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Scientific article

Phylogenetic analysis of some members of the subgenus *Persea* (*Persea*, Lauraceae)

Análisis filogenético de algunos miembros del subgénero Persea (Persea, Lauraceae)

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Abstract: The avocado belongs to the genus *Persea*, which is one of the most controversial genera of the Lauraceae family, since the relationships within the subgenus *Persea* are not clear and only recognized two species, *Persea americana* and *Persea schiedeana*. Its relationship with the subgenus *Eriodaphne* is also complex and there is a debate as to whether it is an independent genus. For this reason, the study aims to analyze the phylogenetic relationships within the genus *Persea*, with an emphasis on the subgenus *Persea*, using maximum parsimony and bayesian inference with the sequence of eight different fragments from nuclear, chloroplast and mitochondrial DNA. Sequences of the chloroplast *ndhF*, *rbcL*, *matK, rpoC*, *trnH-psbA*; mitochondria *atp4* and *cox3* and nuclear 18S rRNA were used. Fourteen fixed mutations were found in species of the subgenus *Eriodaphne*. The maximum parsimony and bayesian phylogenetic analyses of the super-matrices of the five chloroplast sequences and the eight concatenated ones, separated the members of both subgenera into two different clades with high bootstrap and posterior probability support, suggesting that the origin of *Persea* is not monophyletic and therefore both subgenera, *Persea* and *Eriodaphne*, could be recognized as phylogenetically independent genera.

Keywords: "aguacatillo", avocado, phylogeny, chloroplast DNA, mitochondrial DNA.

Resumen: El aguacate pertenece al género *Persea*, el cual es uno de los más controversiales de la familia Lauraceae debido a que las relaciones entre el subgénero *Persea* no están claras, y solo se reconocen dos especies, *Persea americana* y *Persea schiedeana*. Su relación con el subgénero *Eriodaphne* también es compleja, y existe un debate sobre si este es un género independiente. Por ello, el objetivo de esta investigación fue analizar las relaciones filogenéticas dentro del género *Persea*, con énfasis en el subgénero *Persea*, utilizando la máxima parsimonia e inferencia bayesiana con la secuencia de ocho fragmentos diferentes de ADN nuclear, cloroplástico y mitocondrial. Se emplearon secuencias del cloroplasto *ndhF, rbcL, matK, rpoC, trnH-psbA*, mitocondrias *atp4* y cox3, y *18S rARN* nuclear. Se encontraron 14 mutaciones fijas en especies del subgénero *Eriodaphne*. La máxima parsimonia, los análisis filogenéticos bayesianos de las supermatrices de las cinco secuencias de cloroplastos y las ocho

concatenadas separaron a los miembros de ambos subgéneros en dos clados diferentes con un alto *bootstrap* y soporte de probabilidad posterior. Lo anterior sugiere que el origen de *Persea* no es monofilético y, por lo tanto, ambos subgéneros, *Persea* y *Eriodaphne*, podrían ser reconocidos como géneros filogenéticamente independientes.
Palabras clave: "aguacatillo", aguacate, filogenia, ADN de cloroplastos, ADN mitocondrial.

Introduction

Avocado (*Persea americana* Mill.) is today among the most economically important subtropical/tropical fruit crops in the world (Bost, Smith, & Crane, 2013), with a production of avocado fruit that now exceeds 3.5 million tons, of which about 20 % is traded internationally (Schaffer, Wolstenholme, & Whiley, 2013). Chanderbali et al. (2008) consider avocado as the most important commodity from the Lauraceae.

The conservation of avocado genetic resources and their relatives is important to deal with the potential problems of the avocado industry in the future. Threats to the avocado industry have appeared recently, such as laurel wilt, caused by the fungus *Raffaelea lauricola* symbiont of the ambrosia beetle (*Xyleborus glabratus*) that has been responsible for the extensive death of native Lauraceae in the United States since 2000, when it was first detected (Fraedrich et al., 2008). In August 2011, a dooryard avocado tree immediately north of the focus was affected by laurel wilt (Ploetz et al., 2015), close to the center of avocado production in Florida, USA. Resistance to this disease is now of high priority; the pool to search for this resistance is in the genetic resources of the genus *Persea*.

Germplasm banks have tried to conserve the existing diversity of avocado and its relatives (Barrientos, 2010), one of them located in the Fundación Salvador Sánchez Colín-CICTAMEX, S.C., which is considered the richest in respect to diversity and variability, and which started to concentrate more diversity in 1988 (Barrientos, 1999). The variability of this germplasm bank has been reported (López-López, Barrientos-Priego, & Ben-Ya'acov, 1999), as well its potential (Ben-Ya'acov & Barrientos, 2003), along with molecular characterization of some accessions with RAPD (Reyes-Alemán, Valadez-Moctezuma, Simuta-Velázco, Barrientos-Priego, & Gallegos-Vázquez, 2013), ISSR (Reyes-Alemán, Valadez-Moctezuma, & Barrientos-Priego, 2016), SSR (Gutiérrez-Díez, Barrientos-Priego, & Campos-Rojas, 2015) and with the sequence *trnL-trnF* of cpDNA (Cabrera-Hernández et al., 2017). In these studies, the great variability existing in that germplasm bank was evident, where the accessions represent above all the diversity that exists in the subgenus *Persea*.

