] Including nucleotide sequence and indels

Genetic distances within clade 2 are much higher. *Vitis cinerea* and *V. flexuosa* share the same haplotype and are placed at the base. *Vitis amurensis* differs by one SNP and one indel. Close to *V. amurensis* is a group consisting of *V. betulifolia*, *V. coignetiae*, and *V. palmata* with 3 or 2 SNPs/indels difference from the base, respectively. *Vitis jaquemontii* and *V. quinquangularis* show the same haplotype differ by 3 SNPs/indels from the base. *Vitis ficifolia* and *V. thunbergii* show the same haplotype and differ by 6 SNPs/indels from the base.

Clade III is sister to the root of clades I and II. It contains most haplotypes from North America (*V. aestivalis* "F1" and "F2", *V. monticola*, *V. riparia*, *V. rupestris*, *V. cordifolia*, *V. trelasei*, *V. acerifolia*, *V. girdiana*, and *V. labrusca* "B"), the Meso and South American haplotype of *V. tiliifolia*, two haplotypes of North American grapevine cultivars, an unknown haplotype erroneously designated as *V. vinifera* subsp. *sylvestris* (*VsylUS*), and a *V. yenshanensis* haplotype (Asia). The genetic distances are heterogeneous. *Vitis monticola*, *V riparia*, *V. aestivalis* "F2", *V. tiliifolia*, the Asian accession *V. yenshanensis*, the accession *VsylUS*, and the cultivar Solaris (result of a complex series of backcrosses between different grapevine cultivars and different wild grape species from North America, and

