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Tannin phenotyping of the Vitaceae reveals a phylogenetic linkage of epigallocatechin in berries and leaves

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- **Background and Aims** Condensed tannins, responsible for berry and wine astringency, may have been selected during grapevine domestication (Narduzzi *et al.*, 2015). This work examines the phylogenetic distribution of condensed tannins throughout the Vitaceae phylogenetic tree.
- **Methods** Green berries and mature leaves of representative true-to-type members of the Vitaceae were collected before “véraison”, freeze-dried, pulverised, and condensed tannins measured following depolymerization by nucleophilic addition of 2-mercaptoethanol to the C4 of the flavan-3-ol units in an organic acidic medium. Reaction products were separated and quantitated by UPLC/DAD/MS.
- **Key Results and Conclusions** The original ability to incorporate epigallocatechin (EGC) into grapevine condensed tannins was lost independently in both the American and Eurasian/Asian branches of the Vitaceae, with exceptional cases of reversion to the ancestral EGC phenotype. This is particularly true in the genus *Vitis*, where we now find two radically distinct groups differing with respect to EGC content. While *Vitis* species from Asia are void of EGC, 50% of the New World *Vitis* harbour EGC. Interestingly, the presence of EGC is tightly coupled with the degree of leaf margin serration. Noticeably, the rare Asian EGC-forming species are phylogenetically close to *Vitis vinifera*, the only remnant representative of *Vitis* in Eurasia. Both the wild ancestral *V. vinifera* subsp. *sylvestris* as well as the domesticated *V. vinifera* subsp. *sativa* can accumulate EGC and activate galloylation biosynthesis that compete for photoassimilates and reductive power.

Key words: Condensed tannins, epigallocatechin, phenotype, pericarp, leaf, Vitaceae, *Vitis vinifera*, Asian wild grapevines, American wild grapevines, leaf margin teeth, EGC⁺ vines, EGC⁻ vines.

INTRODUCTION

The Vitaceae Juss. (order Vitales) is a vast family of dicotyledonous flowering plants comprising 16 genera and some 950 known species (Wen *et al.*, 2018a), most of them growing in intertropical regions. Of peculiar economic importance is the genus *Vitis*, whose most emblematic member is the domesticated grapevine (*Vitis vinifera* subsp. *sativa*), whose berries, fermented to produce wines, have been consumed by humans for at least eight millenia (McGovern *et al.*, 2017). The phylogeny of Vitaceae is now approaching a consensus, due to constant improvements in the density of chloroplastic, mitochondrial and nuclear markers in the past decade (Péros *et al.*, 2011; Miller *et al.*, 2013; Aradhya *et al.*, 2013; Wan *et al.*, 2013; Liu *et al.*, 2016; Wen *et al.*, 2018b; Klein *et al.*, 2018; Fu *et al.*, 2019; Liang *et al.*, 2019; Ma *et al.*, 2018a, 2018b, 2020, 2021; Zecca *et al.*, 2020).

However, the implemented techniques of phylogenomics [*e.g.* high-throughput genotyping-by-sequencing (Klein *et al.*, 2018), RAD-seq (Ma *et al.*, 2020), and whole genome resequencing (Liang *et al.*, 2019)] are only as good as the identity of the specimens has been properly authenticated. Unfortunately, this is far from trivial in the Vitaceae leading to obvious misidentifications persisting in phylogenetic trees [*e.g.* *Ampelocissus erdvendbergiana* in Liu *et al.* (2016); *Vitis wilsoniae* and *V. flexuosa* placed by Wan *et al.* (2013) in a North American clade; *Vitis lanata* and *Vitis jacquemontii* which are actually a unique species, *Vitis heyneana* subsp. *heyneana*, but appear as two genetically close but separated taxa in Wen *et al.* (2018b)]. On top of that, the full interfertility between *Vitis* species poses a particular challenge to the taxonomy of this genus (Klein *et al.*, 2018; Ma *et al.*, 2020). These difficulties have led Wen *et al.* (2018b) to underline the need for an extended interdisciplinary approach, *sic* “integrative systematic with evidence from morphology, anatomy, phylogenomics, ecology, biogeography, fossils and bioinformatics”.

As contribution to this integrative approach, we use here chemotaxonomy of condensed tannins for the Vitaceae, an approach that has been successful previously in banana (Uclés Santos *et al.*, 2011).

In the Vitaceae, the edible part of the fruit, the pericarp, as well as the leaf contain condensed tannins, also called proanthocyanidins (thereafter abbreviated as PAs), that are polymers composed of 5 to >100 units of C4-C8 and C4-C6 linked flavanols, termed catechins (**Fig. 1**). Upon wounding and rupture of the plant tissue, PAs instantaneously interact with endogeneous and exogeneous proteins forming insoluble complexes in a kind of a healing reaction, and the astringency provided by PAs will discourage any animal with minimal cognitive ability from eating green berries.

Although several studies have investigated the diversity of the composition for condensed tannins (Monagas *et al.*, 2003; Huang *et al.*, 2012; Liang *et al.*, 2019; Koyama *et al.*, 2017; Kedrina-Okutan *et al.*, 2019), it is difficult to compile and compare them, because the techniques employed for extraction and depolymerisation differ. To overcome this limitation, our international network presents a vast phenotyping of condensed tannins in the family of Vitaceae (*ca* 250 accessions) with emphasis on the genus *Vitis*. To ensure comparability, all the analyses were performed by a single group of people based on identical and validated protocols for sample collection, preparation, and analysis (Brillouet *et al.*, 2017).

MATERIALS AND METHODS

Plant materials

The herbaceous tier was chosen for collecting berries because (i) PAs content/berry was at its maximum and their composition was stable until full maturity, (ii) PAs can only be analyzed by the mercaptoethanol technique (Brillouet *et al.*, 2017) in the absence of sugar inside the berries, and (iii) fresh, hard, and green berries at the herbaceous tier can withstand several

days at room temperature. Around ten healthy fruits from each accession were hand-picked at the herbaceous tier of the plant during the vegetational seasons of 2016-2020. Sampling represented different taxa of the family Vitaceae coming from America, Africa, East Asia and Europe. The genera, species, subspecies, accessions, and sites of collection are listed in [Supplementary Data Table S1, Fig. S1] and include some previously genotyped accessions [see Wan *et al.* (2013) and Klein *et al.* (2018)].

As a comparator to the well established developmental programme of *Vitis vinifera* berries (Ollé *et al.*, 2011) [Supplementary Data Fig. S2], ten berries of equal diameters (measured with a caliper on the equator) were periodically collected from *Vitis piasezkii* (accession 8057Mtp2) over the period between anthesis and berry maturity. In addition, healthy, green, fully-developed leaves were collected from some of the species; a few leaf exsiccata were added from herbaria [MNHN(P), MPU] to be analysed for condensed tannins.

Preparation of samples

After removal of the pedicel, fresh green fruits were cross-sectioned in the equator with a scalpel, rapidly deseeded, the pericarp immediately weighed, and shock-frozen in liquid nitrogen. Pericarp fragments were then very finely pulverized in liquid nitrogen with a pre-cooled stainless steel bead grinder. The frozen powders were then lyophilised overnight and stored in plastic vessels at -80°C. After removal of the petiole, fully-developed leaf samples were treated in the same way and then lyophilized.

Acid-catalysed depolymerisation of condensed tannins

Aliquots (around 10-20 mg) of freeze-dried fruits [< 60 days after anthesis (DAA)] or adult leaves were resuspended in 1 mL of methanol containing 0.1 M HCl, and 10% 2-mercaptoethanol, sonicated for 2 min, and subsequently heated for 2 h at 40°C. After centrifugation ($10,000 \times g$, 5 min), the flavan-3-ol mercaptylated adducts in the supernatants,

resulting from the depolymerisation of condensed tannins, were analysed by UPLC–DAD/ESI–MS: briefly, the liquid chromatography system was an Acquity UPLC (Waters, USA) equipped with a photodiode array detector. The column (HSS T3, 100 × 2.1 mm, 1.8 mm) was filled with Nucleosil 120-3 C18 endcapped as solid phase (Macherey-Nagel, Sweden). The flow rate was 0.40 mL·min⁻¹, and the gradient conditions were as follows: solvent A (H₂O-HCOOH, 99/1, v/v), solvent B (CH₃CN-H₂O-HCOOH, 80/19/1, v/v/v); initial 0.1% B; 0–5 min, 60% B linear; 5–7 min, 99% B linear; 7–8 min, 99% B isocratic; and 8–9 min, 0.1% B linear. The Acquity UPLC system was coupled online with an AmaZon X ESI Trap mass spectrometer (Bruker Daltonics, Germany). The mass spectra were acquired over a mass range of 90–1500 Th in the positive ionisation mode. Compounds were measured at λ_{\max} 280 nm, and standardisation against epicatechin using the molar response coefficients 4.16 for epigallocatechin (EGC), 1.00 for epicatechin (EC), 0.27 for epicatechin 3-*O*-gallate (ECG), and 0.33 for epigallocatechin 3-*O*-gallate (EGCG). Data were expressed as relative abundance based on molarity. This means that the peak area of the respective individual compound was given as proportion to the sum of all four measured constituents, corrected for the respective molar response expressed in %. The mean degree of polymerisation (mDP) was not assessed.

