ANNALS OF
BOTANY
Founded 1887

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. 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 and pulverized, and condensed tannins were 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 quantified by ultrahigh pressure liquid chromatography/diode array detection/mass spectrometry.
• **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.*, 2018*a*), most of them growing in intertropical regions. Of peculiar economic importance is the genus *Vitis*, whose most emblematic member is the

EC = epicatechin; ECG = epicatechin-3-*O*-gallate; EGC = epigallocatechin

EGCG = epigallocatechin-3-*O*-gallate; *n* = number of units.

GALL = galloyl = degree of galloylation, *i.e.* (ECG + EGCG)/sum of units.

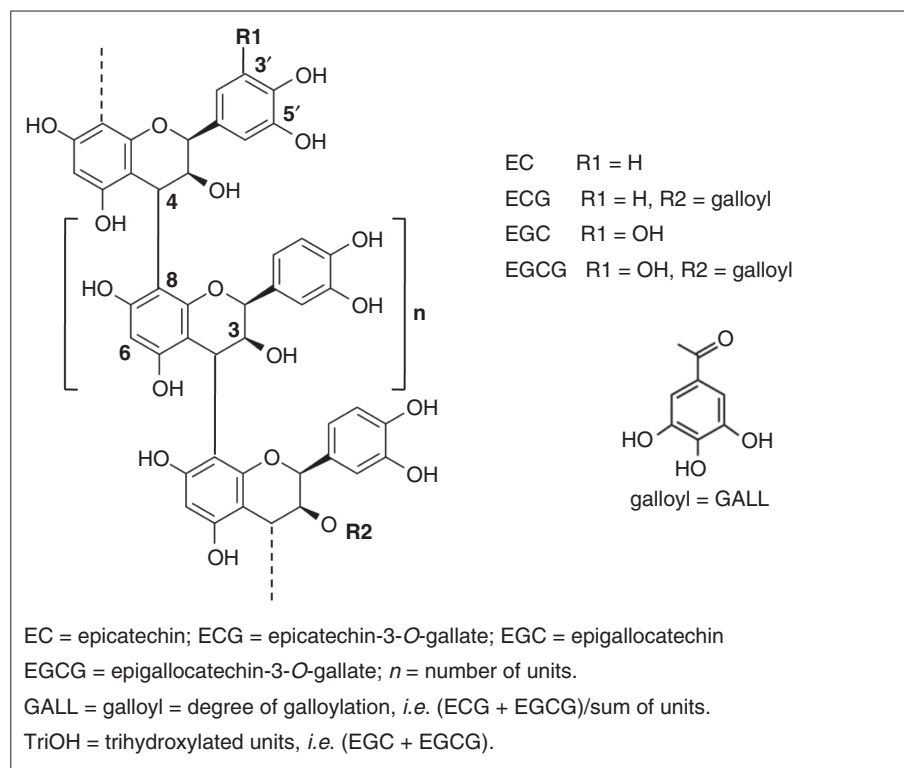TriOH = trihydroxylated units, *i.e.* (EGC + EGCG).

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

domesticated grapevine (*Vitis vinifera* subsp. *sativa*), whose berries, fermented to produce wines, have been consumed by humans for at least eight millennia (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.*, 2018*b*; Klein *et al.*, 2018; Fu *et al.*, 2019; Ma *et al.*, 2018*a*, 2018*b*, 2020, 2021; Liang *et al.*, 2019; 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 proper authentification of the identity of the specimens. Unfortunately, this is far from trivial in the Vitaceae leading to obvious misidentifications persisting in phylogenic 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; and *Vitis lanata* and *Vitis jacquemontii* which are in fact a unique species, *Vitis heyneana* subsp. *heyneana*, but appear as two genetically close but separated taxa in Wen *et al.* (2018*b*)]. Additionally, the complete 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.* (2018*b*) to underline the need for an extended interdisciplinary approach: 'integrative systematic with evidence from morphology, anatomy, phylogenomics, ecology, biogeography, fossils and bioinformatics' (*sic*). As a contribution to this integrative approach, we here use the chemotaxonomy of condensed tannins for the Vitaceae, an approach that has

been successful previously in banana (Uclés Santos *et al.*, 2010).