The knowledge of the phylogenetic relationships of the subgenus *Persea* with the subgenus *Eriodaphne* is important to take decisions in relation to management and organization of germplasm banks and to guide future collections, in addition to defining actions with respect to genetic improvement.

The genus *Persea* L. (Lauraceae) consists of about 85 species distributed in America (Barrientos-Priego, Muñoz-Pérez, Borys, &

Martínez-Damián, 2015), some new species have been described (Lorea-Hernández, 2002; van der Werff, 2002) and there are probably over a 100 species. The genus is distributed from the southern United States (*Persea borbonia* [L.] Spreng) to Chile (*Persea lingue* Ruiz & Pavon), with one species in the Canary Islands (*P. indica* [L.] Spreng.) and probably some representatives in South Asia (Barrientos-Priego et al., 2015); nevertheless, it is controversial as to whether *Persea* should be treated as including species from Asia since results suggest that *Persea* is strictly American (Li et al., 2011). The genus is divided into the subgenera *Persea* and *Eriodaphne* (Kopp, 1966); the first one has fruits known as real avocados (~ 5 to 20 cm) and the second tiny avocados known as "aguacatillos" (< 5 cm).

Within subgenus *Persea*, *P. americana* Mill. is the most studied species, mainly for its importance as a human food resource, and especially for its high oil content. For these reasons, and considering the graft compatibility among species, attempts to use species of subgenus *Eriodaphne* as a rootstock for *P. americana* to improve resistance to *Phytophthora cinnamomi* Rands. have been explored; however, the unsuccessful results revealed a vegetative incompatibility between species of both subgenera (Frolich, Schroeder, & Zentmyer, 1958).

There is a great controversy about the monophyletic origin of the genus *Persea*, indicating that phylogenetic studies based on morphological characters are not conclusive (Rohwer et al., 2009), and the subgenera *Persea* and *Eriodaphne* might perhaps be recognized as independent genera. However, a recent study by Li et al. (2011) shows *Persea* as monophyletic again, if *Apollonias* is included and a few aberrant species excluded. Several studies of the Lauraceae family based on molecular data give some information about *Persea* phylogeny (Chanderbali, van der Werff, & Renner, 2001); nevertheless, the inclusion of few species and specimens made the results uninformative for the *Persea-Eriodaphne* clade. The subgenus *Eriodaphne* has been studied by sequencing fragments of nuclear and chloroplast DNA more extensively by other authors (Chanderbali et al., 2001; Li et al., 2011; Rohwer et al., 2009), while the subgenus *Persea* has not. Cabrera-Hernández et al. (2017) in their study indicated that other sequences (chloroplast, mitochondrial and nuclei) must be studied in a concatenated way to have a better resolution of the subgenus *Persea*.

Specifically, within *Persea*, the cladistic analysis of Campos-Rojas, Terraza, and López-Mata (2007), the ITS phylogenetic study of Rohwer et al. (2009) and the *trnL-trnF* of cpDNA study of Cabrera-Hernández et al. (2017) could separate into different clades the species of the subgenus *Persea* from the species of *Eriodaphne*, supporting the hypothesis of a polyphyletic origin of the genus *Persea*, and providing an explanation of the vegetative (Frolich et al., 1958) and gametic (Lahav & Lavi, 2013) incompatibility between the two subgenera. However, controversy still exists on this issue, because the phylogenetic relationships between the two subgenera are very complex (Kopp, 1966), and so far, there is

insufficient evidence from molecular DNA data for the separation of the two subgenera of *Persea*.

In several families of angiosperms, DNA sequences of coding regions, intergenic spacers and internal transcribed spacers of the chloroplast, mitochondria, and nucleus have been used in a concatenated form to obtain a better understanding of the phylogenetic relationships of the taxa analyzed. Among the most used genes are: *rbcL* (Kress & Erickson, 2007), *ndhF* (Beilstein Nagalingum, Clements, Manchester, & Mathews, 2010), *matK, rpoC1* (Chase et al., 2007), and the intergenic spacer region *trnH-psbA* (Dong, Liu, Yu, Wang, & Zhou, 2012) from chloroplast DNA. Also, fragments of mitochondrial DNA, such as *atp4* gene (Duminil, Pemonge, & Petit, 2002), and the nuclear *18S rRNA* gene have been considered. With these novel analyses, it is evident that information from different DNA genes of several *Persea* species is necessary to reconstruct the phylogenetic history of this genus. For this reason, the study aims to analyze the phylogenetic relationships within the genus *Persea*, with an emphasis on the subgenus *Persea*, using maximum parsimony and bayesian inference with the sequence of eight different fragments from nuclear, chloroplast and mitochondrial DNA.