This standard method had to be modified in case of fruits from *Vitis piasezkii* from 70 to 120 DAA since these fruits, having accumulated sugars, produced a pasty, methanol-insoluble matrix upon freeze-drying, impairing the depolymerisation. Instead, around 100-200 mg of fresh pericarp were placed in 1 mL of 6% sulfur dioxide, then heated at 100°C for 150 min, spun down as described above, and the supernatants analysed by UPLC as described above.

Leaf morphometry

The surface of leaf margin teeth was measured with Image J (USA) as follows: using the pointer, we integrated teeth surface from valley to valley (between the bottom of the sinus preceding the tooth top to the bottom of the following sinus).

RESULTS

Developmental and environmental stability of the pericarp tannin composition

Preliminary experiments were conducted in order to ascertain that the composition of the PAs was stable enough to characterize a given genotype, and exclude significant developmental and environmental effects. The accumulation of PAs and their compositional changes were analyzed throughout one complete development cycle of *Vitis piasezkii* pericarp (accession code number 8052Mtp2), from fruit-setting to fully ripe stage [Supplementary Data Fig. S2]. *V. piasezkii* pericarp displayed a first green growth period, followed by abrupt softening, indicating the onset of ripening, coloration and growth resumption, according to the double sigmoidal growth pattern classically observed in *V. vinifera*, with the exception that berry mass did not double during ripening. The relative EGC mole ratio increased from fruit-set until 42 DAA, *i.e.* 7 days before the herbaceous tier was attained, then it was found constant till full ripeness. Other tannin constituents (EC, and ECG) followed decreasing kinetics symmetric to EGC with stabilization at 42 DAA (data not shown). Finally, the composition of PAs from pericarp was found to be stable from a little before the onset of the herbaceous tier until full maturity.

Green berries (10) were also collected from one bunch of the same plant of *Vitis berlandieri* x *Vitis vinifera* subsp. *sativa* (cv. Colombard), and of *V. piasezkii*, weighed, and their PAs analyzed [Supplementary Data Table S2]: the mole percents of the PAs units were found to vary as a function of the units abundance from *ca.* 2% (major unit) to 5-9%

(intermediate units), to 25-35% (minute units), this ranking varying with the genus and species (*e.g.* EGCG is the major form in *Muscadinia*, and minute in *Vitis*). Thus sampling 10 berries per accession was found sufficient to provide representative mole ratio percentages.

In order to ensure that differences in tannin composition were of genetic origin and before performing massive analyses of the PAs from pericarps of Vitaceae harvested worldwide, we also ruled out a significant impact of the environment. Berries from diverse genera and species of Vitaceae were collected two successive years in a row in the Experimental Vineyard of SupAgro (Montpellier, France), and the condensed tannins from their pericarps analyzed. The mole ratios of the four units were very similar from one year to the next (slope and $R^2 \sim 1$) [Supplementary Data Fig. S3]; thus, owing to its stability, the tannin composition can be ascertained through a single collection place.

Since in some cases female plants were either unavailable or lacked fruits, PAs on leaves from male plants were compared with those from leaves and berries on female plants of the same species. A general rule was established as follows: when EGC and EGCG were absent in berries of a given accession (thereafter called EGC⁻), they were also absent in their corresponding leaves (checked on 24 species, see [Supplementary Data Table S1], and *vice versa*, when they were present in the berries (EGC⁺), there were also present in leaves; thus one can reasonably state that when neither EGC nor EGCG were found in leaves from a male plant, no EGC and EGCG would have been found in berries from a female of the same species; this rule is also valid for leaves.

Compositional diversity within the Vitaceae

Berries and leaves from diverse genera (12) and species (91) of Vitaceae with emphasis on the *Vitis* genus were analyzed for their condensed tannins by the mercaptolysis technique and results are presented thereafter (**Supplementary Data Table S1, Figs. 2 and 4**).

*The Vitaceae tribes*_We studied genera and species from five tribes of the Vitaceae (Ma *et al.*, 2021) plus the genus *Leea* (sister family Leeaceae) as an outgroup. The first diverging Ampelopsidae tribe (**Fig. 2**), *i.e.* genera *Ampelopsis*, *Rhoicissus*, and *Nekemias*, is characterized by very high EGC contents (30-80%) and high degrees of galloylation (10-40%), more similar to the outgroup *Leea* which is almost pergalloylated.

The genera *Cyphostemma*, and *Tetrastigma* from the Cayratiae tribe lost their ability to synthesize gallic acid. *Cayratia* forms only EC, *Cyphostemma* and *Tetrastigma* also form EGC, and *Tetrastigma* and *Cissus* showed EGC⁺ and EGC⁻ species.

The Cisseae tribe shows a high diversity with species forming EGC and GALL (degree of galloylation, *i.e.* EGCG + ECG) and other devoid of these two constituents. The Parthenocisseae tribe and two *Ampelocissus* species (Viteae tribe) seemingly lost their ability to form EGC in their PAs as the genus *Cayratia*; their species were galloylated at 10-30% levels. Finally, the *Vitis* genus (Viteae tribe) which diverged later is formed by two groups, an EGC⁻/GALL⁻ body plus an EGC⁺/GALL⁺ one with a low (5-10%) degree of galloylation.

The Muscadinia genus from the Vitae tribe Muscadinia rotundifolia (also called the muscadine) and its sister variety *M. rotundifolia* var. *munsoniana* exhibits condensed tannins where EGC and its galloylated form (EGCG) represented ~80-90% of total units, and the degree of galloylation was high at ~30-50% (**Fig. 2**), the four analyzed accessions showing a fair compositional homogeneity [**Supplementary Data Table S1**].

The genus Vitis from the Vitae tribe One must note an observation made incidentally during this work on tannins: EGC⁺/GALL⁺ species bear on their leaves doubly serrate (rarely serrate) margins with long sharp teeth while EGC⁻/GALL⁻ ones show serrulate margins with minute regular teeth (**Fig. 3**). A Pearson's Chi-squared test performed on **Fig. 3** (EGC presence/absence vs. doubly serrate/serrulate margins) gave a 5.4 10⁻⁶% probability that the two characters would not be linked; they could be linked genetically and inheritable, epigenetically, or environmentally. A morphometric analysis of various leaves (**Fig. 3**) revealed two groups further designated as EGC⁺ vines containing EGC and GALL with doubly serrate margins and EGC⁻ vines devoid of EGC and GALL with serrulate margins.

We analyzed 23 species, and varieties of North American *Vitis* and 23 Eurasian/Asian species (**Supplementary Data Table S1; Fig. 4**), *i.e.* ca 77% of known *Vitis*. When PAs composition was overlaid onto consensus phylogenetic trees extracted from the recent literature [Zecca *et al.* (2020) for American *Vitis*, and Ma *et al.* (2020) for the Eurasian/Asian *Vitis*] (**Fig. 4**), it is easy to see that most of the Asian vines belongs to the group of EGC⁻ vines. Additionally, EGC⁻ species are also found represented in North America. The vines of the EGC⁺ vines group are less numerous and spread between America and Eurasia/Asia [**Supplementary Data Fig. S4**].