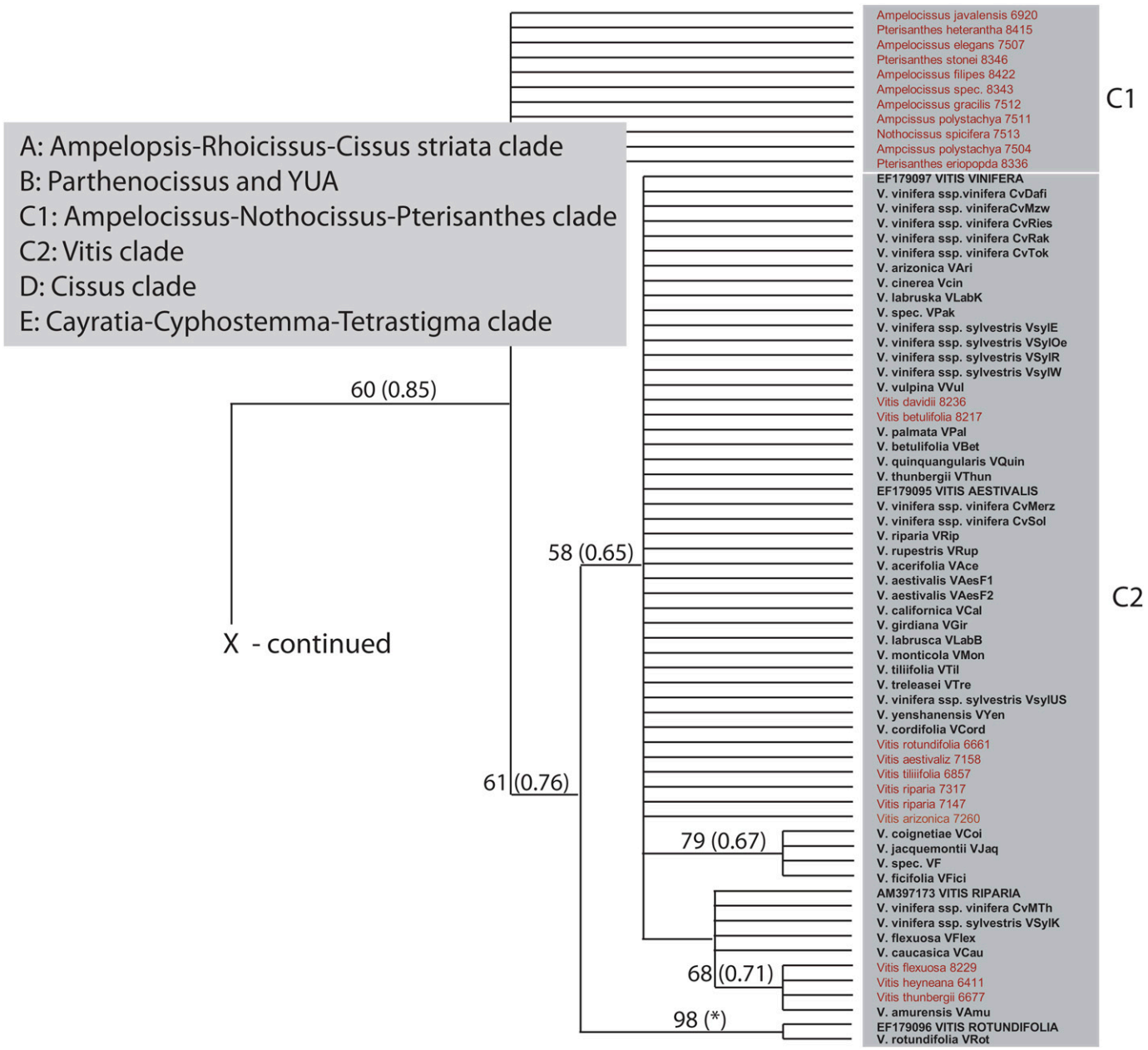

Fig. 1. Strict consensus tree from 10 000 most parsimonious trees based on the assembled *trnL-F* sequence data for the 30 species of the genera *Vitis*, *Ampelopsis*, and *Rhoicissus rhomboidea* analyzed in the present study, merged with data of Soejima and Wen (2006), and supplemented by data of Worberg et al. (2007) and Rossetto et al. (2007). The total alignment is given in online Appendix S1. Bootstrap support from 1000 replicates is indicated along the branches (posterior values from the corresponding Bayesian analysis are provided in brackets; with: * = 1.00; -: node not recognized). Definition of clades A–E follows Soejima and Wen (2006). Details on the taxa are given in Appendix 1.

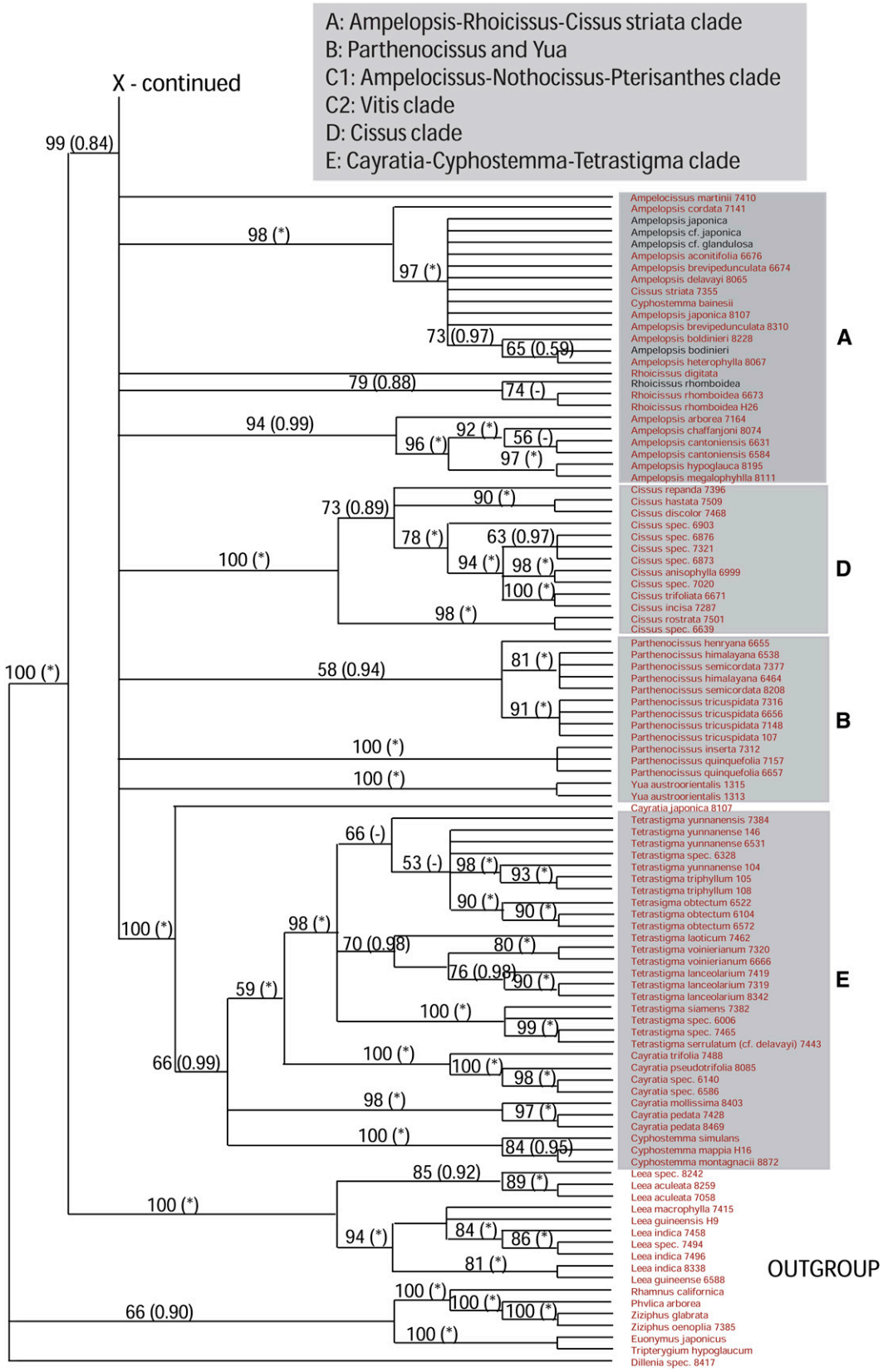

Fig. 1. Continued.