In the Vitaceae, the edible part of the fruit, the pericarp, as well as the leaf contain condensed tannins, also called proanthocyanidins (PAs), that are polymers composed of five 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 of condensed tannins (Monagas *et al.*, 2003; Huang *et al.*, 2012; Koyama *et al.*, 2017; Narduzzi *et al.*, 2015; Kedrina-Okutan *et al.*, 2019; Liang *et al.*, 2019), it is difficult to compile and compare them, because the techniques employed for extraction and depolymerization differ. To overcome this limitation, our international network presents a vast phenotyping of condensed tannins in the family Vitaceae (~250 accessions) with an emphasis on the genus *Vitis*. To ensure comparability, all the analyses were performed by a single group of indivduals 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 (1) PA content per berry was at its maximum and their

composition was stable until full maturity, (2) PAs can only be analysed by the mercaptoethanol technique (Brillouet *et al.*, 2017) in the absence of sugar inside the berries, and (3) 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 from America, Africa, East Asia and Europe. The genera, species, subspecies, accessions and sites of collection are listed in Supplementary Data Table S1 and Fig. S1, and include some previously genotyped accessions (see Wan *et al.*, 2013; 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 and rapidly deseeded, and the pericarp was 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 lyophilized 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 depolymerization of condensed tannins

Aliquots (around 10–20 mg) of freeze-dried fruits [<60 d after anthesis (DAA)] or adult leaves were resuspended in 1 mL of methanol containing 0.1 м HCl and 10 % 2-mercaptoethanol, sonicated for 2 min, and subsequently heated for 2 h at 40 °C. After centrifugation (10 000 *g*, 5 min), the flavan-3-ol mercaptylated adducts in the supernatants, resulting from the depolymerization of condensed tannins, were analysed by ultrahigh pressure liquid chromatography/diode array detection/mass spectrometry : 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 (H_2O–HCOOH, 99 : 1, v/v), solvent B (CH_3CN–H_2O–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 90–1500 Th in positive ionization mode. Compounds were measured at λ_{max} 280 nm, and

standardization 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 a proportion to the sum of all four measured constituents, corrected for the respective molar response expressed in %. The mean degree of polymerization (mDP) was not assessed.

This standard method had to be modified for fruits from *Vitis piasezkii* from 70 to 120 DAA because these fruits, having accumulated sugars, produced a pasty, methanol-insoluble matrix upon freeze-drying, impairing the depolymerization. Instead, around 100–200 mg of fresh pericarp was placed in 1 mL of 6 % sulphur 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 ImageJ (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 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 analysed throughout one complete development cycle of *Vitis piasezkii* pericarp (accession code number 8052Mtp2), from fruit-setting to fully ripe stage (Supplementary Data Fig. S2). *Vinus 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 molar ratio increased from fruit-set until 42 DAA, i.e. 7 d before the herbaceous tier was attained, then remained constant until full ripeness. Other tannin constituents (EC and ECG) followed decreasing kinetics similar to EGC with stabilization at 42 DAA (data not shown). Finally, the composition of PAs from pericarp was found to be stable from just 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* × *Vitis vinifera* subsp. *sativa* (cv. Colombard), and of *V. piasezkii*, weighed, and their PAs analysed (Supplementary Data Table S2): molar percentages of the PA units were found to vary as a function of the units' abundance from *ca.* 2 % (major units) to 5–9 % (intermediate units), to 25–35 % (minor units), this ranking varying with the genus and species (e.g. EGCG is the major form in

Muscadinia, and minor in *Vitis*). Thus, sampling 10 berries per accession was sufficient to provide representative molar ratio percentages.

To ensure that differences in tannin composition were of genetic origin and before performing large-scale 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 in two successive years in a row in the Experimental Vineyard of SupAgro (Montpellier, France), and the condensed tannins from their pericarps were analysed. The molar 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 location.