Material and methods

Plant material

Plant material from 35 specimens of the genus *Persea*, 29 of *Persea* subgenus and five of *Eriodaphne* subgenus, and one from *Beilschmiedia anay* (Blake) Kosterm, were obtained from *Fundación Salvador Sánchez Colín-CICTAMEX*, S.C. germplasm bank (Coatepec Harinas, Mexico), and from specimens deposited at the herbarium of the Forestry Department at *Universidad Autónoma Chapingo*, Mexico (CHAP). The specimens are from locations inhabited by the genus in Mexico and other countries (Table 1). The accessions included in the study represent practically all the diversity (seven species) of the subgenus Persea, according to the Kopp (1966) classification, although the unrecognized species *Persea zentmayerii* is not included (Schieber & Bergh, 1987). In the case of *Persea americana,* all races or botanical varieties were included, as well as the proposed fourth race *Persea americana* var. *costaricensis*. In addition, some hybrids were considered (Table 1), as well as *Beilschmiedia anay* that was used as an outgroup.

Table 1

Fundación Salvador Sánchez Colín-CICTAMEX collection accession number, place of origin and GenBank accession numbers of the species used in the analysis.

Species name / Nombre de la especie	Accession number / Número de accesión	Location of origin / Lugar de origen	GenBank accession number / Número de accesión del GenBank							
			trnH-psbA	*matK*	*rpoC1*	*cox3*	18S rRNA / 18S rARN	*atp4*	*rbcL*	*ndh*
Genera *Beilschmiedia* / Género *Beilschmiedia*										
Beilschmiedia anay	CG-Hu-56	Puebla, México.	JF966434	JF966448	JF966482	JF966516	JF966550	JF966584	JF966618	JF966644
Género *Persea*										
Subgenera *Eriodaphne* / Subgénero *Eriodaphne*										
P. chamissonis	CHAP 37473[Z]	Hidalgo, México	JF966426	JF966466	JF966500	JF966534	JF966568	JF966602	JF966636	JF966661
P. cinerascens	CH-C-30	Michoacán, México	JF966431	JF966452	JF966486	JF966520	JF966554	JF966588	JF966622	JF966670
P. lingue	CH-Pl-1	Chile	JF966423	JF966445	JF966479	JF966513	JF966547	JF966581	JF966615	JF966641
P. longipes	CH-G-36	Veracruz, México	JF966424	JF966456	JF966490	JF966524	JF966558	JF966592	JF966626	JF966652
P. sp. 'PR'	CH-PR-1	Veracruz, México	JF966432	JF966457	JF966491	JF966525	JF966559	JF966593	JF966627	JF966671
Subgenera *Persea* / Subgénero *Persea*										
Persea americana (*P.a.*)										
P. a. var. americana	CH -CR- 28	Costa Rica	JF966410	JF966454	JF966488	JF966522	JF966556	JF966590	JF966624	JF966650
P. a. var. americana	CH-G-48	Yucatán, México	JF966396	JF966442	JF966476	JF966510	JF966544	JF966578	JF966612	JF966669
P. a. var. americana	CH-G-45	Yucatán, México	JF966416	JF966450	JF966484	JF966518	JF966552	JF966586	JF966620	JF966646
P. a. var. americana	CH-I-6	Veracruz, México	JF966403	JF966458	JF966492	JF966526	JF966560	JF966594	JF966628	JF966653
P. a. var. drymifolia x *P. a.* var. guatemalensis	'Hass'	California, Estados Unidos	JF966409	JF966447	JF966481	JF966515	JF966549	JF966583	JF966617	JF966643
P. a. var. costaricensis	CH-CR-25	Costa Rica	JF966430	JF966438	JF966472	JF966506	JF966540	JF966574	JF966608	JF966665

Table 1 (Cont.)

Fundación Salvador Sánchez Colín-CICTAMEX collection accession number, place of origin and GenBank accession numbers of the species used in the analysis.