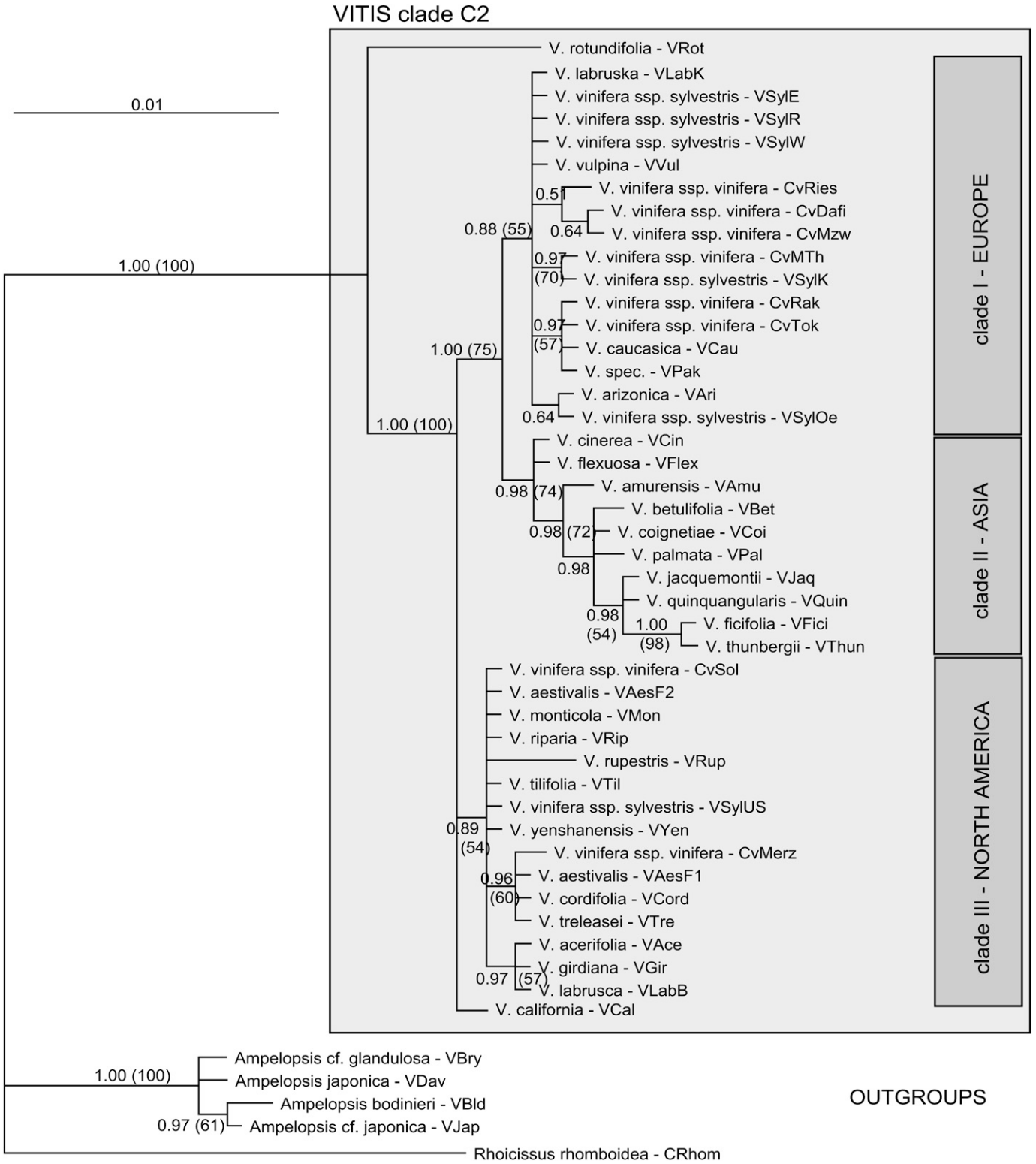

Fig. 2. Bayesian inference consensus tree for the assembled sequences (*trnL* intron, *trnL-F* IGS, *trnK* intron), including posterior clade probabilities, for 30 species of the genus *Vitis*, with *Ampelopsis* species and *Rhoicissus rhomboidea* as outgroups (Bootstrap values from the corresponding maximum parsimony analysis are provided in brackets when >95%.). The whole *Vitis* clade (C2) is also indicated in Fig. 1. The corresponding alignment is given in online Appendix S2. Details on the taxa are given in Appendix 1.

V. amurensis) share the same haplotype, positioned basal at the clade. *Vitis rupestris* stands alone, with greater distance. There are two other groups within the clade: *V. aestivalis* "F1", *V. cordifolia*, and *V. trelasei* share one haplotype, differing by one SNP from the cultivar Merzling (again result of a complex series of backcrosses between different grapevine cultivars and different wild grape species from North America). *Vitis acerifolia*, *V. girdiana*, and *V. labrusca* "B" belong to a different haplotype, differing by oneSNP from the basis. *Vitis californica* (North America) stands at a polytomy, but the neighbor-joining analysis indicates a weak sister relationship with clade 3, and *V. rotundifolia* (North America) stands as sister to the joint root of all three clades.