Because 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 (hereafter 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 was 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 an emphasis on the genus *Vitis* were analysed for their condensed tannins by the mercaptolysis technique (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 Ampelopsideae tribe (Fig. 2), i.e. the 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 tribe Cayratiae lost their ability to synthesize gallic acid. *Cayratia*
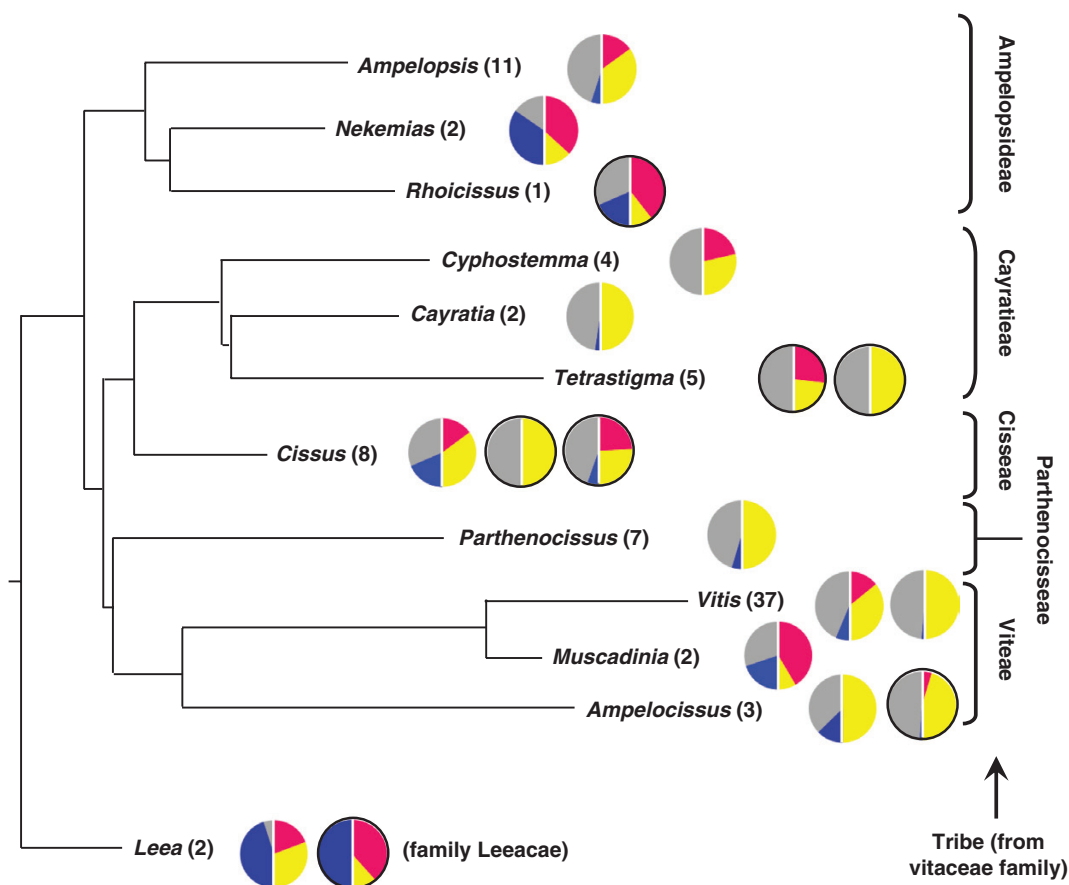

FIG. 2. Tannin composition across Vitaceae tribes and genera worldwide. All accessions of a given species were averaged, then all averaged species of a given genus were averaged. Pie charts depict tannin composition in berries (no outline) or leaves (black-outlined). Right hemisphere indicates the proportion 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 parentheses: number of species per genus.

forms only EC, *Cyphostemma* and *Tetrastigma* also form EGC, and *Tetrastigma* and *Cissus* showed EGC⁺ and EGC⁻ species.

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

The genus Muscadinia *from the tribe Vitae. Muscadinia rotundifolia* (also called the muscadine) and its sister variety *M. rotundifolia* var. *munsoniana* exhibit 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 analysed accessions showing a fair degree of compositional homogeneity (Supplementary Data Table S1).

The genus Vitis *from the tribe Vitae.* A notable observation was 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⁻ species 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 inheritably, epigenetically or environmentally. 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 analysed 23 species, and varieties of North American *Vitis* and 23 Eurasian/Asian species (Supplementary Data Table S1; Fig. 4), i.e. ~77 % of known *Vitis*. When PA composition was overlayed 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 was clear that most of the Asian vines belong to the group of EGC⁻ vines. Additionally, EGC⁻ species are also found represented in North America.

The vines of the EGC⁺ group are less numerous and spread between America and Eurasia/Asia (Supplementary Data Fig. S4).

Hybrids in Vitis. We analysed nine hybrids for which one of the parents was an EGC⁻ *Vitis* (Supplementary Data Table S1); the other parents were EGC⁺ vines, either *Vitis vinifera* subsp. s*ativa* or *V. piasezkii*; all parental accessions from a given species were unknown. 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 (Fig. S5); however, *Vitis piasezkii* is an exception showing dominance upon hybridization in its leaf morphology (J. Londo, pers. comm.).

DISCUSSION

Condensed tannins are a valuable tool in chemotaxonomy

Prior to our comprehensive analysis of tannins, we performed numerous preliminary experiments to ensure that the

data were representative in all aspects of berries and/or leaves from Vitaceae individuals. We considered 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) that 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 analysed as described here and expressed as the percentage molar ratio of the four constituents is unique to 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.

Vitaceae tribes

The tribe Ampelopsidae defined by Ma *et al.* (2021) comprises 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. Note that the observed exclusive distributions of EGC or GALL, which are present or absent in the genera *Cyphostemma* and *Cayratia*, and the tribe Parthenocissae, reflect part of the reality: indeed, if more species per genus had been available, we might have observed genera with and without EGC or GALL in the same genus as in the tribes Cissae and Vitae (see *The Vitis genus*). Note that the genus *Muscadinia*, although clading within the distant tribe Vitae, exhibited a strong resemblance to *Rhoicissus* and *Nekemias*.