Hybrids in Vitis We analyzed nine hybrids of which one of the parents was an EGC⁻ *Vitis* [**Supplementary Data Table S1**]; the other parents were EGC⁺ vines, either *Vitis vinifera* subsp. *sativa* or *V. piasezkii*; all parental accessions from a given species were unknown. One can see that, in all cases, the EGC⁻ character was lost, all hybrids carrying EGC in their condensed tannins and adopting longer teeth than in their EGC⁻ parent [**Supplementary Data Figure S5**]; however, *Vitis piasezkii* is an exception showing dominance upon hybridization in its leaf morphology (Londo, personal communication).

DISCUSSION

Condensed tannins are a valuable tool in chemotaxonomy

Prior to our comprehensive analysis of tannins, we performed numerous preliminary experiments to ensure that data were representative in all aspects of berries and/or leaves from Vitaceae individuals. We thought that PAs, being constructed of four linked distinct units (**Fig. 1**) could complement plant phylogenomics through phenotyping: indeed, and contrary to other plant phenolics (anthocyanins, stilbenes, hydroxycinnamic acids) which are single molecules sensitive to environmental conditions, the internal variations of these units within the polymers would depend not on the environment but on the genetic origin of the plant (Scioneaux *et al.*, 2011). Finally, the composition of PAs analyzed as described here and expressed as percent molar ratio of the four constituents is specific of a sole accession whether true-to-type or hybrid, whatever its botanical, germplasm, or cultivated origin, and whatever the agropedoclimatic growth conditions, notwithstanding the precision of the technique.

The Vitaceae tribes

The Ampelopsidae tribe defined by Ma *et al.* (2021) comprise three genera, namely *Ampelopsis*, *Nekemias*, and *Rhoicissus*, and it was the first to diverge (**Fig. 2**). Blasting the phenotyping data onto the genomic tree revealed that the three genera showed similarities: indeed, this clade is rich in EGC, and GALL. It is worth to note that the observed exclusive distributions of EGC or GALL which are present or absent in the *Cyphostemma* and *Cayratia* genera, and the Parthenocissae tribe, reflect part of the reality: indeed, if more species per genus had been available, maybe we would have observed genera with and without EGC or GALL in the same genus like in the Cissae and Vitae tribes (see further paragraph *The Vitis*

genus). It should be noted that the genus *Muscadinia*, although clading within the distant Vitae tribe, exhibited a strong resemblance with *Rhoicissus* and *Nekemias*.

The genus Muscadinia

The new structural element added to the knowledge of the Muscadine is that EGCG is a major structural component of *Muscadinia* condensed tannins while it is either absent or present at a <4% level in all other studied *Vitis*; within the Vitaceae studied here, this trait is only shared with *Nekemias arborea*, and *Rhoicissus rhomboidea*, members of the tribe Ampelopsidae (Wen *et al.*, 2018^a). Alongside the PAs, we detected caffeoyltartaric and coumaroyltartaric acids ($\lambda = 320$ nm), typical hydroxycinnamic acids of *Vitis* (Liang *et al.*, 2012), in all *Vitis* studied so far, while they are absent in *Muscadinia* as already reported by Singleton *et al.* (1986). This species was recently placed as a subgenus of the *Vitis* genus although possessing $2n = 40$ chromosomes instead of $2n = 38$ for all other *Vitis* which belong to the subgenus *Euvitis*. However, in most phylogenomic studies, it is placed in a separate clade at a very long distance from all *Vitis* (Zecca *et al.*, 2012, 2020; Liu *et al.*, 2016; Wen *et al.*, 2018^b; Klein *et al.*, 2018). The differences between these two subgenera are so marked that Olmo (1978) taking up an idea of Small (1913) proposed to turn the subgenus *Muscadinia* into a separate full-fledged genus of the *Vitis* genus. Our results on tannins also emphasize a clear-cut differentiation of *Muscadinia* from the *Vitis* genus. In other words, as mentioned by Bouquet (1978, 1980), the taxonomic status of this species, in view of these new features added to the already known dramatic differences with *Vitis*, would need to be reexamined.

The *Vitis* genus

The studied 46 species and varieties from the *Vitis* genus represent 77% of known species, and they formed four major clades distributed as one pair in North America and one pair in Eurasia/Asia (**Fig. 4**), in each pair one clade embracing EGC⁻ vines, and another clade comprising EGC⁺ vines. In addition, two other clades were observed, one in Eurasia/Asia with the EGC⁺ *Vitis vinifera* subsp. *sylvestris* and its supposedly derived subsp. *sativa* possessing EGC in its PAs, and in North America, the EGC⁺ *Vitis californica*.

Twenty-three wild and cultivated Eurasian/Asian and Asian *Vitis* were analyzed for their PAs from pericarp and/or leaf [**Supplementary Data Table S1**]; they represent 60% of known wild *Vitis* of this continent (Wan *et al.*, 2008). Nineteen were EGC⁻ vines, *i.e.* 83% of Eurasian/Asian and Asian tested *Vitis* (*V. adenoclada*, *balanseana*, *bellula*, *betulifolia*, *bryonifolia*, *chungii*, *davidii*, *hancockii*, *heyneana* subsp. *ficifolia*, *heyneana* subsp. *heyneana*, *hui*, *pseudoreticulata*, *retordii*, *romanetii*, *sinocinerea*, *thunbergii*, and *wilsoniae*) amongst which nine growing in continental China, and three with ubiquitous distributions [*V. heyneana* subsp. *heyneana* (= *V. lanata* Roxburgh = *V. Jacquemontii* Parker; The Plant List, 2020; Trias-Blasi, 2017) and *heyneana* subsp. *ficifolia* extending to South East Asia, and Northern India, Pakistan, and Afghanistan (Hindu Kush) from sea level to 3,600 m (Tibetan tier), and *flexuosa* which extends from the South East Asia to the Philippines]. Four were EGC⁺ vines (*V. amurensis*, *piasezkii*, *vinifera* subsp. *sylvestris*, and *V. vinifera* subsp. *sativa*), and two EGC⁺ vines were unclassified according to their leaf margin teeth (*V. coignetiae* and *flexuosa*)(**Fig. 3**).

Twenty-two wild North American *Vitis* were analyzed for their condensed tannins from pericarp and/or leaf [**Supplementary Data Table S1**]. Nine were EGC⁻ vines (*V. aestivalis*, *berlandieri*, *blancoi*, *cinerea*, *labrusca*, *lincecumii*, *mustangensis*, *shuttleworthii*, and *simpsonii*). Thirteen were EGC⁺ vines producing EGC and GALL in their PAs (*V.*

acerifolia, *arizonica*, *bourgaeana*, *californica*, *cordifolia*, *girdiana*, *monticola*, *palmata*, *riparia*, *riparia* subsp. *longii*, *rupestris*, *vulpina*, and the two *Muscadinia rotundifolia* and *M. rotundifolia* var. *munsoniana*).

The EGC⁺ Vitis Four Asian and the Eurasian species were distinguished by their ability to form EGC under various proportions, from moderate (*V. amurensis* 1.9% in average, *V. coignetiae* 2.4%) to high (*piasezkii* 30.8%, *V. vinifera* subsp. *sylvestris* 57.9%). It must be noted that *V. amurensis*, and *V. coignetiae*, all growing in North-Eastern China and South-Eastern Russia (formerly Greater Manchuria), the Korean Peninsula, and Japan for the later, clade together in several phylogenetic trees (Wan *et al.*, 2013; Wen *et al.*, 2018b; Ma *et al.*, 2020). The third species, *V. piasezkii*, from Central China (primarily Gansu, Shaanxi) also clades with *V. amurensis* and *V. coignetiae* (Miller *et al.*, 2013; Wen *et al.*, 2018a; Klein *et al.*, 2018; Fu *et al.*, 2019; Ma *et al.*, 2018b, 2020). Finally, the three Asian EGC⁺ vines species are restricted to the great North East quarter of Asia from the Qin Lin Mountains to Northern Manchuria (subclade 5; **Fig. 4**).

At this point, the PAs similarity between *piasezkii* and *vinifera* (the two subspecies) must be highlighted. This is well illustrated in **Fig. 5**: indeed, when EGC was expressed vs. GALL, one sees that *Vitis piasezkii*, *Vitis vinifera* subsp. *sylvestris* and its domesticated form subsp. *sativa* are again linked and differed from other *Vitis* by their 5x ability to form EGC. Péros *et al.* (2011) found these two vines close phylogenetically. Thus, it appears that the clading of these species matches amazingly well with their ability to form the trihydroxylated flavanol EGC and its galloylated form EGCG.