DISCUSSION

In the present study, we constructed the first molecular phylogeny of the economically important genus *Vitis* that is of tremendous importance as genetic resource for grapevine breeding, especially in the context of resistance to pathogens such as *Plasmopara viticola* (Jürges et al., 2009). We used three plastid DNA regions (two introns, one IGS) of 48 accessions comprising 30 *Vitis* species and several cultivars of *V. vinifera*. These data were fitted into the familywide analysis by Soejima and Wen (2006) and confirm that the genus *Vitis* is monophyletic (Fig. 1). Within the *Vitis* clade, we can discriminate three clades, reflecting the geographical distribution of *Vitis* species (Fig. 2): Clade I is dominated by European haplotypes, clade 2 by Asian haplotypes, and clade III by North American haplotypes. However, within each continental clade, haplotypes from other continents are interspersed, indicating relationships across continents. A further limitation is the fact that two of the three clades are not well supported, because the genetic distances within clade I (European haplotypes) and clade III (American haplotypes) and between those clades are relatively low (by maximally 2 SNPs/indels). In contrast, clade II (Asian haplotypes) diverges more (up to 6 SNPs/indels).

Vitis rotundifolia is sister to clades I–III. Within the genus *Vitis*, two subgenera are widely recognized: subg. (*Eu-*)*Vitis* and subg. *Muscadinia*. Whereas most *Vitis* species belong to subg. *Vitis*, *V. rotundifolia* is member of subg. *Muscadinia*. *Vitis rotundifolia* is widely distributed in North America, from Indiana to Texas. It is sympatric with several species of subg. *Vitis*, but at least in this accession there were no indications of hybridization. *Vitis californica* stands at a polytomy with a weak affinity to clade III. It occurs along the west coast of the USA (Oregon and California) and is sympatric with *V. girdiana* in California. Yet, there is a distinct genetic distance between the accessions of *V. californica*, *V. girdiana*, and all other North American accessions in this study, suggesting long-term genetic separation of the various species.

Clade I, Europe—The *V. vinifera* subsp. *vinifera* cultivars form small subgroups that are genetically very close to each other. 'Riesling' is an ancient German grape variety from the lower Rhine region. 'Müller-Thurgau' was bred at the end of the 19th century (Dettweiler et al., 2000), 'Damenfinger', 'Rkatsiteli' and 'Mzwane' are ancient varieties from Georgia. 'Tokajer' is an old and famous Hungarian variety. Most wild accessions of *V. vinifera*, subsp. *sylvestris* "E" (from Alsace, France), "R" and "W" (from autochthonous populations in the upper Rhine region, Germany), stand basal to the clade, with their sequenced plastid DNA regions being identical. Exceptions are *V. vinifera* subsp. *sylvestris* "K" (from a recent population in the upper Rhine region, Germany), and "Oe" (from the Danube region, Austria), *V. vinifera* subsp. *caucasica* (from the Caucasus) and an unidentified wild *Vitis* accession from Pakistan, that all are grouped with the cultivated grape accessions. Leaf shape and seed morphology place these accessions close to cultivated grapevine, indicating that they might be hybrids between autochthonous and cultivated vines (most residual habitats of European wild grapes are situated in regions, where viticulture is common). Because European accessions belong to the same species, *V. vinifera*, genetic distances between these haplotypes are expected to be low. In addition to the European accessions, three haplotypes from North America are also in clade I. *Vitis labrusca* "K" and *V. vulpina* (syn. *V. cordifolia*) have haplotypes identical to the basal subsp. *sylvestris* accessions, and *V. arizonica* is identical to subsp. *sylvestris* "Oe". Leaf morphology of this accession differs from that reported in the literature in which *V. arizonica* is depicted with heart-shaped, dark-green leaves that are coarsely toothed. In our case, however, the leaves are lobed and more delicate with some similarity to leaves of cultivated grapevine. In contrast, the accessions of *V. labrusca* "K" and *V. vulpina* are morphologically congruent with the literature descriptions. Whether these three haplotypes from America have been misidentified (which might be the case in *V. arizonica*) or whether they represent hybrids between autochthonous American species of *Vitis* and anthopogenically introduced grapevine cultivars remains to be elucidated using nuclear markers. Hybridization events should be considered, because these species come from regions where viticulture is common. Although cultivars of *Vitis* are generally thought to be not competitive enough to survive outside a vineyard, there have been reports that hybrids between wild and cultivated grapevines are often morphologically close to their wild ancestors and very successfully invade wild populations (Olmo and Koyama, 1980).