P. a. var. costaricensis	CH-CR-44	Costa Rica	JF966407	JF966437	JF966471	JF966505	JF966539	JF966573	JF966607	JF966664
P. a. var. drymifolia	CH-C-10	Puebla, México	JF966395	JF966441	JF966475	JF966509	JF966543	JF966577	JF966611	JF966668
P. a. var. drymifolia	CH-C-47	Michoacán, México	JF966411	JF966462	JF966496	JF966530	JF966564	JF966598	JF966632	JF966657
P. a. var. drymifolia	CH-C-57	México, México	JF966397	JF966443	JF966477	JF966511	JF966545	JF966579	JF966613	JF966639
P. a. var. drymifolia	CH-C-63	México, México	JF966402	JF966453	JF966487	JF966521	JF966555	JF966589	JF966623	JF966649
P. a. var. drymifolia	CH-Der-2	México, México	JF966401	JF966451	JF966485	JF966519	JF966553	JF966587	JF966621	JF966648
*P. a.*var. guatemalensis	CH-G-7 S2	Chiapas, México	JF966413	JF966464	JF966498	JF966532	JF966566	JF966600	JF966634	JF966659
P. a. var. guatemalensis	CH-G-11 S1	Chiapas, México	JF966412	JF966463	JF966497	JF966531	JF966565	JF966599	JF966633	JF966658
P. a. var. guatemalensis	CH-GU-5	Guatemala	JF966417	JF966455	JF966489	JF966523	JF966557	JF966591	JF966625	JF966651
P. a. var. guatemalensis	CH-GU-6	Guatemala	JF966399	JF966449	JF966483	JF966517	JF966551	JF966585	JF966619	JF966645
P. floccosa	CH-I-3	Veracruz, México	JF966406	JF966435	JF966469	JF966503	JF966537	JF966571	JF966605	JF966647
P. a. var. drymifolia	CH-I-2	México, México	JF966398	JF966444	JF966478	JF966512	JF966546	JF966580	JF966614	JF966640
P. nubigena	CH-G-76	Chiapas, México	JF966414	JF966467	JF966501	JF966535	JF966569	JF966603	JF966637	JF966662
P. nubigena	CH-I-4	Israel	JF966425	JF966459	JF966493	JF966527	JF966561	JF966595	JF966629	JF966654
P. parvifolia	CH-Ve-2	Veracruz, México	JF966408	JF966446	JF966480	JF966514	JF966548	JF966582	JF966616	JF966642
P. schiedeana	CH-Der-1	Veracruz, México	-	JQ352803	-	-	-	-	-	-

Table 1 (Cont.)
Fundación Salvador Sánchez Colín-CICTAMEX collection accession number, place of origin and GenBank accession numbers of the species used in the analysis.

P. schiedeana	CH-Gu-1	Guatemala	JF966420	JF966440	JF966474	JF966508	JF966542	JF966576	JF966610	JF966667
P. schiedeana	CH-H-5	Honduras	JF966404	JF966460	JF966494	JF966528	JF966562	JF966596	JF966630	JF966655
P. schiedeana	CH-H-7	Honduras	JF966418	JF966465	JF966499	JF966533	JF966567	JF966601	JF966635	JF966660
P. schiedeana x *P. a.* var. guatemalensis	CH-C-62	Guatemala	JF966405	JF966461	JF966495	JF966529	JF966563	JF966597	JF966631	JF966656
P. steyermarkii	CH-G-Ch1	Chiapas, México	JF966429	JF966439	JF966473	JF966507	JF966541	JF966575	JF966609	JF966666
P. tolimanensis	Mv1	Chiapas, México	JF966433	JF966468	JF966502	JF966536	JF966570	JF966604	JF966638	JF966663
P. sp. 'Freddy 4'	CH-CR-29	Costa Rica	JF966428	JF966436	JF966470	JF966504	JF966538	JF966572	JF966606	JF966672

DNA extraction, amplification, and sequencing

DNA was extracted from ~ 50 to 100 mg of leaves previously dried in silica gel. In some cases, leaves from herbarium specimens were used. Genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) based method (Gambino, Perrone, & Gribaudo, 2008). At the end of the procedure, the DNA was purified with Qiaquick columns (Qiagen®, USA) following manufacturer's instructions. The quality and quantity of the DNA were evaluated with a NanoDrop® ND-1000 spectrophotometer. The amplification of each of the eight fragments was performed in a total volume of 25 µL containing: 50 to 100 ng of DNA, 200 µM of dNTPs mix, 1X Colorless GoTaq® Flexi Reaction Buffer (Promega, USA), 20 pM of specific primers (Table 2), 2.5 mM of $MgCl_2$ and 2 U of GoTaq® Flexi DNA Polymerase (Promega, USA). Amplification programs consisted of one cycle of an initial denaturation of 4 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at specific melting temperature (Table 2) and 1 min at 72 °C, finally an extension of 5 min at 72 °C. The amplification reactions were performed in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, USA).

Table 2
Primers used in the amplification and sequencing of mitochondrial, nuclear and chloroplast DNA.