Beyond the mountain ridge towards West and bordering the Northern and Western Pakistan, a single EGC⁺ vine, *V. vinifera* subsp. *sylvestris* and its cultivated form, grows from Iran and Afghanistan to Portugal [**Supplementary Data Fig. S4**], and *V. vinifera* subsp.

sativa, the cultivated grapevine, would have derived from it by domestication (This *et al.*, 2007; McGovern *et al.*, 2017).

Our placement of the tannin composition pie chart of *Vitis flexuosa* (**Fig. 4**) brought up an interesting divergence since similarly to subclade 5, and differently from other Asian wild vines of subclade 6 (*V. adenoclada*, *balanseana*, *bellula*, *betulifolia*, *bryonifolia*, *chungii*, *davidii*, *hancockii*, *heyneana* subsp. *heyneana*, *heyneana* subsp. *ficifolia*, *pseudoreticulata*, *retordii*, *romanetii*, *shenxiensis*, *sinocinerea*, *thunbergii*, and *wilsoniae*), *V. flexuosa* forms EGC and its PAs are highly galloylated; hence, it remains unclassified. Peros *et al.* (2011) placed *flexuosa* in a maximum parsimony network of haplotypes with *V. vinifera*, *coignetiae*, and *piasezkii*, *i.e.* Asian species producing EGC.

Eleven species from Central and North America were EGC⁺ vines (*Vitis acerifolia*, *arizonica*, *girdiana*, *monticola*, *riparia*, *bourgaeana*, *cordifolia*, *palmata*, *riparia* subsp. *longii*, *rupestris*, and *vulpina*). The first five group phenotypically in one subclade 3 defined genotypically by Zecca *et al.* (2020) (**Fig. 4**). The position of *Vitis palmata*, the first to diverge in subclade 2, is interesting since it was found at the root of subclade 3 of EGC⁻ American *Vitis*. If we consider our PAs data, a superposition was observed with genomic data from Klein *et al.* (2018) (North American clade I) and Zecca *et al.* (2020) (subclade 3 as shown in **Fig. 4**): indeed, the EGC⁺ vines *V. acerifolia*, *arizonica*, *monticola*, *riparia*, and *rupestris*, clade together in both genomic studies; similar subclades were described by Wan *et al.* (2013), and Miller *et al.* (2013). However, *V. vulpina* is consistently placed with *V. cinerea*, although possessing no leaf serrulate margin. A partial correspondence was found with series *ripariae* (Munson, 1909; Moore, 1991) where *acerifolia*, *riparia*, and *rupestris* nested; however, *monticola* was placed in the series *cordifoliae* with *vulpina* (Moore, 1991). Thus we propose that the ancient classifications in series (Planchon, 1887; Munson, 1909; Bailey, 1934; Galet, 1988; Moore, 1991) be abandoned in favor of a possible new

classification (if useful) based on up-to-date genomic techniques in association with tannin analyses.

Our data show that the leaves of *M. rotundifolia* (clade 1) and EGC⁺ vines from subclade 3 (*V. acerifolia*, *arizonica*, *monticola*, *riparia*, and *rupestris*) are small and cordate to slightly tricuspid with doubly serrate margins bearing long sharp teeth. These characters which are also found in *V. vinifera* subsp. *sylvestris* and its cultivated form (Eurasian clade), and Asian species from subclade 5, but *coignetiae* and *flexuosa* (unclassified), are not found in the other studied Asian wild species [our data and leaf illustrations in e-Flora of China (2020) and Li *et al.* (1996)].

The EGC⁺ vines diverged first in Eurasia and Asia, as well as in America with *V. californica*.

The EGC⁻ Vitis Most species lost their ability to form EGC: 18/23 (*i.e.* 78%) from Asia, and 7/23 (*i.e.* 30%) from North America; they also bear serrulate leaf margins (**Fig. 3**). The disjunction observed within the genus *Vitis* between EGC⁺ and EGC⁻ vines is not specific to this genus since it is also noted in the genera *Cissus* and *Tetrastigma* where EGC⁺ and EGC⁻ coexist in one genus (**Fig. 2**).

Vitis interspecific hybrids In order to test the discriminating character of EGC in disentangling true-to-types from hybrids, we blindly compared the phenotypic data of some accessions and their genetic data. For example, among a small population of four *Vitis labrusca* from NGR [**Supplementary Data Table S1**], three were EGC-free (483145, 483147, 483148) and one contained 13% EGC (483163); by consulting the corresponding circular dendrogram (Klein *et al.*, 2018), we found that this last EGC-containing accession was classified as an hybrid and that the three EGC-free were true *labrusca*. Likewise, two accessions of *Vitis heyneana* subsp. *heyneana* (= *V. jacquemontii*) (DVIT2352, DVIT2355)

provided 5-10% EGC-containing PAs while a true-to-type (DVIT1815) lacked it: again, comparison with genetic data showed that the former were hybrids bearing hermaphrodite flowers (*V. heyneana* x *V. vinifera* subsp. *sativa*) (Wan *et al.*, 2013). These observations were also true for the well known *labrusca* hybrids, Isabelle and Concord (Wen *et al.*, 2020). However, this rationale can only be enforced if reference true-to-type plants are available. The effect of hybridization is also well visible in the leaf margin teeth [**Supplementary Data Fig. S5**].

Moreover, we analyzed separately sets of berries from *ca.* 300 accessions of the domestic grapevine (not shown), and the average EGC content was around 30%. One can see that the average EGC percent of the 9 hybrids was 18%, that is around half of their parent domestic grapevine.

The cases of Vitis piasezkii and Vitis shenxiensis In their study on four Chinese *Vitis*, Ma *et al.* (2018a) amply discussed the taxonomic positions of *Vitis piasezkii* Maxim. and *V. shenxiensis* C. Li, two often sympatric wild vines from the Shaanxi, and nearby Henan provinces: indeed, on one side, they were recognized as distinct species (Li *et al.*, 1996; Kong, 2004; Chen *et al.*, 2007), while, on the other side, Niu and He (1996) argued that *V. shenxiensis* should be seen as a variety of *piasezkii*. Finally, Ma *et al.* (2018a) stated that further integrative systematic studies are needed to assess the taxonomic status of *V. shenxiensis* based on detailed morphological, phylogenomic, ecological, and geographic data. The difficulty comes from the fact that *shenxiensis* appears morphologically as an hybrid between *V. romanetii* (bearing red trichomes on its stem) and *V. piasezkii* (multi-foliolated leaves)(**Fig. 6**).

The examination of leaf margin teeth and the analysis of leaf PAs from these two species allow to easily decide which is which: indeed, *V. shenxiensis* is an EGC⁻ vine while

piasezkii is an EGC⁺ vine (**Fig. 6**). Moreover, as consistently reported in diagnoses (Maximowicz, 1881; Romanet du Caillaud, 1884; Hui and Wen, 2007; Ma *et al.*, 2016), *piasezkii* specimens do not bear glandular trichomes on their branches, while *shenxiensis* shows some (Li *et al.*, 1996); an additional trait must be added: juvenile leaflets of *shenxiensis* consistently show a reddish color, while *piasezkii*'s are always pale green. Thus, although showing morphological similarities, *V. piasezkii* and *V. shenxiensis* appear as radically distinct species; the status of *Vitis shenxiensis* C.L. Li (Li *et al.*, 1996) as a true species is thus reinforced. Finally, it would be of great interest to analyse the tannins from accessions (Liu 048, 406, 423, 683, 691, and 694) presented in a circular dendrogram by Ma *et al.* (2018^a): indeed, they appear as *V. piasezkii* nested within a *V. shenxiensis* population. This could result from misidentifications and/or true hybrids which would be easily seen if they contain EGC.