Clade II, Asia—Clade II comprises mostly accessions from Asia with *V. flexuosa* (syn. *V. indica*, *V. parvifolia*, *V. wallichii*), found in East Asia, India, and Indochina being close to the base. The other members of this clade stand in progressive genetic distance (that parallels geographical distance), with highly supported branches. *Vitis amurensis* (East Siberia, China), *V. betulifolia* (China), and *V. coignetiae* (East Siberia, East Asia) form a group. A second, more derived group is formed by *V. jaquemontii* (India) and *V. quinquangularis* (syn. *V. heyneana*, *V. kiusiana*, *V. pentagona*) from East Asia, India, and Indochina. Both accessions share the same haplotype, although the morphology is quite distinct, especially with respect to leaf shape. Another strongly derived group is formed by *V. ficifolia* and *V. thunbergii.* Both taxa are listed as synonyms (Wan et al., 2008) for *V. kaempferi* (Ohwi, 1965), occur in East Asia, and share one single haplotype. However, their leaf morphologies differ distinctly. Thus, these taxa are sympatric and genetically very close, but morphologically often quite different. It remains to be elucidated to what extent this correlation between genetic similarity and morphological diversity is caused by interspecies hybridization. Two accessions from North America fall into clade II: *Vitis cinerea* stands basal to the clade, having the same haplotype as the Asian *V. flexuosa*; and *V. palmate*, which is close to the Asian species *V. betulifolia* and *V. coignetiae* in the phylogenetic trees. Again, whether these accessions originated from Asian populations of

Vitis populations and have been introduced to North America either by anthropogenic or by natural dispersal has to be tested. In case of the *V. palmata* accession, the leaves are ovate-elliptic, and almost unlobed, which contrasts with the deep lobing reported for this species and places this accession also morphologically close to *V. betulifolia*. Again, this poses the question, whether these American haplotypes represent either misidentified Asian neophytes (*V. palmata*) or whether they resulted from hybridization events (*V. cinerea*). Generally, genetic diversity within main clade 1 is maximal in clade 2 (Asia). This indicates Asia as a center of a rapid, possibly recent, diversification.

Clade III, America—Clade III consists mainly of North American haplotypes. Basal to the clade sharing the same haplotype are *V. aestivalis* "F2" (syn. *V. rufotomentosa*), occurring in eastern Canada and the United States; *V. monticola*, indigenous only to Texas; *V. riparia*, with the largest distribution of all American *Vitis* species over Canada and eastern United States; and *V. tiliifolia* (syn. *V. caribbea*), occurring in Mexico, Mesoamerica, the Caribbean and western South America. Two groups can be distinguished within the remaining taxa of clade III. The first group implies the identical haplotype of *V. aestivalis* "F1" (clearly differing from *V. aestivalis* "F2", also in leaf morphology), *V. cordifolia* and *V. trelasei*. *Vitis cordifolia* is considered synonymous to *V. vulpine*, which fell into clade 1, indicating that the *V. vulpina* accession in this study might have arisen from a hybridization between autochthonous American species and a *Vitis* cultivar, whereas the *V. cordifolia* haplotype might represent the indigenous wild form. *Vitis trelasei* occurs only in three southern states of the United States. The identical haplotype of these three accessions indicates that they still maintain gene flow.

The second group comprises *V. acerifolia* (syn. *V. longii*), occurring in northwestern and central United States; *V. girdiana*, occurring only in North Mexico; and *V. labrusca* "B", distributed throughout eastern United States. These three accessions have the same haplotype, hinting at gene flow between them. *Vitis labrusca* "B" falls into the American clade, in contrast to *V. labrusca* "K" that nests within the European clade and possibly arose from hybridization with cultivated grapevine. *Vitis labrusca* "B", also because of its morphology, most likely represents an autochthonous haplotype unaffected by hybridization with *Vitis* cultivars. However, both accessions are very similar with respect to leaf morphology. The only haplotype in clear distance to all other haplotypes is *V. rupestris*, distributed over eastern and central United States. This haplotype seems to be clearly delineated from the other *Vitis* species. Two *Vitis vinifera* subsp. *vinifera* cultivars are located within the American clade. Both cultivars are results of complex backcrosses with wild *Vitis* species from North America to breed for pathogen resistance, such as grapevine downy mildew (*Plasmopara viticola*). For instance, the resistant cultivar Merzling derives from crosses with *V. rupestris* and *V. lincecumii* (a synonym of *V. aestivalis* var. *lincecumii*), explaining why the Merzling haplotype is close to *V. aestivalis* "F1". The cultivar Solaris descends from Merzling and is located near the base of the American clade. As in clades I and II, there are accessions from other continents found basal to the American clade III. The accession for *V. yenshanensis* originates from China and is morphologically clearly *V. yenshanensis*, but shares the same haplotype with American *Vitis* species as well as the accession designated *V. vinifera* subsp. *sylvestris* "US". This accession was collected in the United States, but is described as *V. vinifera* subsp. *sylvestris* by its habit (e.g., its deeply lobed leaves) and is morphologically close to European grapevine. The most straightforward explanation is that it represents a hybrid form between an American wild *Vitis* species and an introduced European cultivar. Generally, similar to the other two clades, most accessions (with exception of *V. rupestris*) within the American clade have only three very closely related haplotypes. This points to migration as mirrored by the maternally inherited plastid genome between continents, but also within America, which might be explained by the north–south orientation of the mountains, that allowed for free and rapid migration during glaciation and deglaciation cycles.