The genus Muscadinia

The new structural element within the Muscadine is that EGCG is a major structural component of *Muscadinia* condensed tannins while it is either absent or present at < 4 % 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 Ampelopsideae (Wen *et al.*, 2018*a*). Alongside the PAs, we detected caffeoyltartaric and coumaroyltartaric acids (λ = 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 genus *Vitis* 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 make the subgenus *Muscadinia* into a separate fully fledged genus of the genus *Vitis*. Our results on tannins also emphasize a clear-cut differentiation of *Muscadinia* from the genus *Vitis*. In other words, as mentioned by Bouquet (1978, 1980), the taxonomic status of this species, given these
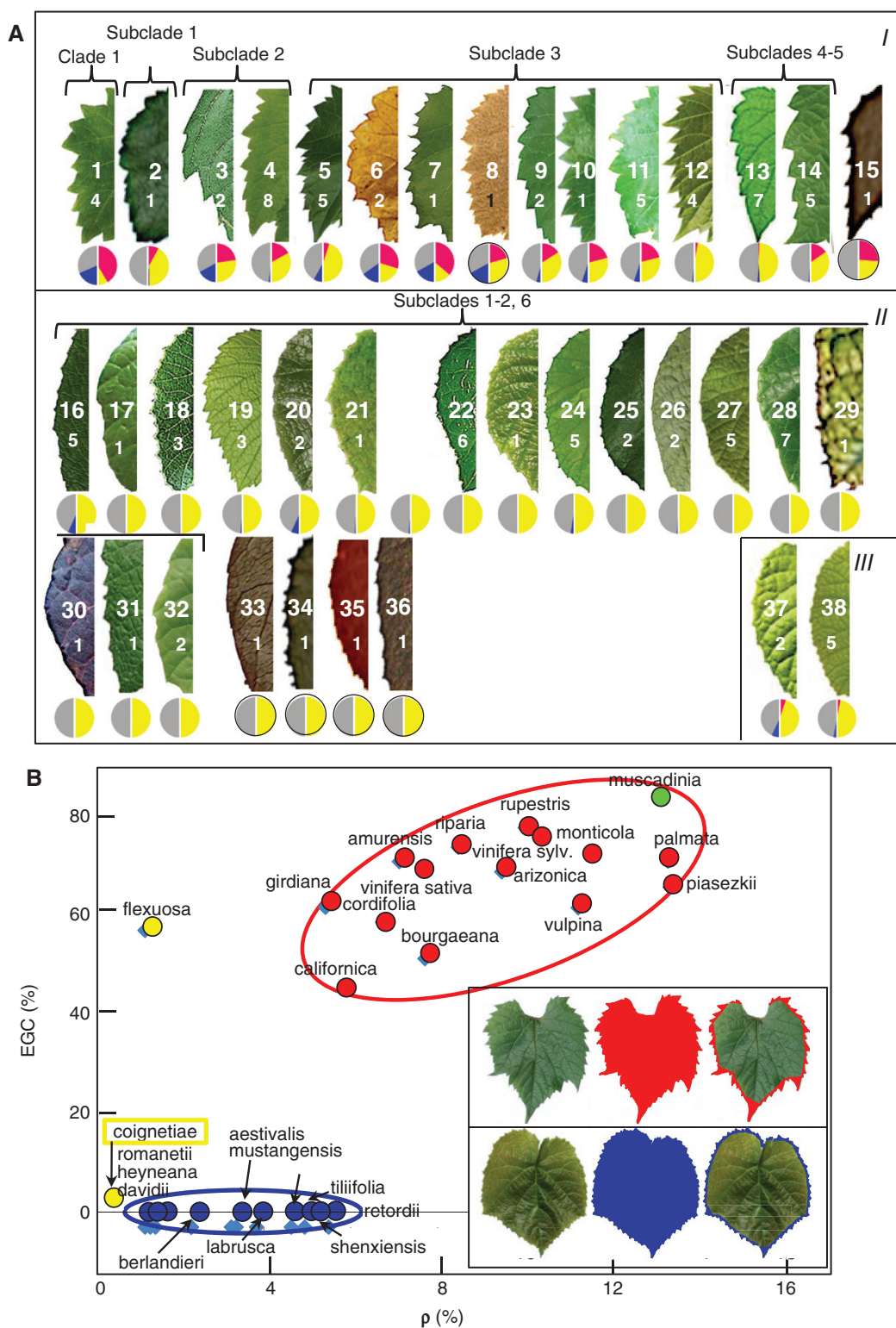
FIG. 3. (A) Views of adaxial faces of some adult leaves from the studied American and Eurasian/Asian *Vitis* species showing teeth lining the margins. Teeth are shown at equal scales, and the significance of the pie-charts is given in Fig. 2. White number above: species no.; below: number of accessions 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. handcokii*; 