Locus/ segment / Locus/ segmento	Name / Nombre	Sequence 5'-3' / Secuencia 5'-3'	Tm (°C) / Tf (°C)	Reference / Referencia
n^z *18S rRNA* / n^z *18S rARN*	*NS1*	GTAGTCATATGCTTGTCTC	56	White, Bruns, Lee, & Taylor (1990)
	NS4	CTTCCGTCAATTCCTTTAAG	56	White et al. (1990)
	NS5	AACTTAAAGGAATTGACGGAAG	56	White et al. (1990)
	NS8	TCCGCAGGTTCACCTACGGA	56	White et al. (1990)
cp *rpoC1*	1f	GTGGATACACTTCTTGATAATGG	56	Ford et al. (2009)
	4r	TGAGAAAACATAAGTAAACGGGC	56	Ford et al. (2009)
cp *trnH-psbA*	trnH2	CGCGCATGGTGGATTCACAATCC	51	Tate & Simpson (2003)
	psbAF	GTTATGCATGAACGTAATGCTC	51	Tate et al. (2003)
cp *rbcL*	1f	ATGTCACCACAAACAGAAAC	56	Olmstead, Michaels, Scott, & Palmer (1992)
	724r	TCGCATGTACCTGCAGTAGC	56	Fay, Swensen, & Chase (1997)
cp *ndhF*	389f	CTGCBACCATAGTMGCAGCA	59	This study / Presente estudio
	461r	GATTRGGACTTCTRSTTGTTCCGA	59	This study / Presente estudio
cp *matK*	1326R	TCTAGCACACGAAAGTCGAAGT	48	Schmitz-Linneweber et al. (2001)
	390F	CGATCTATTCATTCAATATTTC	48	Schmitz-Linneweber et al. (2001)
mt *atp4*	Orf1	AAGACCRCCAAGCYYTCTCG	50	Duminil et al. (2002)
	Orf2	TTGCTGCTATTCTATCTATT	50	Duminil et al. (2002)
mt *cox3*	Cox3r	CTCCCCACCAATAGATAGAG	51	Duminil et al. (2002)
	Cox3f	CCGTAGGAGGTGTGATGT	51	Duminil et al. (2002)

The amplified DNA fragments were visualized on a 1.2 % agarose gel stained with ethidium bromide. The polymerase chain reaction (PCR) products were cleaned using Qiaquick® PCR Purification Kit columns (Qiagen, USA), following the instructions provided by the manufacturer. The PCR products were sequenced directly using the same primers (Table 2) in an automated sequencing system in Macrogen Inc., South Korea. The sequences were edited and assembled with the BioEdit version 7.0.9.0 program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Sequence alignment

The 34 sequences obtained from the intergenic spacer *trnH-psbA*, *ndhF*, *rbcL*, *rpoC1*, 18S rRNA, *cox3*, and *atp4* genes, and 35 from the *matK* gene (Table 2) were aligned with MUSCLE version 3.8 (Edgar, 2004). Additionally, 16 sequences of *matK* were aligned with 36 sequences downloaded from GeneBank (http://ncbi.nlm.nih.gov): two of *Persea* and 18 from the closely related genera (*Sassafras*, *Litsea*, *Lindera*, *Ocotea*, *Cinnamomum*, *Nectandra*, *Actinodaphne*, *Parasassafras*, *Sinosassafras*, *Neolitsea*, *Iteadaphne*, *Endlicheria*, *Aniba*, *Laurus*, *Umbellularia*, *Alseodaphne*, *Phoebe* and *Machilus*). Afterward, two super-matrices, the first one with the chloroplast DNA sequences: *ndhF* + *rbcL* + *matK* + *rpoC1* + *trnH-psbA* and the second with all eight, were built manually.

Phylogenetic analysis

The 52 aligned sequences of *matK*, and the two super-matrixes mentioned above were analyzed with maximum parsimony (MP) using PAUP ver. 4.0b10 software (Swofford, 2001) and bayesian inference (BI) using MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck, 2003). The mitochondrial genes and the nuclear rDNA data were not analyzed separately since they did not show sufficient informative characters. In each analysis of MP, all the characters were weighted equally, and gaps treated as missing data. A set of the most parsimonious trees from the different datasets was obtained through heuristic searches of 1,000 replicates with random stepwise sequence addition, tree bisection-reconnection branch (TBR) swapping, "MulTrees" option in "effect", and saving 10 trees from each random sequence addition. Robustness of clades was estimated by a bootstrap analysis with 1,000 replicates with simple sequence addition, TBR swapping and holding only 10 trees per replicate to reduce time spent in swapping on large numbers of suboptimal trees. The BI was performed using the GTR + G model and two independent replicates of four chains with a maximum of 10 million generations, with trees sampled every 100 generations.