Several accessions (Vbry, Vdav, VBld, and Vjap) originally identified in germplasm centers as members of the genus *Vitis* did not cluster into the main clade C2 (Fig. 1), which would lead to the (false) conclusion that *Vitis* is polyphyletic. Based on their morphology, they could be later identified as members of the genus *Ampelopsis*. This redetermination was then confirmed by their position in the familywide *trnL-F* analysis, where they were positioned in clade A together with the other *Ampelopsis* accessions. These accessions, obtained from public collections, emphasize the need for careful redetermination of accessions even if they have been obtained from widely used germplasm centers. This caveat is especially important in case of taxa that are located at key cladistic positions.

In conclusion, our phylogenetic analysis of a combined data set of three plastid DNA markers of 47 accessions representing 30 *Vitis* species allowed us to reconstruct a phylogeny of the genus *Vitis*. The phylogenetic trees demonstrated monophyly of this genus and a separation into three clades that mostly mirror geographic distribution of the taxa. However, two of these clades are not well supported. For the few cases in which single accessions from other continents clustered into the clade of a different continent, the morphology indicates either recent hybridization between cultivated grapevine and autochthonous species or discrepancies with the traits described in the literature indicating misidentification. In the familywide analysis by Soejima and Wen (2006), 79 species representing 12 genera of Vitaceae had been sequenced for the *trnL-F* spacer, 37 of which were subsequently sequenced for the *atpB-rbcL* spacer and the *rps16* intron. These authors cooncluded that *Vitis* is monophyletic. In an earlier study of the *rbcL* marker for 20 species of the Vitaceae, Ingrouille et al. (2002) concluded that the genus *Vitis* is paraphyletic; however, they used only three species (*V. aestivalis, V. vinifera*, and *V. rotundifolia*). Our findings confirm the conclusions by Soejima and Wen (2006) — the genus *Vitis* is monophyletic.

In contrast to previous studies, the present work was on the genus level, and we included additionally (30) species of the genus *Vitis* into our analysis. Due to this higher coverage, we were able to discriminate three clades within the genus that basically can be assigned to individual continents. The genetic diversity within the Asian clade is high, but it is low within North America and Europe with suggestions of hybridization between cultivated grapevine and autochthonous accessions. In the terminology of Ernst Mayr (1963), *Vitis* would be in an early stage of allopatric speciation, where genetic barriers are not yet established. However, there seem to be reproductive barriers between *Vitis* and *Ampelopsis* because they are represented by well-supported evolutionary lineages. Similar to previous studies, the present study was based on plastid markers, which do not allow assessments of hybridization. Such assessments await data from nuclear markers.

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APPENDIX 1. Abbreviations and voucher numbers (*Vitis* collection of the Botanical Garden of the University of Karlsruhe), geographic distribution, and synonyms for the taxa used in this study are given. All accessions are kept as living plants in the Botanical Garden of the University of Karlsruhe, and vouchers have been deposited at the herbarium at the Botanical Garden Heidelberg.

Taxon abbreviation - Voucher numbers - *Taxon* - Geographic distribution - Synonyms.

CRhom - 001-004 - *Rhoicissus rhomboidea* - South America (Mexico [Chiapas], Panama, Trinidad and Tobago [Trinidad], French Guiana, Guyana, Venezuela, Bolivia, Colombia, Ecuador, Peru) - Syn.: *Cissus alata* Jacq., *Vitis rhombifolia* (Vahl) Baker, *Cissus rhombifolia* Vahl.

CvDafi - 005-008 - *Vitis vinifera* L. subsp. *vinifera* cv. Damenfinger - Georgia. **CvMerz** - 009-012 - *Vitis vinifera* L. subsp. *vinifera* cv. Merzling - Germany. **CvMTh** - 013-016 - *Vitis vinifera* L. subsp. *vinifera* cv. Mueller-Thurgau - Germany. **CvMzw** - 017-020 - *Vitis vinifera* L. subsp. *vinifera* cv. Mzwane - Georgia. **CvRies** - 021-024 - *Vitis vinifera* L. subsp. *vinifera* cv. Riesling - Germany. **CvRak** - 025-028 - *Vitis vinifera* L. subsp. *vinifera* cv. Rkatsiteli - Georgia. **CvSol** - 029-032 - *Vitis vinifera* L. subsp. *vinifera* cv. Solaris - Germany. **CvTok** - 033-036 - *Vitis vinifera* L. subsp. *vinifera* cv. Harslevelu/Tokajer (Hungary). Syn.: *Vitis vinifera* L.

VAce - 037-040 - *Vitis acerifolia* Raf. - North America (USA [Kansas, Oklahoma, Colorado, New Mexico, Texas]) - Syn.: *Vitis longii* W. Prince, *Vitis longii* var. *microsperma* (Munson) L. H. Bailey, *Vitis solonis* var. *microsperma* Munson.

VAesF1 - 041-044, **VAesF2** - 045-048 - *Vitis aestivalis* Michx. - North America (Canada [Ontario], USA [Connecticut, Indiana, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Ohio, Pennsylvania, Rhode Island, Vermont, West Virginia, Illinois, Iowa, Kansas, Minnesota, Missouri, Nebraska, Oklahoma, Wisconsin, Alabama, Delaware, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, Texas]) - Syn.: *Vitis rufotomentosa* Small.