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. percentage of teeth area in the leaf (r). Inset: mode of measurement of the surfaces of entire leaf and margin teeth using Image J (Schneider *et al.*, 2012) (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 (×100) of the (sum of the teeth surfaces)/(entire leaf surface).
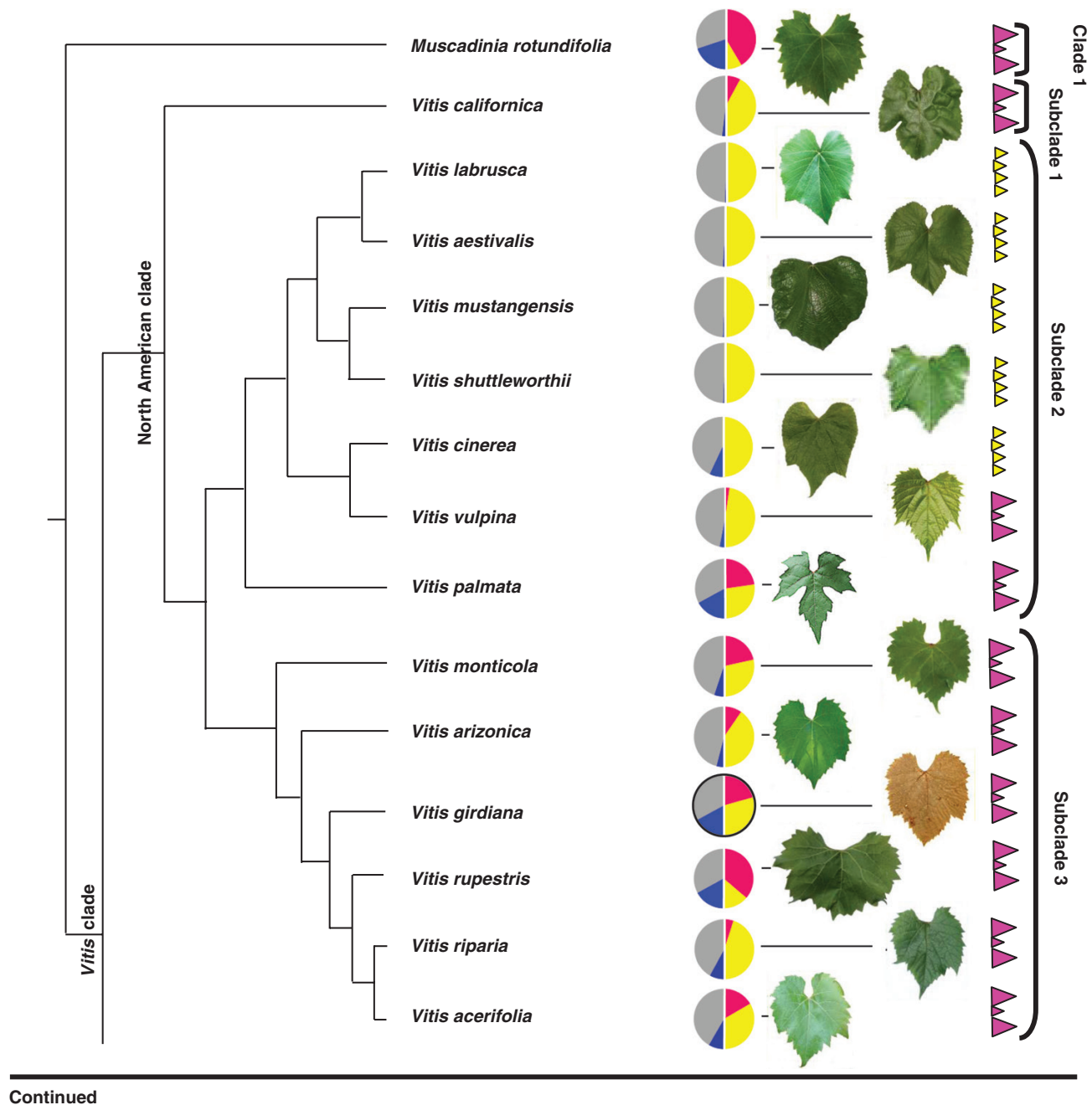
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FIG. 4. Tannin composition across *Vitis* species from North American (A) and Eurasian/Asian (B) subclades. Pie charts depict tannin composition in berries (no outline) or leaves (black-outlined). Right hemisphere indicates the proportion out of 100 % of dihydroxylated (EC + ECG; yellow) and trihydroxylated (EGC + EGCG; pink) units. The left hemisphere indicates the degree of galloylation in blue [(EGCG + ECG)/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. Consensus phylogenomic dendrograms taken from (A) Zecca *et al.* (2020) and (B) Ma *et al.* (2018*b*). Leaf sizes have been equalized. Doubly serrate with EGC; serrulate with minute teeth without EGC; serrulate with minute teeth with EGC.