The GALL/TriOH bifurcation On the (EGC+EGCG) vs. GALL graph (**Fig. 5**), berries from the *Vitis* genus are distributed in three groups, according to their capacity to accumulate trihydroxylated subunits in PAs. Clearly, the rare Eurasian/Asian EGC⁺ vines, *i.e.* both *vinifera* subspecies and *V. piasezkii*, were characterized by an aptitude to form EGC vs. GALL at least 4.9 times higher than the two other groups. Again is shown a proximity of *piasezkii* with the two *vinifera*. The biosynthesis of GALL and EGC are both achieved by the shikimate pathway, more precisely by the action on 3-dehydroshikimate of the bifunctional enzyme DHQ dehydratase-shikimate dehydrogenase (Hermann and Weaver, 1999); it is a bifurcation leading on one side to GALL, and on the other side to the aromatic amino acids and the flavonoid core including EGC. Thus, it is not surprising that both GALL and EGC being competitors show correlations of rather good quality; this peculiar behavior of *V. vinifera* and *piasezkii* needs further research.

CONCLUSIONS AND PERSPECTIVES

Phenotyping of condensed tannins and examination of leaf margins allowed a new classification of *Vitis* into two radically distinct groups, the EGC⁺ and EGC⁻ vines possessing respectively doubly serrate or serrate leaf margins with long teeth, and serrulate margins with minute teeth, differently from phylogenomics which identify two clades, namely American and Eurasian/Asian clades (Klein *et al.*, 2018); this classification crosses the continental boundaries showing transcontinental distributions. The Eurasian/Asian vines are essentially free of EGC and bear serrulate leaf margins while the American ones are a *ca.* 50/50 mixture of EGC⁺ and EGC⁻ vines with doubly serrate and serrulate leaf margins, respectively. Although not understood, this new result could prove to be a useful tool and offer a new perspective to scientists interested in the evolution of the genus *Vitis*. This usefulness of tannins phenotyping was precedently illustrated in the *Musa* genus (Uclès Santos *et al.*, 2010): analysis of banana flesh PAs allowed the AA diploid “Pisang Lilin” cultivar (*Musa acuminata* subsp. *malaccensis*), the only member of its AA sub-group separated from other AA diploid members in a dendrogram built with AFLP markers (Ude *et al.*, 2002), to be also well separated from other AA diploids by its (EGC/EC) ratio.

Further integrative systematic studies (Wen *et al.*, 2015, 2017, 2018^b; Ma *et al.*, 2020) are needed to assess the taxonomic status of certain species (*e.g.* *V. shenxiensis*) based on detailed morphological, phylogenomic, ecological, and geographic data. On top of that and in agreement with Wen *et al.* (2018^b), we suggest the production of an integrative systematics of grapevines and their close relatives, particularly seeking to supply up-to-date taxonomic

revisions of the *Vitis* genus and other Vitaceae on an international basis and to generate informatics tools for soft public access online. This could be nicely achieved by massively extending the analyses of condensed tannins from green berries and leaves to populations from all members of the Vitaceae, a promising technique still in its infancy; this would possibly help in clarifying some uncertainties.

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FIGURE CAPTIONS

FIG. 1. Composition and structure of a condensed tannin.

FIG. 2. Tannin composition across Vitaceae tribes and genera worldwide. All accessions of a given species were averaged, then all averaged species of a given genus were averaged. Pie charts depict tannin composition in berries (no outline) or leaves (black-outlined). Right hemisphere indicates the proportion out of 100% of dihydroxylated (EC+ECG; yellow) and trihydroxylated (EGC+EGCG; pink) units. The left hemisphere indicates the degree of esterification in blue (EGCG+ECG = sum of *O*-galloylated units) out of 100%, with its complement in grey. Phylogenomic consensus dendrogram taken from Ma *et al.* (2021). Bold numbers in brackets: number of species/genus.

FIG. 3. A- Views of adaxial faces of some adult leaves from the studied American and Eurasian/Asian *Vitis* species showing teeth lining the margins. Teeth are shown at equal scales, and the significance of the pie-charts is given in Fig. 2. White number above: species n°; below: number of accessions observed. **I.** EGC⁺ vines: 1- *Muscadinia rotundifolia*; 2- *Vitis californica*; 3- *V. palmata*; 4- *V. vinifera* subsp. *sativa* (cv. Riesling); 5- *V. riparia*; 6- *V. cordifolia*; 7- *V. rupestris*; 8- *V. girdiana*; 9- *V. arizonica*; 10- *V. monticola*; 11- *V. acerifolia*; 12- *V. vulpina*; 13- *V. amurensis*; 14- *V. piasezkii*; 15- *V. bourgaeana*; **II.** EGC⁻ vines: 16- *V. cinerea*; 17- *V. mustangensis*; 18- *V. labrusca*; 19- *V. aestivalis*; 20- *V. shuttleworthii*; 21- *V. berlandieri*; 22- *Vitis heyneana* subsp. *ficifolia*; 23- *V. romanetii*; 24- *V. pseudoreticulata*; 25- *V. adenoclada*; 26- *V. betulifolia*; 27- *V. heyneana* subsp. *heyneana*; 28- *V. davidii*; 29- *V. handcockii*; 30- *V. wilsoniae*; 31- *V. simpsonii*; 32- *V. bryonifolia*; 33- *V. retordii*; 34- *V. tiliifolia*; 35- *V. blancoi*; 36- *V. chungii*; **III.** Unclassified: 37- *V. flexuosa*; 38- *V. coignetiae*. B-Expression of the EGC

content vs. percent of teeth area in the leaf (r). Insert: mode of measurement of the surfaces of entire leaf and margin teeth using Image J (red: *V. riparia*, an EGC⁺; blue: *V. romanetii*, an EGC⁻). The exceptions *V. flexuosa* and *coignetiae* are in yellow. ρ (rho) is defined as the ratio (x 100) of the (sum of the teeth surfaces)/(entire leaf surface).

FIG.4. Tannin composition across *Vitis* species from North American (A) and Eurasian/Asian (B) subclades. Pie charts depict tannin composition in berries (no outline) or leaves (black-outlined). Right hemisphere indicates the proportion out of 100% of dihydroxylated (EC+ECG; yellow) and trihydroxylated (EGC+EGCG; pink) units. The left hemisphere indicates the degree of galloylation in blue [(EGCG+ECC)/sum of units] out of 100%, with its complement in grey. Tannin composition of *Vitis vinifera* subsp. *vinifera* subsp. *sativa*^a and *sylvestris*^b and leaf morphologies of *V. heyneana* subsp. *heyneana*^a and *ficifolia*^b are labelled. Phylogenomics consensus dendrograms taken from (A) Zecca *et al.* (2020), and (B) from Ma *et al.* (2018b). Zoom to see minor constituents better. Leaves sizes equalized.  Doubly serrate with EGC;  serrulate with minute teeth without EGC;  serrulate with minute teeth with EGC.

FIG. 5. Variation, among the *Vitis* species, of (EGC + EGCG) vs. GALL in berries. GALL is the percent of galloylation [*i.e.* (EGCG + ECG)/sum of units]. Red: *Vitis piasezkii*, *V. vinifera* subsp. *sylvestris* and subsp. *sativa*, blue: other species, high (EGC+EGCG) accumulators, black: other species, low (EGC+EGCG) accumulators.

FIG. 6. Compared morphologies of leaves and stems of *Vitis piasezkii* and *Vitis shenxiensis*.