VAmu - 049-052 - *Vitis amurensis* Rupr. - Asia (Russia [Amur, Primorye], China [Anhui, Hebei, Heilongjiang, Jilin, Liaoning, Shandong, Shanxi, Zhejiang], Japan [Honshu], Korea).

VAri - 053-056 - *Vitis arizonica* Engelm. - North America (USA [New Mexico, Texas, Arizona, Utah], Mexico [Sonora, Tamaulipas]) - Syn.: *Vitis arizonica* var. *glabra* Munson.

VBld - 057-060 - *Vitis berlandieri* Planch. - North America (USA [Texas]) - Syn.: *Vitis cinerea* (Engelm.) Engelm. ex Millardet var. *helleri* (L. H. Bailey) M. O. Moore. Redetermined as *Ampelopsis bodinieri*.

VBet - 061-064 - *Vitis betulifolia* Diels & Gilg - Asia (China [Gansu, Henan, Hubei, Hunan, Shaanxi, Sichuan, Yunnan]).

VBry - 065-068 - *Vitis bryonifolia* Bunge - Asia. Redetermined as *Ampelopsis cf. glandulosa.*

VCal - 069-072 - *Vitis californica* Benth. - North America (USA [Oregon, California]).

VCau - 073-076 - *Vitis caucasica* - Europe (Upper Rhine, Switzerland, Balkan peninsula) - Syn.: *Vitis vinifera caucasica*.

VCin - 077-080 - *Vitis cinerea* (Engelm.) Engelm. ex Millardet - North America (USA [Indiana, Ohio, Pennsylvania, West Virginia, Illinois, Iowa, Kansas, Missouri, Oklahoma, Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, Texas]).

VCoi - 081-084 - *Vitis coignetiae* Pulliat ex Planch. - Asia (Russia [Sakhalin], Japan [Hokkaido, Honshu, Shikoku], Korea).

VCord - 085-088 - *Vitis cordifolia* Michx. - North America (USA [Indiana, Massachusetts, Michigan, New Jersey, New York, Ohio, Pennsylvania, West Virginia, Illinois, Iowa, Kansas, Missouri, Nebraska, Oklahoma, South Dakota, Wisconsin, Alabama, Arkansas, Delaware, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, Texas]) - Syn.: *Vitis vulpina* L.

VDavB - 089-092 - *Vitis davidii* (Rom. Caill.) Foëx - Asia (China [Anhui, Fujian, Gansu, Guangdong, Guangxi, Guizhou, Hubei, Hunan, Jiangsu, Jiangxi, Shaanxi, Sichuan, Yunnan, Zhejiang]) - Syn.: *Vitis armata* Diels & Gilg, *Spinovitis davidii* Rom. Caill. Redetermined as *Ampelopsis japonica*.

VFici - 093-096 - *Vitis ficifolia* Bunge - Asia (China [Hebei, Henan, Jiangsu, Shaanxi, Shandong, Shanxi], Japan [Hokkaido, Honshu, Kyushu, Ryukyu Islands, Shikoku], Korea, Taiwan) - Syn.: *Vitis kaempferi* K. Koch, *Vitis thunbergii* Siebold & Zucc.

VFlex - 097-100 - *Vitis flexuosa* Thunb. - Asia (China [Anhui, Fujian, Gansu, Guangdong, Guangxi, Guizhou, Henan, Hubei, Hunan, Jiangsu, Jiangxi, Shaanxi, Shandong, Sichuan, Yunnan, Zhejiang], Japan [Hokkaido, Honshu, Kyushu, Ryukyu Islands, Shikoku], Korea, Taiwan, India [Assam, Himachal Pradesh, Jammu and Kashmir, Manipur, Uttar Pradesh, West Bengal], Nepal, Pakistan) - Syn.: *Vitis flexuosa* var. *parvifolia* (Roxb.) Gagnep., *Vitis indica* Thunb., *Vitis parvifolia* Roxb., *Vitis wallichii* DC.

VGir - 101-104 - *Vitis girdiana* Munson - North America (USA [California], Mexico [Baja California]).

VJaq - 105-108 - *Vitis jacquemontii* R. Parker - Asia (India [Himachal Pradesh, Uttar Pradesh], Nepal, Pakistan).

VJap - 109-112 - *Vitis japonica* Thunb. - Asia (China [Anhui, Fujian, Gansu, Guangdong, Guangxi, Guizhou, Hainan, Henan, Hubei, Hunan, Jiangsu, Jiangxi, Shaanxi, Shandong, Sichuan, Yunnan, Zhejiang], Japan [Hokkaido, Honshu, Kyushu, Ryukyu Islands, Shikoku], Korea, Taiwan, Bangladesh, Bhutan, India [Assam, Goa, Karnataka, Kerala, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, Tamil Nadu, Tripura, West Bengal, Andaman and Nicobar], Nepal, Cambodia, Myanmar, Thailand, Vietnam, Indonesia, Malaysia) - Australasia (Australia [Queensland]) - North America (USA, as neophyte) - Syn.: *Cayratia japonica* (Thunb.) Gagnep. Redetermined as *Ampelopsis* cf. *japonica*

VLabB - 113-116, **VLabK** - 117-120 - *Vitis labrusca* L. - North America (USA [Connecticut, Indiana, Maine, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Ohio, Pennsylvania, Rhode Island, Vermont, West Virginia, Illinois, Missouri, Alabama, Arkansas, Delaware, Georgia, Kentucky, Maryland, Mississippi, North Carolina, South Carolina, Tennessee, Virginia]).