new features added to the already known dramatic differences with *Vitis*, would need to be re-examined.

The genus Vitis

The studied 46 species and varieties from the genus *Vitis* 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 analysed for their PAs from pericarp and/or leaf
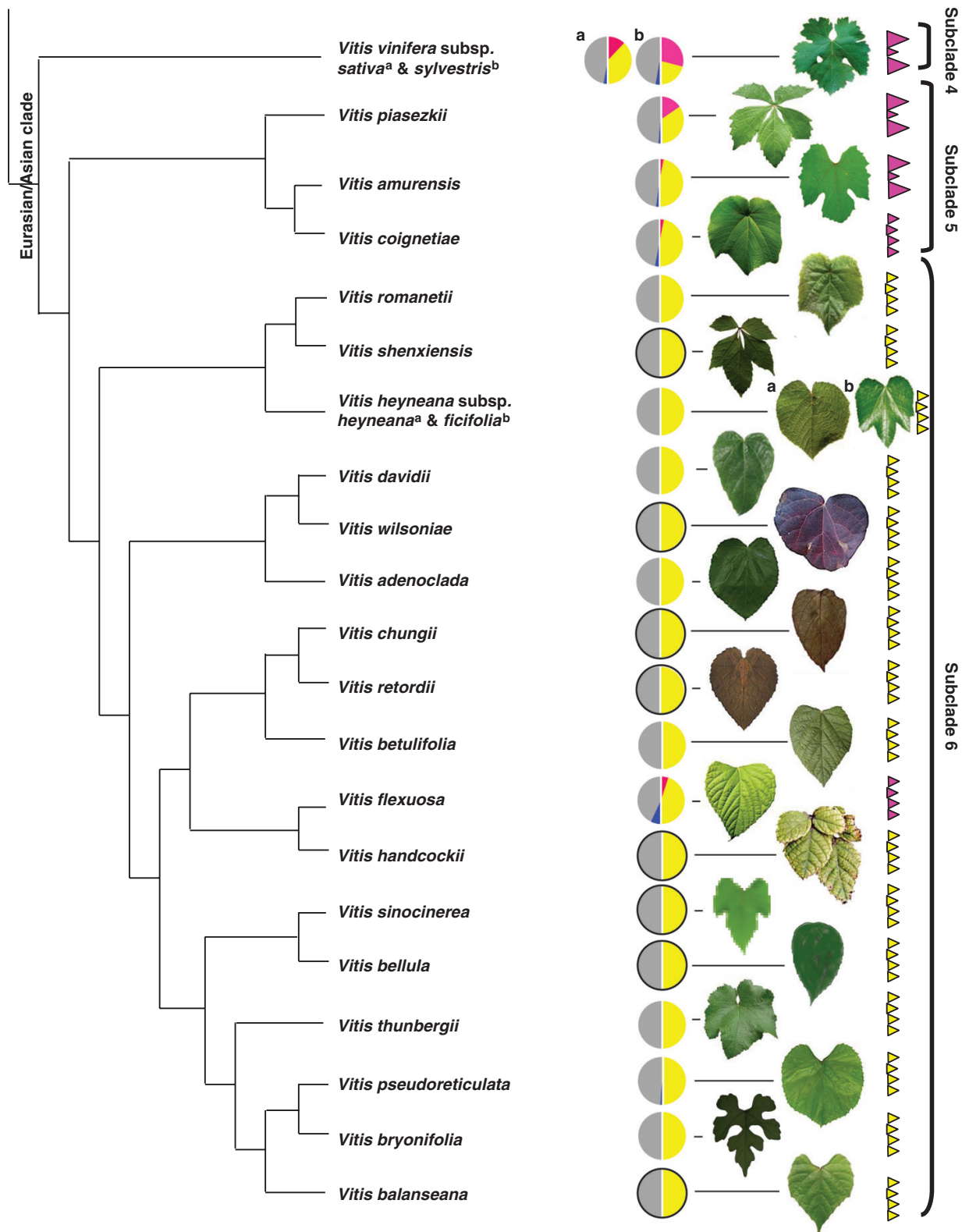
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(Supplementary Data Table S1); they represent 60 % of known wild *Vitis* from Eurasia/Asia (Wan *et al.*, 2008). Nineteen were EGC⁻ vines, i.e. 83 % of Eurasian/Asian and Asian tested *Vitis*

(*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 grow 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 3600 m (Tibetan tier), and *flexuosa* which extends from 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 analysed 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 at various proportions, from moderate (*V. amurensis* 1.9 % on average, *V. coignetiae* 2.4 %) to high (*piasezkii* 30.8 %, *V. vinifera* subsp. *sylvestris* 57.9 %). Note that *V. amurensis* and *V. coignetiae*, both growing in north-eastern China and south-eastern Russia (formerly Greater Manchuria), the Korean Peninsula, and Japan for the latter, group 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 groups 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).