Figure 1

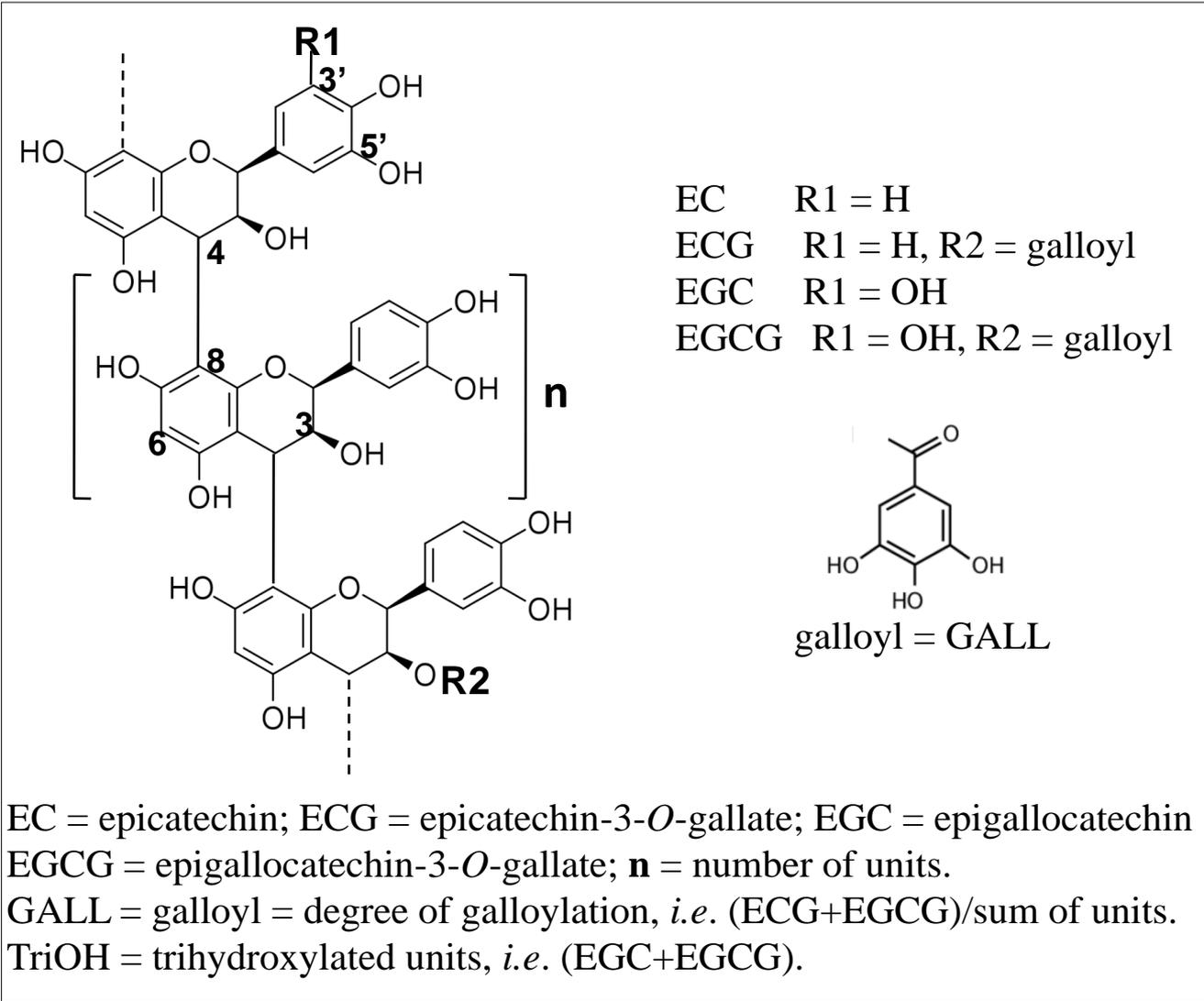


Figure 2

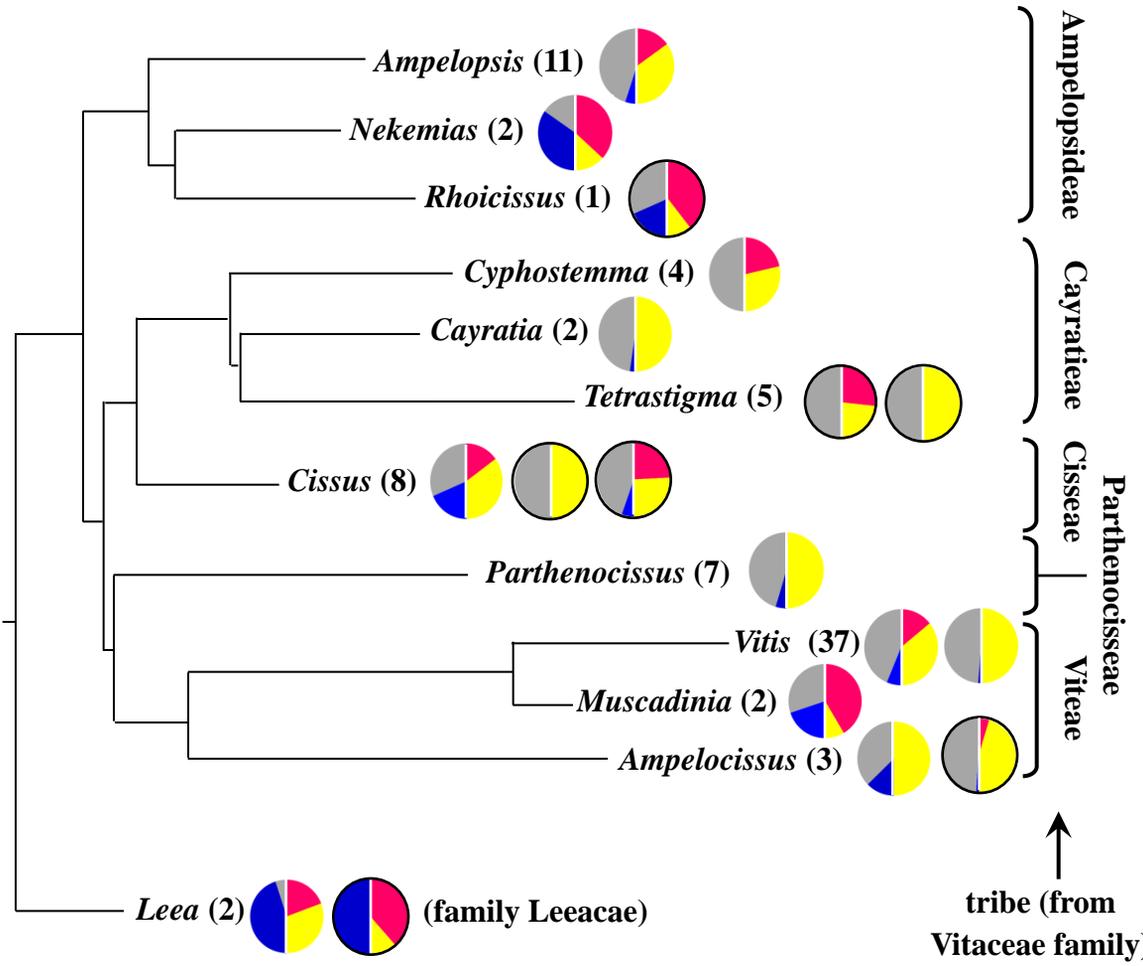


Figure 3.

