Molecular barcode and morphological analysis of ~~the medicinal plant~~ *Smilax purhampuy* Ruiz~~, collected in~~ Ecuador

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Abstract

~~**Background.**~~
Smilax plants are distributed in tropical, subtropical and temperate regions in both hemispheres ~~throughout~~ of the world~~, . Common names of Smilax include sarsaparilla, vine of life and stick of life; and~~ are extensively used in different countries for traditional medicine. ~~Despite of the medicinal use of Smilax~~However, morphological and molecular barcodes analysis are lacking in Ecuador~~, which . Furthermore, molecular barcodes~~ could be used to assist ~~as a complement tool~~ in the ~~analysis and~~ taxonomic identification of species. ~~**Methods.**~~ For the evaluation of the micromorphological characteristics, cross sections of the leaves were performed by the manual method of S. *purhampuy*. The ~~powder drug of the~~ rhizomes was ~~also~~ analyzed for what?. All the samples were clarified with 1% sodium hypochlorite. Tissues were colored with 1% safranin in water, following fixation with glycerinated gelatin. For molecular barcode

characterization, total DNA extraction was performed from leaves using a modified CTAB method, and PCR was performed using primers for amplification of different *loci* including plastid genome regions *atpF-atpH* spacer, *matK* gene, *rbcL* gene, *rpoB* gene, *rpoC1* gene, *psbK–psbI* spacer, and *trnH–psbA* spacer; and the nuclear DNA sequence ITS2. DNA sequence similarity search was performed using blastn in the GenBank nr database and phylogenetic analysis was performed using the Maximun Likelihood method according to the best model found by MEGAX using bootstrap test (100 replicates). R**esults showed that … First report on** micromorphological characteristics of the leaves, 2nd report on rhizome analysis, 3rd report on molecular barcode characterization – as per sequence of method reported above .Amplicons were detected for all the barcodes tested except for the *trnH–psbA* spacer. Blast analysis revealed that the best hit for all the samples for each barcode were Smilax species; therefore, the its taxonomicy classification of Smilax was confirmed. Furthermore, the barcodes sequences *psbK-psbI, atpF-atpH, and (why typeset in italics?) ITS2* indicated a better resolution at the species level than the other barcodes tested in this study.

Introduction

1. The Introduction cannot consist of just one long paragraph with a number of different topics – each topic = separate paragraph. This reviewer attempted to create a number of self-contained paragraph, each with their own focal area.
2. The Introduction section provide NO information on *Smilax purhampuy* in Ecuador, which is the core species of this paper. Is it indigenous to Ecuador? What medicinal value does it have? Is it taxonomic position uncertain? etc etc.– remedy this by inserting information on *S. purhampuy* /or/ replace some of the general information of the genus with more specific information related to *S. purhampuy.*

The genus Smilax (all occurrences of Smilax should be typeset in *italics* – correct throughout paper), *genus* placed in the (family Smilacaceae,) consists of 310 species that are distributed in tropical, subtropical and temperate regions in both hemispheres throughout of the world (Cameron & Fu, 2006; Judd et al., 2009; Qi et al., 2013). Plants are dioecious, vines or herbaceous, or rarely, sub-shrubs or shrubs. The lLeaves are simple, alternate and contain petioles with tendrils; the primary venation of acrodrome. T; thickened stems are rippled stems, while and aerial stems are generally aculeate (Martins et al., 2013). In Ecuador, the genus is not monographed, although Gaskin (1999) indicates that around ten species of this genus have been reported in the country. Smilax is found predominantly in humid and very humid forests, from sea level to 2,500 meters above sea level in all the coastal provinces, Amazon and Andean areas (indicate source of this information). Reviewer inserted paragraph (above = morphology/taxonomy; below = ethnomedicinal uses)