Results

Features of the sequence alignments

A total of 273 sequences were obtained from *ndhF*, *rbcL*, *matK*, *rpoC1*, *trnH-psbA*, 18S rRNA, *atp4* and *cox3*; all of them were deposited at GenBank under Accession numbers JF966395-JF966399, JF966401-JF966414, JF966416-JF966418, JF966420, JF966423-JF966426, JF966428-JF966672, and JQ352803 (Table 1). The *trnH-psbA* alignment held the highest variation, with 32 parsimony-informative sites (Pi, 6.44 %), and 67 variable sites (VS, 13.48 %) (Table 3). The mitochondrial genes *atp4* and *cox3* held the least variation, with 0 to 1 Pi sites, and 0.18 and 0.43 % VS, respectively (Table 3); despite the low informative sites obtained, it was decided to include them. *Beilschmiedia anay* CG-Hu-56 had the most divergent sequence in the eight sequences, by a variation of 0-4 % with *P. americana* sequences. *B. anay* CG-Hu-56 was used as an outgroup in the phylogenetic analysis.

Table 3

Description of sequence alignments of 34 materials of *Persea* genus and one of *Beilschmiedia anay*.

Locus/segment/ Locus/segmento	Alignment length (bp)/ Longitud de alineación (bp)	CR[z]/ RC[z]	NCR/ RNC	Pi (%)/ IP (%)	CS (%)/ SC (%)	VS (%)/ SV (%)	S	EFM/ MFE
n 18S rRNA/n 18S rARN	1748	0	1748	6 (0.34)	1719 (98.34)	29 (1.69)	23	2
cp *rpoC1*	599	599	0	2 (0.33)	577 (96.33)	22 (3.67)	20	2
cp *trnH-psbA*	497	98	399	32 (6.44)	428 (86.12)	67 (13.48)	41	5
cp *rbcL*	1481	1428	53	10 (0.67)	1390 (93.86)	91 (6.14)	81	4
cp *ndhF*	739	739	0	4 (0.54)	707 (95.67)	32 (4.33)	28	0
cp *matK*	909	909	0	7 (0.77)	866 (95.27)	43 (4.73)	36	1
mt *atp4*	507	507	0	1 (0.20)	501 (99.82)	6 (1.18)	5	0
mt *cox3*	695	695	0	0 (0.00)	692 (99.57)	3 (0.43)	3	0
matK+rbcL+ndhF+rpoC1+ trnH-psbA	4236	3773	463	55 (1.30)	3965 (93.60)	261 (6.16)	206	12
18S rRNA+*cox3+atp4+matK+ rbcL+ ndhF+rpoC1+trnH-psbA*	7183	4983	2200	62 (0.86)	6874 (95.69)	299 (4.16)	237	14

Phylogenetic analysis of matK

A large phylogenetic analysis was performed with the *matK*. To place the subgenera *Persea* and *Eriodaphne* inside the Lauraceae family, representatives of 18 closely related genera were included in the analysis. Both the BI and the MP approaches resulted in relatively congruent topologies concerning subgenus *Eriodaphne* and the *Litsea-Ocotea* clade, and although *Persea* subgenera species were grouped with a weak Posterior Probability (PP) in BI, the bootstrap (BS) majority rule consensus tree from MP does not support this clade (Figure 1). The MP and BI recovered the subgenus *Eriodaphne* and the *Litsea-Ocotea* clade with weak BS and strong PP, BS values for these clades are 52 and 66 %, and BI support for the same branches is 86 and 96 %, respectively. Within the *Eriodaphne* clade, both analyses support the subclade *P. lingue-P. longipes*, with 63 and 100 % of BS and PP, respectively. In the *Litsea-Ocotea* clade, both analyses support the formation of eight different subclades, mainly with species of the same genera, with 63 to 98 % of BS values and 71 to100 % of PP (Figure 1). *Beilschmiedia anay* JF966448 and *Machilus rimosa* AB259098 are separated from the main core (100 % PP).