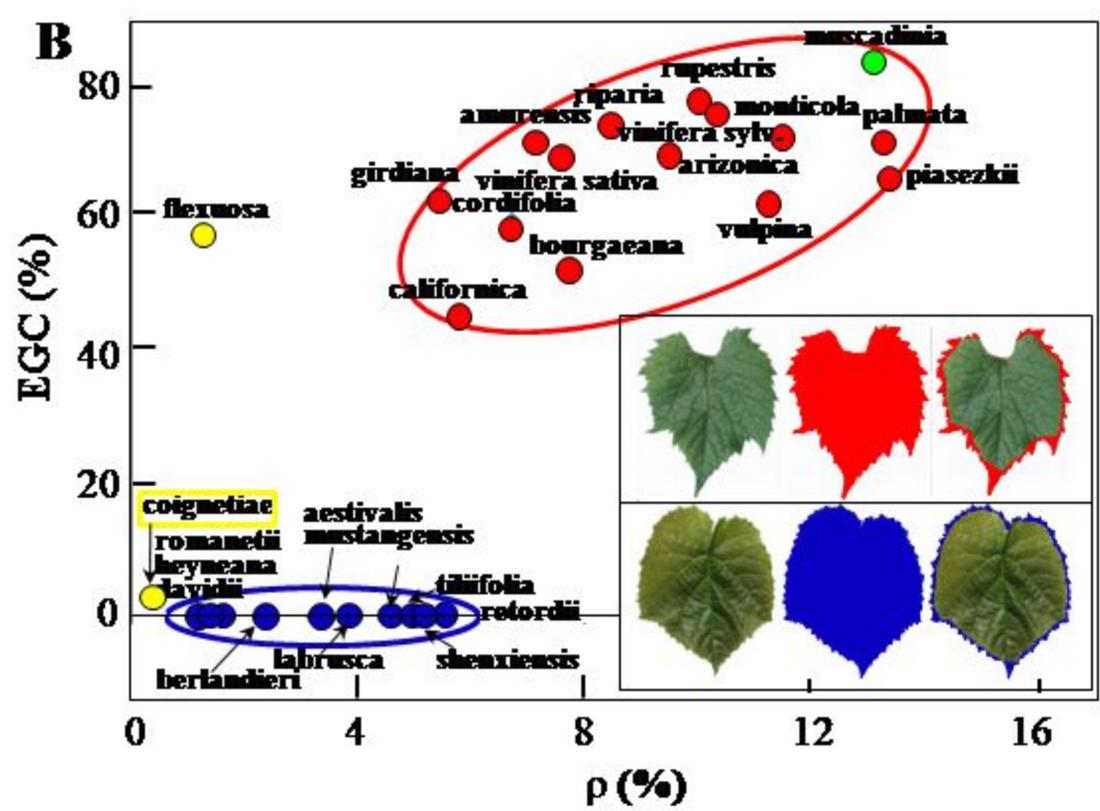
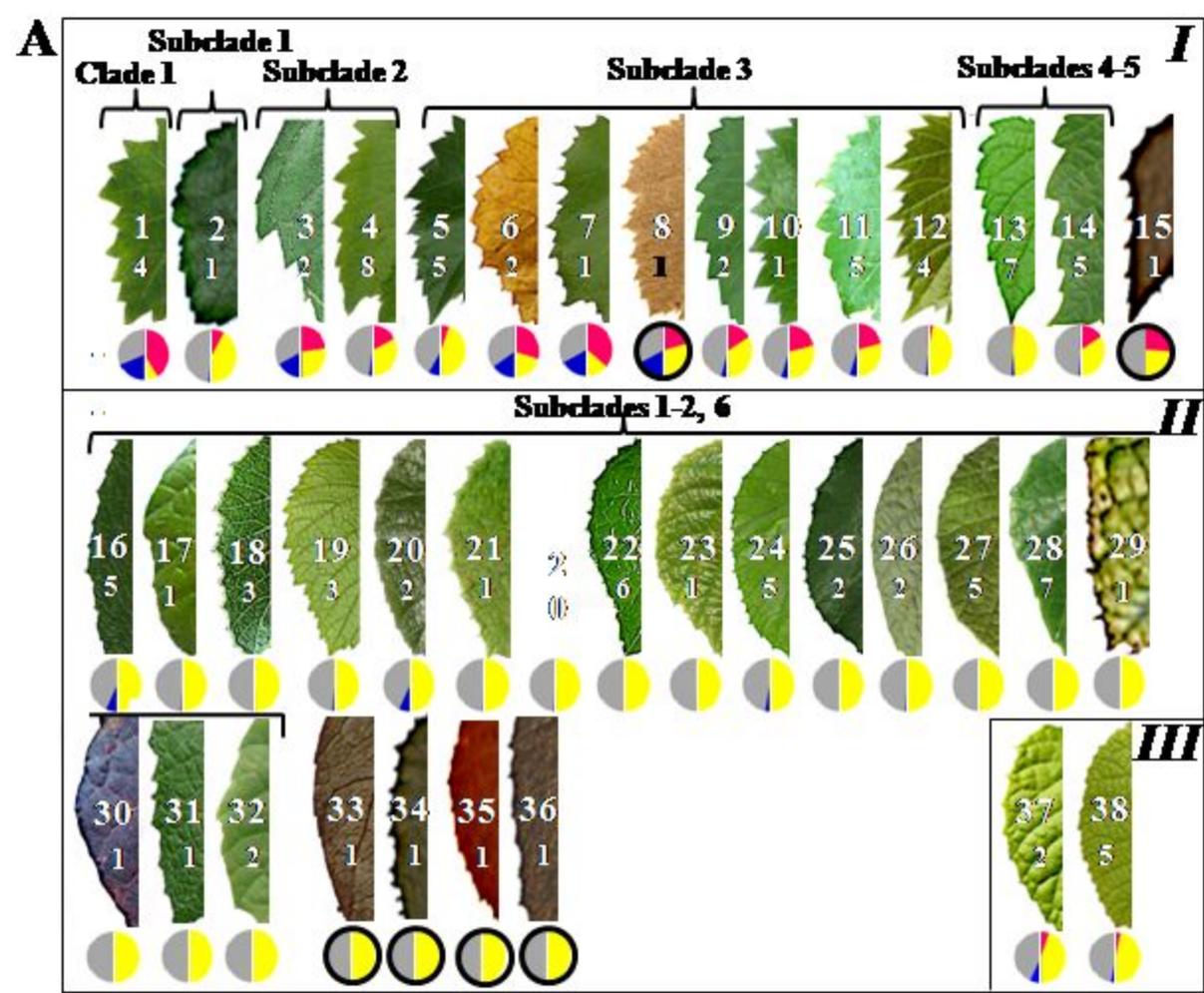
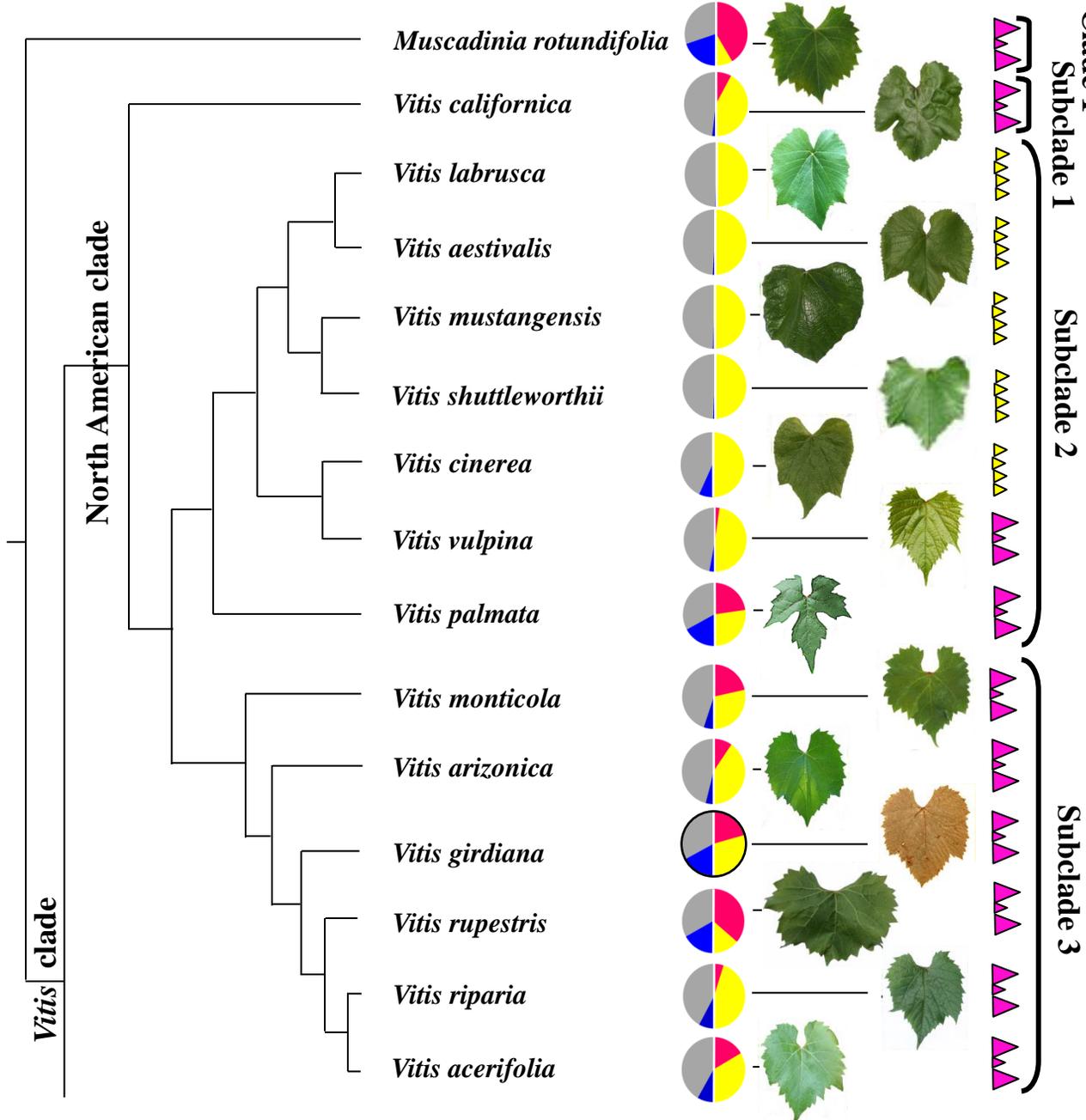