~~Common names of~~ Smilax ~~include sarsaparilla, vine of life and stick of life; and~~ have~~are attributed~~ different uses in traditional medicine. For instance, in Brazil, Smilax (indicate specific species of Smilax used) is used as a tonic for rheumatism and as an anti-syphilitic (Andreata, 1997), ~~;~~ while in Central America, several species of Smilax is used for diuretic problems, dermatological infections, gastrointestinal disorders, rheumatism, vaginitis, contraception, for menstrual regulation, anemia, snake bites and arthritis. In Ecuador, Smilax species Re~~i~~s used for the elimination of excess of cholesterol and triglycerides, arthritis, intestinal, stomach, and prostate inflammations, chronic gastritis, and cysts (Ferrudino, 2004). From these uses, among the pharmacological activities demonstrated could be mentioned: glucose-lowering (Romo-Pérez et al., 2019), anti-hyperuricemic (Huang et al., 2019), anti-inflammatory and analgesic (Khana et al., 2019), diuretic (Pérez-Ramírez et al., 2016), antioxidant (Fonseca et al., 2017), among others. Among the chemical compounds identified for the *genus* include polysaccharides (Zhang et al., 2019), steroidal saponins (Luo et al., 2018), flavonoids (Wang et al., 2019), and others.
Reviewer inserted paragraph (above = ethnomedicinal uses; below paragraph = molecular barcoding)

Despite of the medicinal use of Smilax, morphological and molecular barcodes analysis are lacking in Ecuador. Molecular barcodes could be used as a complementary tool in the analysis and taxonomic identification of species. For instance, the plastid genome regions *atpF–atpH* spacer, *matK* gene, *rbcL* gene, *rpoB* gene, *rpoC1* gene, *psbK–psbI* spacer, and *trnH–psbA* spacer have been tested as universal plant barcodes (CBOL Plant Working Group, 2009). The chloroplast genes *rbcL* and *matK*, are recommended for characterization for land plants as a 2-*locus* combination (CBOL Plant Working Group, 2009). On the other hand, other *loci* have been proposed for the characterization of medicinal plants, including the nuclear sequence ITS2 (~~Techen et al., 2014;~~ Zhang et al., 2016). The present work ~~has as objective the~~investigated the micromorphological and the molecular barcode characterization of *Smilax purhampuy* Ruiz collected in Ecuador.

Materials ~~&~~ and Methods

Study area

The climate of the area is classified as rainy mega thermal with an average monthly temperature between 22°C and 26°C, and rainfall in a range of 2,000 to 3,000 mm per year. The study area consists of a tropical humid forest.

Collection of plant material

Plant material was collected from three specimens of *Smilax purhampuy* Ruiz in the Francisco de Orellana Province in Ecuador, (coordinates 1°10’03.7’’S 76°56’30.9’’W) during ~~the months of~~ March and April 2019 (Indicate~~. The climate of the area is classified as rainy mega thermal with an average monthly temperature between 22°C and 26°C, and rainfall in a range of 2,000 to 3,000 mm per year with a characteristic of tropical humid forest.~~ A branch of the species containing leaves, fruits and rhizome was taken to the

GUAY herbarium of the Faculty of Natural Sciences of the University of Guayaquil for its taxonomic characterization, being ~~characterized~~ identified as *Smilax purhampuy* Ruiz (voucher number 13,117).

Plant material preparation

The collected leaves and tubers were washed with plenty of water. The leaves for the micromorphological study were used fresh and for DNA extraction leaves were stored at -80°C. The tubers were dried in a Mettler Toledo brand stove at a temperature of 40°C. Once dried, samples of tubers were crushed with a manual knife mill and stored in amber glass jars for the corresponding analysis.

Micromorphological analysis (incomplete – see example below)

For the evaluation of the micromorphological characteristics, cross sections of the leaves were performed ~~by~~ according to the ~~the~~ manual method of ??? (reference needed for the manual method). The powder drug of the rhizomes was analyzed. All the samples were hydrated and clarified with 1% sodium hypochlorite. Tissues were colored with 1% safranin in water, following fixation with glycerinated gelatin according to Gattuso ~~&~~ and Gattuso (1999) as well as ~~nd~~ Miranda ~~&~~ and Cuellar (2000). Histochemical reaction with the Lugol reagent for the detection of starch was performed on the powdered drug from the rhizomes (Gattuso & Gattuso, 1999). Morphological analysis was performed using a light microscope NOVEL with 10x lens and coupled to HDCE-50B digital camera model HDCE-50B.