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

Beyond the mountain ridge towards the west and bordering northern and western Pakistan, a single EGC⁺ vine, *V. vinifera* subsp. *sylvestris* and its cultivated form grow from Iran and Afghanistan to Portugal (Supplementary Data Fig. S4), and *V. vinifera* susbp. *sativa*, the cultivated grapevine, would have been 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) revealed an interesting divergence in that, similarly to subclade 5, and differently from other Asian wild vines of subclade 6 (*V. adenoclada*, *balanseana*, *bellula*, *betulifolia*, *bryonifolia*, *chungii*, *davidii*, *hancockii*, *heyneana* susbp. *heyneana*, *heyneana* subsp. *ficifolia*, *pseudoreticulata*, *retordii*,
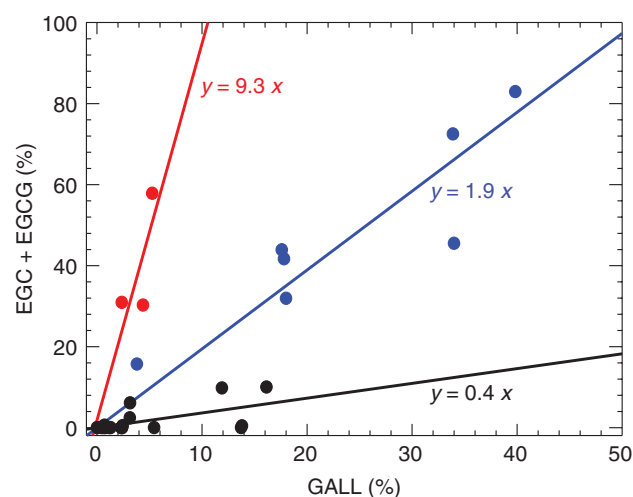

Fig. 5. Variation, among the *Vitis* species, of (EGC + EGCG) vs. GALL in berries. GALL is the percentage of galloylation [i.e. (EGCG + ECG)/sum of units]. Red: *Vitis piasezkii*, *V. vinifera* subsp. *sylvestris* and subsp. *sativa*, blue: other species, high (EGC + EGCG) accumulators, black: other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

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 because it was found at the root of subclade 3 of EGC⁻ American *Vitis*. If we consider our PA 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* group 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 into series (Planchon, 1887; Munson, 1909; Bailey, 1934; Galet, 1988; Moore, 1991) be abandoned in favour 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, except *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 because it is also noted in the genera *Cissus* and *Tetrastigma* where EGC⁺ and EGC⁻ coexist in one genus (Fig. 2).

Vitis interspecific hybrids. 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 a hybrid and that the three EGC-free accessions 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* × *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 clearly visible in the leaf margin teeth (Supplementary Data Fig. S5).

Moreover, we separately analysed sets of berries from ~300 accessions of the domestic grapevine (data not shown), and the average EGC content was around 30 %. The average EGC content of the nine 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.* (2018*a*) amply discussed the taxonomic positions of *Vitis piasezkii* Maxim. and *V. shenxiensis* C. Li, two often sympatric wild vines from Shaanxi province and nearby Henan provinces: indeed, on the one side, they were recognized as distinct species (Li *et al.*, 1996; Kong, 2004; Chen *et al.*, 2007), while, on the other, Niu and He (1996) argued that *V. shenxiensis* should be seen as a variety of *piasezkii*. Finally, Ma *et al.* (2018*a*) 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. Difficulties arise from the fact that *shenxiensis* appears morphologically as a hybrid between *V. romanetii* (bearing red trichomes on its stem) and *V. piasezkii* (multi-foliolated leaves) (Fig. 6).