Figure 4.



continued

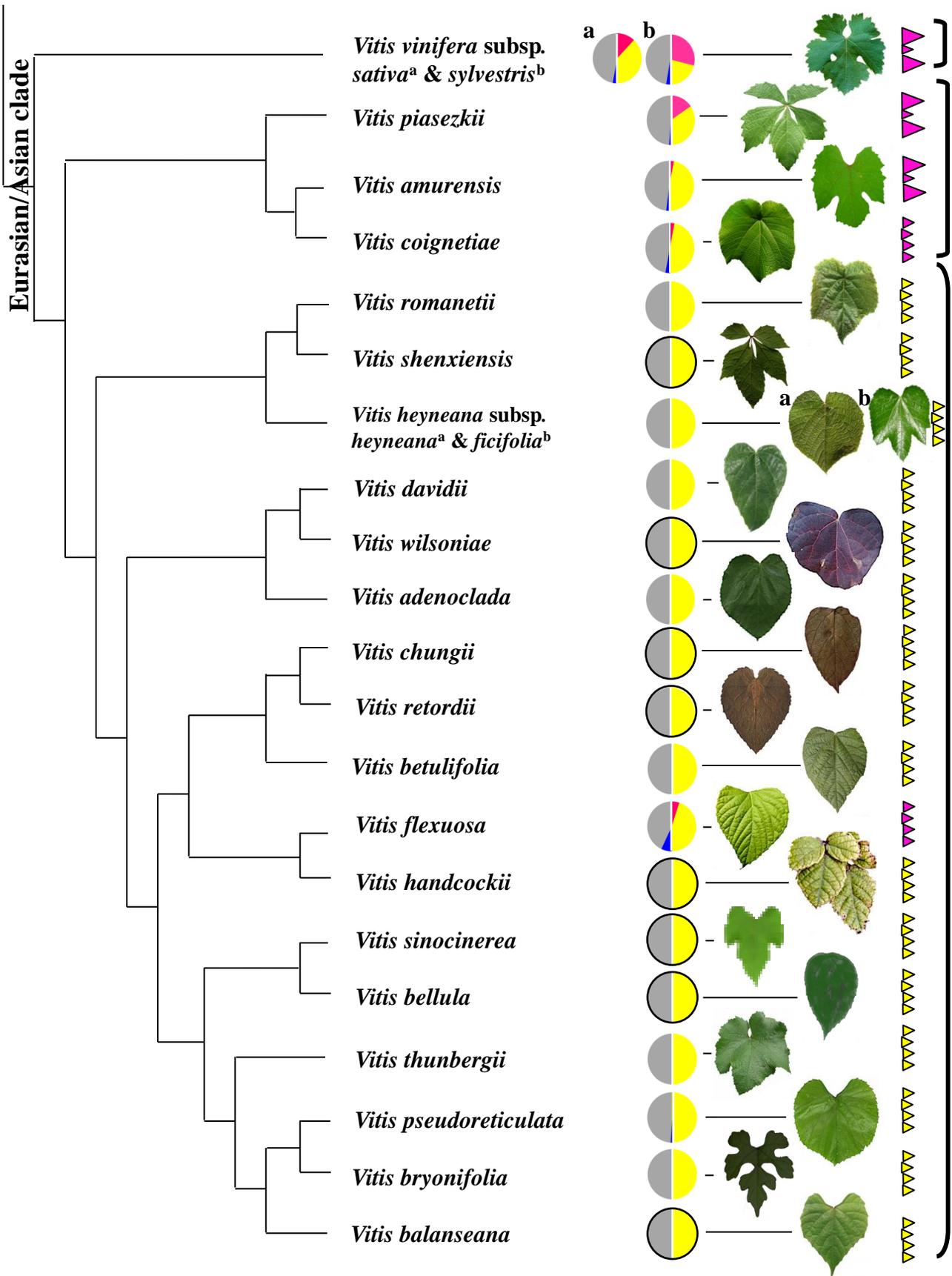


FIGURE 5

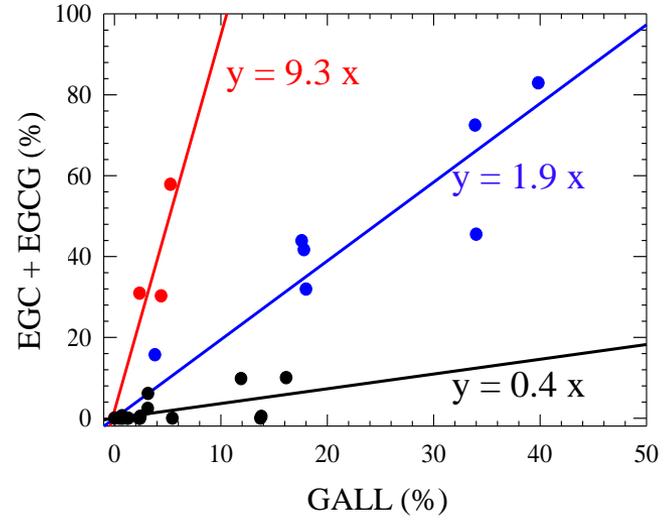
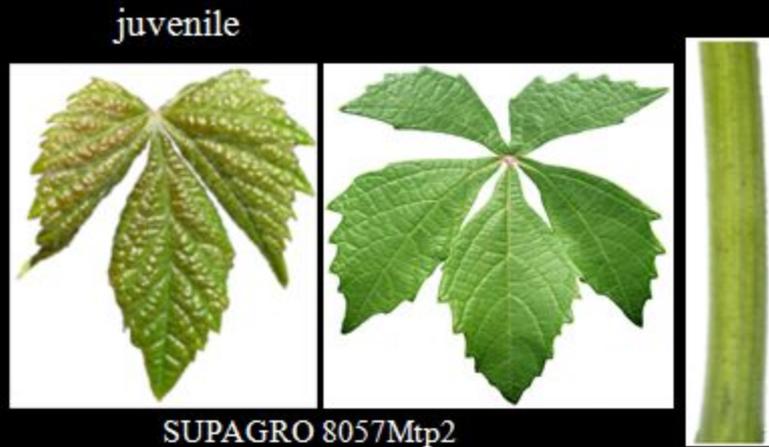


Figure 6

A



SUPAGRO 8057Mtp2

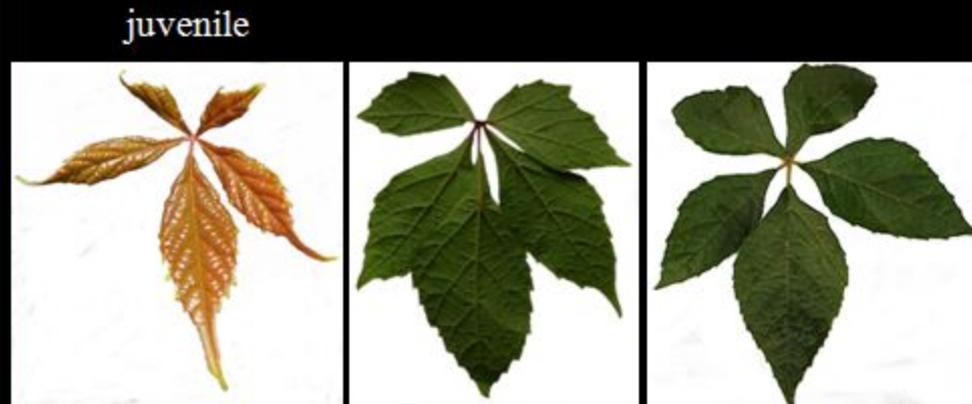
Vitis piasezkii Maxim.

=



no trichomes on stem
(arrows), juvenile consistently
green, and classified as an EGC⁺
Vitis (60.0%) (photo from Cheng *et al.*, 2012)

B



PM 19961192-52

PM 19961192-52

ZFRI Lingbao06

Vitis shenxiensis C.L. Li



=



tentatively attributed to *Vitis piasezkii*: bears red
trichomes on stem (red arrows), juvenile
consistently red bronze, and classified as an EGC⁻
Vitis (0.0%) (photo from Cheng *et al.*, 2012)

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