Example:

Anatomical preparation

The leaf samples were taken in the middle of the lamina, cutting the mid-rib transversely. Due to sampling limitations the maximum sample width was 5 mm, so that whole leaves were cut across when their widths were less than 5 mm, whereas only a part of the lamina, including the mid-rib, was considered for larger leaves. The samples were embedded in 5% agar and progressively dehydrated in 50, 70 and 95% ethanol (2 h per solution). The small blocks of agar were then infiltrated for 15 days with resin JB 4 (Polysciences). After polymerisation of the resin 2-µm-thick cross-sections were obtained with a glass ultra-microtome; then they were stained with 5% toluidine blue and permanently mounted onto slides with DPX (further details in Castro-Díez et al. 1998).

DNA extraction and PCR

Leaf samples from the three collected *Smilax purhampuy* Ruiz plants (with codes CIBE-010, CIBE-011, CIBE-012) were collected and ground with the MM400 (Retsch, Haan, Germnay) with liquid nitrogen and stored at -80°C. The DNA extraction was performed for each smilax plant independently. A modified CTAB protocol was used for total DNA extraction, according to ~~(~~Pacheco Coello et al.~~,~~ (2017). The master mix GoTaq® 2x (Cat# M7123, Promega) was used for PCR according to manufacturer instructions using 0.5 µM for each primer according to each barcode (Table 1) in a 50 µL PCR reaction. The conditions for the PCR includes 95°C for 3 min for initial denaturation; 35 cycles of: 95°C for 30 s, 50°C/56°C/60°C (depending of the barcode, Table 1) for 60 s, 72°C for 60 s; and a final extension of 72°C for 10 min. Amplicons were detected by sampling 5 µL in agarose gel (1.5%) electrophoresis. The remaining 45 µL was purified and sequence commercially

(Macrogen, Rockville, MD, USA). At least two technical replicates were sequences and a consensus was generated for each biological replicate.

Bioinformatic analysis

Sequences were processed using MEGAX (Stecher et al., 2020). Technical replicates were aligned with MUSCLE and a consensus sequences was generated for each barcode. Consensus sequences were blast (Zhang et al., 2000) in the GenBank nucleotide database (nr). Based on the blast analysis, different accessions were selected for phylogenetic analysis. For each barcode, the accessions and samples sequences were aligned using MUSCLE, and the recommended model from MEGAX was used. The Maximun Likelihood method was performed according to the best model found by MEGAX using bootstrap test (100 replicates).

Results

Morphological analysis (incomplete – see example at end of manuscript)

The micromorphological evaluation of a cross section of the leaf (Fig. 1A) showed a concave arrangement of the central vain (or mid rib)~~nerve~~ (nerve – conducts electrical impulses), being more pronounced in the lower part with a slight protuberance. The mesophyll with well-defined adaxial and abaxial epidermis was observed on the lateral sides of the central ~~nerve~~vein. Below the adaxial epidermis, a palisade parenchyma and the spongy parenchyma that borders with the abaxial epidermis were observed. An enlargement of the central nerve (Fig. 1B) showed the fundamental parenchyma formed by many isometric cells. Towards the center of the central nerve, the sclerenchyma tissue surrounding the well-defined vascular system (xylem and phloem) was observed.

The micromorphological analysis of the powder drug of rhizome (Fig. 2) allowed the visualization of a group of cells of the parenchyma of variable size (Fig. 2A), thickened xylematic vessels associated with other elements of the vascular system (Fig. 2B). Elongated, fusiform and pointed structures were also visualized, which correspond to fibers, and may suggest a type of filiform sclerides (Fig. 2C). In another sample of the powder drug (Fig. 2D), xylematic thickening vessels with holes were observed ~~(Khandelwal, 2004)~~ (results do not have citations). Numerous starch granules of variable size were observed, which had a blackish coloration with the Lugol reagent (Fig. 2E).

Molecular barcodes for *Smilax purhampuy* Ruiz plants

PCR amplification was detected for all barcodes except for *trnH-psbA* (data not shown). Blast analysis was performed for each barcode sequence (Data S1, Data S2) and the best hit for all the samples for each barcode indicates Smilax species (Table 2). The best hits in blastn for the different species includes: *S. nipponica* for *psbK-psbI* (96.57%, 96.56% and 93.83% of identity for the three biological replicates respectively); *S. nipponica* and *S. china* for *rpoB* (99.73% for the three biological replicates); *S. nipponica*, *S. china* and *S. aspera* for *rpoC1* (100%); *S. sieboldii* f. *inermis* for *atpF-atpH* (90.66%, 90.69% and 96.47%)~~,~~; *S. fluminensis* (99.65%, 100%), *S. bona-nox* (99.88%) and *S. laurifolia* (99.88%) for *matK*; *S. aspera* (99.82%, 99.82%) and *S. laurifolia* (99.82%) for *rbcL*; and~~,~~ *S. excelsa* (80.49%, 80.05%) for ITS2 (only two biological replicates were sequenced successfully for ITS2).