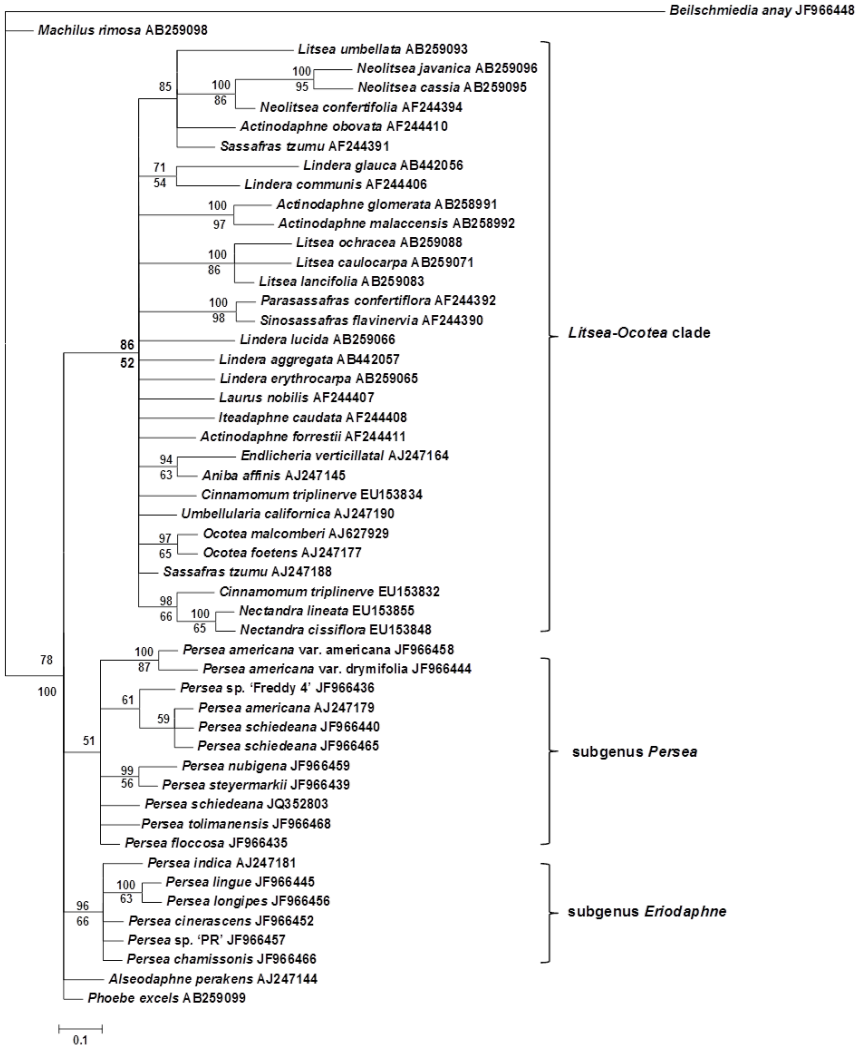

Figure 1

Bayesian 50 % majority rule consensus phylogram resulting from the analysis of partial sequences of the *matK* gene of *Persea* and other genera of Lauraceae. Posterior probabilities are indicated above the nodes, and maximum parsimony bootstrap support values (where 50 %) appear below the nodes. In the parsimonious analysis, 133 equally parsimonious trees with a length of 121 steps, and a consistency index of 0.88, homoplasy index of 0.12 and a retention index of 0.88 were obtained.

Analysis of the concatenated chloroplast sequences

The phylogenetic analysis of the five chloroplast sequences was performed with sequences of 34 different plant accessions evaluated in this study, with members of the subgenera *Persea* and *Eriodaphne*, plus *Beilschmedia anay*. The BI and MP analyses resulted in relatively congruent topologies (Figure 2). The analyses recovered two major clades, subgenus *Eriodaphne* and subgenus *Persea*, with well-supported BS/PP (88/100 %) and moderate values (82/84 %), respectively. This indicates that the additional parsimony informative characters from the other chloroplast sequences may have improved the phylogenetic signal.

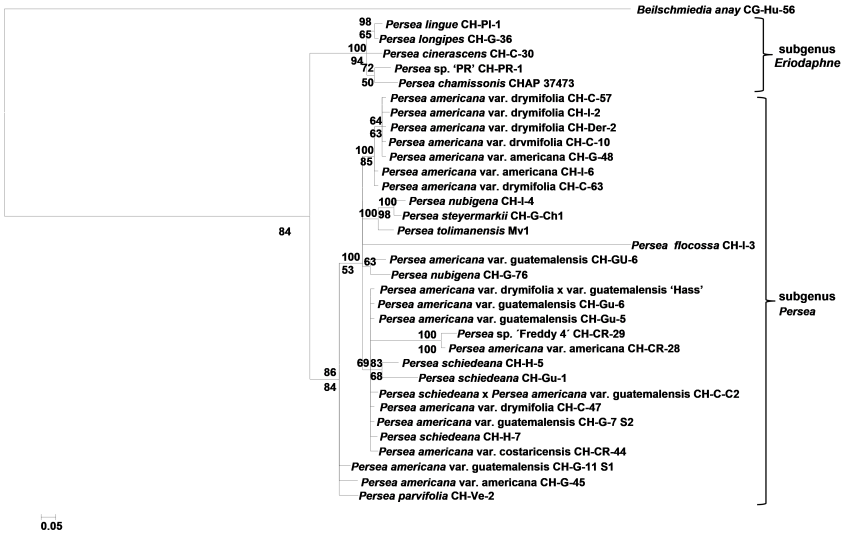

Figure 2

Bayesian 50 % majority rule consensus tree resulting from the analysis of the concatenation of the five chloroplast sequences *matK+rbcL+ndhF+rpoC1+trnH-psbA* of *Persea* and *Beilschmiedea anay* (Lauraceae). Posterior probabilities are indicated above the nodes, and maximum parsimony bootstrap support values (where 50 %) appear below the nodes. In the parsimonious analysis, 160 equally parsimonious trees with a length of 311 steps, and a consistency index of 0.87, homoplasy index of 0.13 and a retention index of 0.82 were obtained.