Examination of leaf margin teeth and the analysis of leaf PAs from these two species allow them to be identified: 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 colour, 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 genus *Vitis* 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, proximity of *piasezkii* with the two *vinifera* is shown. 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); this is a bifurcation leading on the 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 good correlations; this peculiar behaviour 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, in contrast to phylogenomics which identify two clades, namely American and Eurasian/Asian (Klein *et al.*, 2018); this classification crosses the continental boundaries, revealing transcontinental distributions. The Eurasian/Asian vines are essentially free of EGC and bear serrulate leaf margins while the American vines are an ~50/50 mixture of EGC⁺ and EGC⁻ vines with doubly serrate and serrulate leaf margins, respectively. Although not yet clearly understood, this new result could prove to be a useful tool and offers a new perspective to scientists interested in the evolution of the genus *Vitis*. This usefulness of tannin phenotyping was previously illustrated in the genus *Musa* (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 based on 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
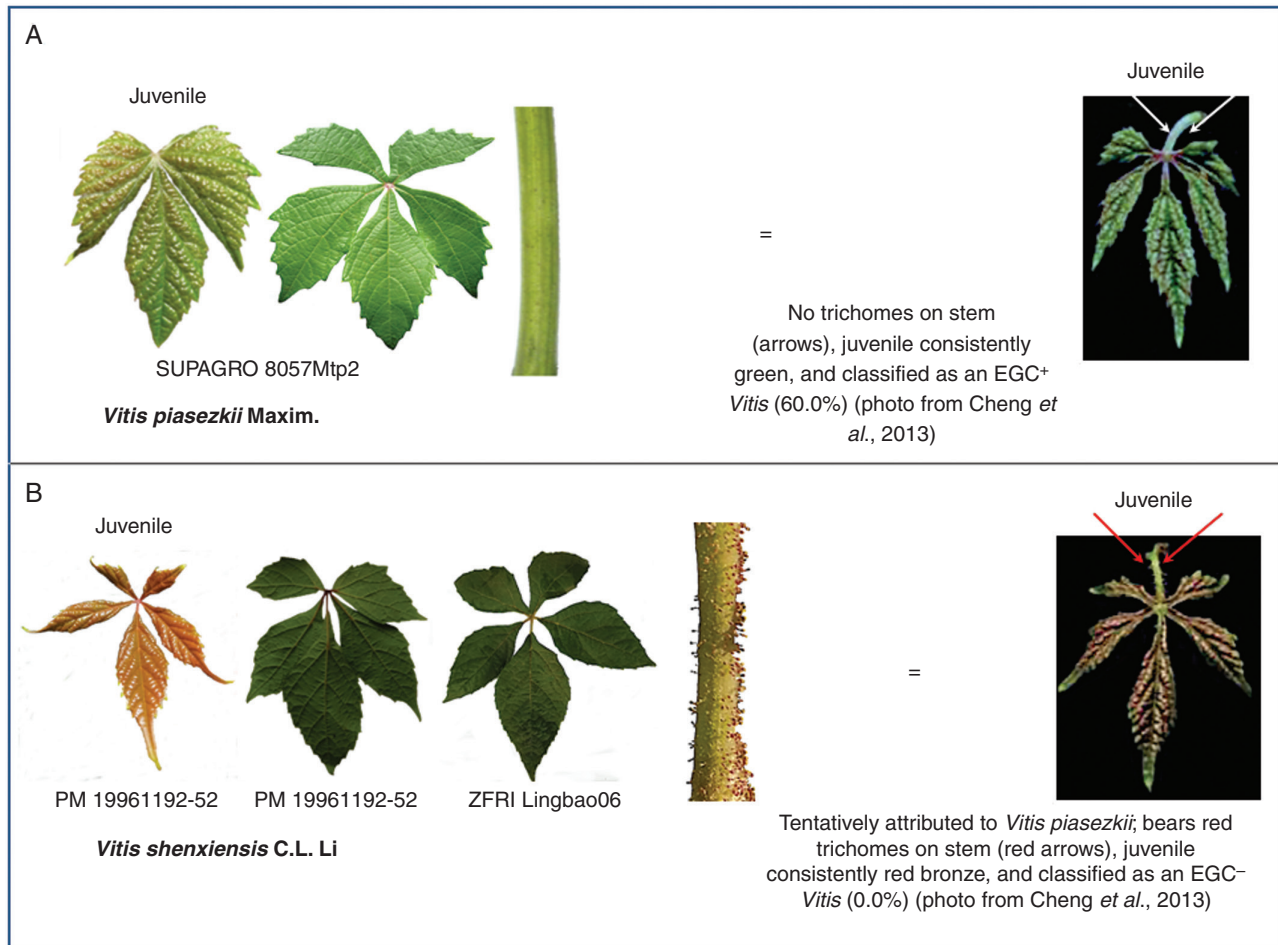
FIG. 6. Comparison of morphologies of leaves and stems of *Vitis piasezkii* and *Vitis shenxiensis*.

on detailed morphological, phylogenomic, ecological and geographic data. Additionally, and in agreement with Wen *et al.* (2018*b*), we suggest the production of an integrative systematics of grapevines and their close relatives, in particular seeking to supply up-to-date taxonomic revisions of the genus *Vitis* and other Vitaceae on an international basis and to generate informatics tools for soft public access online. This could be 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 that is still in its infancy; this could help in clarifying some uncertainties.

SUPPLEMENTARY INFORMATION

Supplementary data are available online at www.aob. oxfordjournals.org and consist of the following. Fig. S1. Leaves and stems of the studied genera and species from the Vitaceae. Fig. S2. Development of grape berries from *Vitis piasezkii* from fruit onset to full ripeness. Fig. S3. Variations of the molar ratios of the four PA constituents from berries of various species and accessions collected in the same place over two consecutive

years. Fig. S4. Worldwide distributions of vines. Fig. S5. Views of leaves from three true-to-type *Vitis* species and their hybrids with EGC + *Vitis*. Table S1. Molar composition of condensed tannins from green berry pericarps and leaves of the Vitaceae under study. Table S2. Variation of tannin molar ratios amongst berries from a unique cluster.

from different Institutes and Botanical Gardens for their help in organizing the sampling of berries and leaves. We are grateful to Profs. L. Torregrosa and T. Lacombe (SupAgro, Montpellier, France), and Drs A. Bervillé and J.-P. Péros (INRAe), and J. Mutzinger (IRD, Montpellier) for their advice.

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