After alignment of the barcodes sequences between the Smilax sequences samples from this study with different accessions including other *genera*, the best models for phylogenetic analysis were T92+G (*psbK-psbI, atpF-atpH*), T92 (*rpoB*, ITS2), JC *(rpoC1*), T92+I (*mat*K), and JC+G (*rbc*L). Phylogenetic analysis revealed for the *psbK-psbI*, that the samples shared a clade (100 bootstrap) with different Smilax species including *S. china*, *S. nipponica*, and *S. glycophylla*; and with two accessions from another *genus (why typeset in italics?)* which includes *Hemidesmus indicus* (Fig. S1). However, the three samples of Smilax are grouped in a subclade (bootstrap 76). The other major clades in the phylogenetic tree correspond to other *genera (why typeset in italics?)*. Similarly, for the *rpoB* the phylogenetic tree revealed that the Smilax samples were in a clade (99 bootstrap) with other Smilax species; while other *genera* (indicate names of genera) were in other clades (Fig. S1). For the *rpoC1*, the Smilax samples were in a clade with different species of Smilax, including *S. aspera*, *S. nipponica*, *S. china*, and *S. herbacea* (Fig. S1) (where were these other Smilax species obtained?). The *atpF-atpH* phylogenetic analysis revealed that the Smilax species are in the same clade. H~~; h~~owever, the Smilax samples from this study are in a subclade (bootstrap 100). For the *mat*K and *rbc*L barcodes, the same pattern was observed where a clade sharing~~e~~ different Smilax species (Fig. S1), although a subclade is form with the two Smilax samples (CIBE-010, CIBE-011) and *S. fluminensis*; while for the Smilax CIBE-012 sample, a branch is shared with *S. aspera*. Furthermore, in the *rbc*L phylogenetic tree, a subclade is form for Smilax CIBE-010 and CIBE-011, while for the Smilax CIBE-012 a clade is shared with *S. domingensis* and *S. lauriflora*. On the other hand, the ITS2 phylogenetic tree revealed that the two Smilax samples (name them) are in a different clade (bootstrap 100) from the other Smilax species including *S. aspera*, *S. stans*, *S. menispermoidea*, *S. trachypoda*, *S. aberrans*, *S. retroflexa*, *S. excelsa*, *S. lunglingensis*, *S. hispida*, *S. japonica*, *S. china*, and *S. pumila*.

Discussion

Microscopic analysis

Microscopic evaluation involves the detailed assessment of the herbal drugs and is used to recognize them~~ organized drugs~~ based on their known histological characters (~~Mr.~~ Shailesh et al., 2015). Micromorphological studies are thus essential in quality control of plant drugs, since significant details are revealed to confirm the identity of the plant and identify possible adulterants.

The cross section~~s~~ of ~~the~~ *S. purhampuy* leaf indicated that the abaxial and adaxial epidermis are uniseriate, with the existence of an easily perceptible cuticle (how thick was the cuticle?) – not mentioned in results section. Morphoanatomic studies carried out on the leaves of various *Smilax* species (*S. brasiliensis*, *S. campestris*, *S. cisoides*, *S. fluminensis*, *S. goyazana*, *S. oblongifolia* and *S. rufescens*) revealed a non-stratified epidermis with thick cuticle (Martin ~~and col~~et al., 2013), in correspondence with the studies of the present investigation, which could be a distinctive anatomical characteristic~~on~~ of the ~~*Smilax*~~ genus.