VMon - 121-124 - *Vitis monticola* Buckley - North America (USA [Texas]).

VPal - 125-128 - *Vitis palmata* Vahl - North America (USA [Indiana, Illinois, Missouri, Oklahoma, Alabama, Arkansas, Florida, Kentucky, Louisiana, Mississippi, Tennessee, Texas]).

VQuin - 129-132 - *Vitis quinquangularis* Rehder - Asia (China [Anhui, Fujian, Gansu, Guangdong, Guangxi, Guizhou, Henan, Hubei, Hunan, Jiangxi, Shaanxi, Shandong, Shanxi, Sichuan, Xizang, Yunnan, Zhejiang], Japan [Kyushu], Bhutan, India [Andhra Pradesh, Assam, Himachal Pradesh, Meghalaya, Sikkim, West Bengal], Nepal, Myanmar - Syn.: *Vitis heyneana* Roem. & Schult., *Vitis kiusiana* Momiy., *Vitis pentagona* Diels & Gilg.

VRip - 133-136 - *Vitis riparia* Michx. - North America (Canada [New Brunswick, Nova Scotia, Ontario, Quebec, Manitoba, Saskatchewan], USA (Connecticut, Indiana, Maine, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Ohio, Pennsylvania, Rhode Island, Vermont, West Virginia, Illinois, Iowa, Kansas, Minnesota, Missouri, Nebraska, North Dakota, Oklahoma, South Dakota, Wisconsin, Alabama, Arkansas, Delaware, Kentucky, Louisiana, Maryland, Mississippi, Tennessee, Virginia, Texas]).

VRot - 137-140 - *Vitis rotundifolia* Michx. - North America (USA [Indiana, West Virginia, Missouri, Oklahoma, Alabama, Arkansas, Delaware, Florida, Georgia, Kentucky, Louisiana, Maryland, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, Texas]).

VRup - 141-144 - *Vitis rupestris* Scheele - North America (USA [Indiana, Pennsylvania, West Virginia, Missouri, Oklahoma, Arkansas, Kentucky, Maryland, Tennessee, Texas]).

VPak - 145-148 - *Vitis spec.* - Asia (Pakistan [wild-growing forest population]).

VThun - 149-152 - *Vitis thunbergii* Siebold & Zucc. - Asia (China [Hebei, Henan, Jiangsu, Shaanxi, Shandong, Shanxi], Japan [Hokkaido, Honshu, Kyushu, Ryukyu Islands, Shikoku], Korea, Taiwan) - Syn.: *Vitis ficifolia* Bunge, *Vitis kaempferi* K. Koch.

VTil - 153-156 - *Vitis tiliifolia* Humb. & Bonpl. ex Schult. - North America (Mexico) - South America (Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, Panama, Cuba, Dominican Republic, Guadeloupe, Haiti, Jamaica, Puerto Rico, Virgin Islands [St. Croix, St. John], Colombia, Ecuador) - Syn.: *Vitis caribaea* DC.

VTre -157-160 - *Vitis treleasei* Munson ex L. H. Bailey - North America (USA [New Mexico, Texas, Arizona]).

VSylE - 161-164, **VSylK** - 165-168, **VSylOe** - 169-172, **VSylR** - 173-176, **VSylUS** - 177-180, **VSylW** - 181-184 - *Vitis vinifera* L. subsp. *sylvestris* (C. C. Gmel.) Hegi - Africa (Algeria, Morocco, Tunisia) - Asia (Iran, Iraq, Israel, Syria, Turkey, Russia [Ciscaucasia, Dagestan], Turkmenistan, Armenia, Azerbaijan, Georgia) - Europe (Austria, Czechoslovakia, Germany, Hungary, Switzerland, Moldova, Ukraine [incl. Krym], Albania, Bulgaria, Greece, Italy [incl. Sardinia, Sicily], Romania, Yugoslavia, France [incl. Corsica]) - Syn.: *Vitis sylvestris* C. C. Gmel.

VVul - 185-188 - *Vitis vulpina* L. - North America (USA [Indiana, Massachusetts, Michigan, New Jersey, New York, Ohio, Pennsylvania, West Virginia, Illinois, Iowa, Kansas, Missouri, Nebraska, Oklahoma, South Dakota, Wisconsin, Alabama, Arkansas, Delaware, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, Texas]) - Syn.: *Vitis cordifolia* Michx.

VYen - 189-192 - *Vitis yenshanensis* - Asia.