In most plants, the leaf mesophyll is made up of palisade and spongy tissue, which differ in location, cell morphology, and function. In plants with a dorsiventral mesophyll, the palisade tissue is located on the adaxial side and the spongy tissue on the abaxial side, this distribution makes a greater contribution to the photosynthesis process (~~Ivanova and~~

P'yankov, 2002; *Yahia et al., 2019* - *why typeset in italics?*). Studies by various researchers have shown that the mesophyll can vary from one leaf to another of the same individual or of different individuals, depending on light intensity and salt concentration (Gapińska and Glińska, ; 2014). In *S. purampuy*, a dorsiventral mesophyll was observed where the palisade parenchyma (PP) is towards the adaxial side and the spongy parenchyma (SP) was located towards the abaxial side, in agreement with studies carried out by Martins and Appezzato-da-Glória (2006) to *S. polyantha* and Martin et al. (2013) for (which species?).

A peculiarity of the species studied was the fact of findingpresence of six vascular bundles of variable size at the level of the central nerve vein of the leaf (was this indicated as such in the results section?), which is in discrepancy with other *Smilax* species (indicate which species) that present three vascular bundles (Martin et al., 2013). Cannot have a 1 sentence paragraph

In other species (indicate which species) of the Smilax genus from Cuba and Guatemala, the presence of parenchyma tissue; xylematic vessels, fibers and starch granules are reported (Cáceres et al., 2012; González et al., 2017), which corresponds with the present study. This information which is in correspondence with the results of *S. purhampuy*. The difference lies within the morphology and arrangement of these structures. For example, in *S. domingensis* thickened scalariformly xilematic vessel and fibrotraqueids (fibers) were observed, while thickened xilematic vessel with holes and filiform sclerides (fibers) were visualized in the studied species.

Molecular barcode sequences

Genetic analysis has proven to be an important tool in the standardization of medicinal plants. The genotypic characterization of plant species is important since most plants, although they belong to the same genus and species, could show considerable variation in what?. DNA analysis is useful for identification of cells, individuals, or species; and could help distinguish genuine from adulterated drug (Kumari et al., 2016). The use of molecular barcodes could be used as a complementary analysis for identification of plants species. The recommended barcodes for species identification are the core 2-*locus* rbcL and matK (CBOL Plant Working Group, 2009). Generally, for species identification, the blastn analysis relies in the presence of those species in the GenBank. For this scenario, the three samples taxonomically identified as *Smilax purhampuy* Ruiz (CIBE-010, CIBE-011, CIBE-012) were more similar between them than other Smilax species (name them), as observed in the phylogenetic trees for *psbK-psbI* spacer, *atpF-atpH* spacer, and ITS2. Therefore, the other barcodes could identify accurately at the *genus* level. Furthermore, the ITS2 revealed low percentage (be specific) of identity at the blastn, suggesting that species differentiation could be detected using the ITS2. Similar results indicate a better resolution for species identification using the ITS2 in medicinal plants (Techen et al., 2014; Zhang et al., 2016; Bustamante et al., 2019; Sarmiento et al., 2020).

Conclusions

The morphological characteristics and the molecular barcodes analyses of the *Smilax purhampuy* Ruiz plants collected in Ecuador were performed. In this work, micromorphological characteristics of the leaves and rhizomes were described for the first

time which constitutes a novel contribution to the botanical characterization of the species. The taxonomy classification of Smilax was confirmed by the molecular barcodes used, including *psbK-psbI, rpoB, rpoC, atpF-atpH, mat*K, *rbc*L, and ITS2. Furthermore, the barcodes sequences *psbK-psbI, atpF-atpH, and ITS2* indicated a better resolution at the species level than the other barcodes tested in this study. However, further molecular barcode characterization could be performed in *Smilax* spp. from Ecuador, to analyze diversity and as a complement for taxonomic classification. Medicinal properties will be studied in the Smilax plants used in this study.

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Tables

Table 1. Primers used for amplification of the barcodes *psbA-trnH* spacer*, psbK-psbI* spacer*, rpoB, rpoC1, atpF-atpH* spacer*, rbc*L, *mat*K, and ITS2

Table 2. Blastn analysis for six different barcodes of *Smilax purhampuy* Ruiz plants (CIBE-010, CIBE-011, CIBE-012). Results were ranked for the first three with the highest percentage of identity.

Figures

Figure 1. Microscopic characteristics of the leaf from *Smilax purhampuy* Ruiz. Transversal section of the central nerve of the leaf (A, B). AbE, abaxial epidermis; AdE, adaxial epidermis; FP, fundamental parenchyma; Me, mesophyll; PP, palisade parenchyma; SP, spongy parenchyma; ST, sclerenchyma tissue; Vs, vascular system.

Figure 2. Microscopic characteristics of the powder drug from *Smilax purhampuy* Ruiz rhizome. A: parenchyma cells. B: xylematic veseels and other elements of the vascular system. C: fibers (filiform sclerides). D: xylematic veseels, E: starch granules

Supplemental information

Fig. S1. Phylogenetic analysis of molecular barcode sequences.

Data S1. Barcode DNA sequences of *Smilax purhampuy* Ruiz.

Data S2. Accession numbers of Smilax purhampuy Ruiz (GUAY 13117) barcode sequences at GenBank.

3. Results

3.1. Leaf anatomy

The ground-plan of all maple leaves is similar (Figs 1–6), despite considerable variation in overall leaf shape. All species have an upper epidermis, palisade mesophyll, 2–3 layers of spongy mesophyll, and lower epidermis. In all species, the minor veins form a reticulum which encloses blocks or areoles of mesophyll cells. Some minor veins terminate in the center of areoles. In *A. saccharum*, most veins form a closed network.

The percentage volume occupied by epidermis, vascular tissue, mesophyll and air for each species are given in Tab. 2. *Acer negundo* has a higher percentage volume of mesophyll than other species, and sun leaves of *A. saccharum* have the highest proportion of vascular tissue, but otherwise, all species are similar in quantitative tissue allocation. Species differed most strongly in the extent of deposition of chemicals within particular tissues: lignification of bundle sheath cells, and the occurrence of tannin or crystalline deposits in mesophyll and other tissues.

Most maple leaf veins are surrounded by a continuous layer of parenchyma or sclerenchyma forming a bundle sheath that separates vascular tissue from mesophyll. The bundle sheaths of *A. saccharum* veins (in both sun and shade leaves) are characterized by thickened walls forming a lignified fibrous encasement for the vascular tissues. Leaves of other maples have less sclerenchyma around the veins. *Acer negundo* veins show no sclerenchyma sheath (Fig. 1). Other cells form transcurrent bundle sheath extensions that connect veins with both upper and lower epidermis. Bundle sheath extensions vary in composition. In *Acer saccharum* a layer of cells in the lower bundle sheath extension is rich in crystalline material, possibly calcium oxalate (Fig. 4).

Tannin deposits occur in the mesophyll vacuoles of all species, except *A. negundo*. Tannins are not uniformly distributed between leaves, or within leaves. Some palisade cells may contain large amounts, appearing as large black deposits within the vacuoles, while nearby cells may contain much less. Mesophyll cell vacuoles may also contain other structurally different materials of uncertain nature.

The internal wall of upper epidermal cells is often swollen or distended with material ("slime"), probably an acidic polysaccharide. *Acer negundo,* alone among the maples, lacks all wall expansion.

3.2. Leaf toughness

Results for three leaf traits related to sclerophylly (puncture resistance, specific leaf weight, and leaf lamina thickness) are shown in Tab. 3. Puncture resistance probably most closely reflects true sclerophylly from the perspective of a leaf-chewing insect. Specific leaf weight is averaged over relatively large leaf areas, while lamina thickness is estimated from single points on the leaf surface; however, both can contribute to puncture resistance.

There are significant differences in puncture resistance among the maple species. *A. saccharum* sun and shade leaves are toughest, while the two understory species (*A. spicatum* and *A. pensylvanicum)* are least sclerophyllous. However, there is considerable overlap in the puncture resistances of all the species, suggesting a continuum in sclerophylly among maples.

Within each species, there is significant variability in puncture resistance among trees and among leaves, but the largest source of variance within species occurs within individual leaves (Tab. 3). The fine spatial scale of variation in puncture resistance suggest that an insect feeding on a single maple leaf may encounter very different leaf toughnesses, depending on its precise location.

Two components of sclerophylly, leaf lamina thickness and specific leaf weight, are not well correlated with differences in puncture resistance among the maples (Tab. 3). *Acer saccharum* shade leaves are thin and have low specific weight, yet have higher mean puncture resistance than other species. The specific leaf weight of *A. saccharum* sun leaves is nearly twice that of shade leaves, but there is no significant difference in the puncture resistance of these leaves.

The puncture resistance of all 5 maples is low, relative