On the other hand, the five genes have a total of 261 VS, with 22 in *rpoC1* to 91 of *rbcL*; of these, 55 are Pi sites, with two in *rpoC* to 32 in *trnH-psbA* (Table 3). Also, it is important to note the presence of 12 fixed mutations in the five species of subgenus *Eriodaphne* so far investigated, which have led to the formation of a very solid clade (Table 3).

Within the five accessions of the subgenus *Eriodaphne* clade, the BI supports two groups, in the MP-BS majority rule consensus tree, although just the *Persea chamissonis-Persea* sp. 'PR' clade has a weak support of 61 %. This clade was also supported in the *matK* analysis. Within the *Persea* clade, there was a basal polytomy of two accessions of species of *Persea americana* (var. americana, CH-G-45 from Yucatán, Mexico and var. guatemalensis CH-G-11 S1 from Chiapas, Mexico), *Persea parvifolia* (CH-Ve-2 from Veracruz, Mexico) and a clade comprising the rest of the accessions (Figure 2). In this subclade, the BI tree shows five clades; two of them strongly supported one with all the *Persea americana* var. drymifolia accessions and another with *Persea nubigena* CH-I-4*, Persea steyermarkii* CH-G-Ch1 *and P. tolimanensis* Mv1; one with weak support; another with negligible; plus, one consisting of the single *Persea floccosa* CH-I-3 (Figure 2).

Analysis of the eight concatenated sequences

The phylogenetic analysis of the eight sequences was performed with plant accessions of 29 members of the subgenus *Persea*, five of the subgenus *Eriodaphne* and *Beilschmiedia anay*. The BI and MP analyses also resulted in relatively congruent topologies (Figure 3), and in general very similar to the BI and MP tree of the concatenated chloroplast sequences. The subgenera *Eriodaphne* and *Persea* clades were also obtained, but with slightly higher BS/PP support, 94/100 % for *Eriodaphne* and 84/86 % for *Persea* (Figure 3). The addition of *18S rRNA*, *cox3,* and *atp4* genes provided 38 VS, seven of which are Pi (Table 3). This information was not able to significantly improve the phylogenetic signal. The *Eriodaphne* fixed mutations increased from 12 to 14, by the addition of two mutations of the *18S rRNA* gene (Table 3).

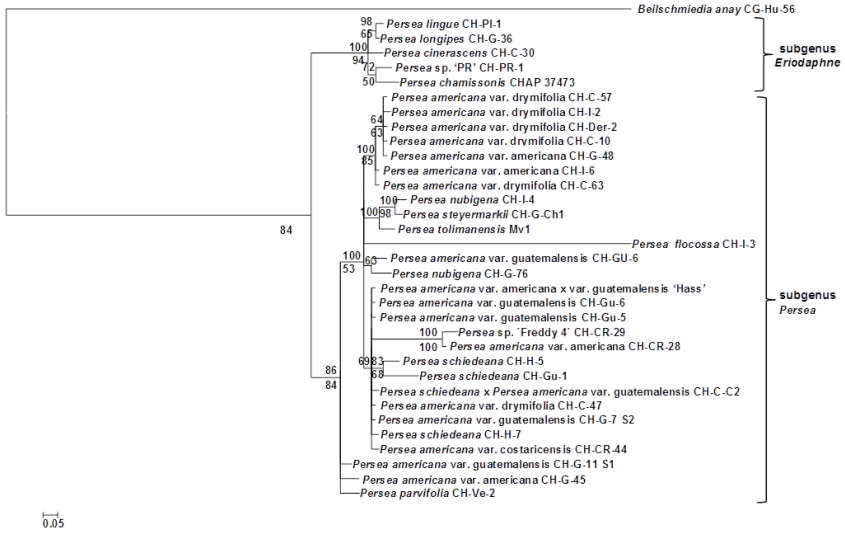

Figure 3

Bayesian 50 % majority rule consensus phylogram resulting from the analysis of the concatenation of *18S rRNA+cox3+atp4+matK+rbcL+ndhF+rpoC1+trnH-psbA* sequences of *Persea* and *Beilschmiedea anay* (Lauraceae). Posterior probabilities are indicated above the nodes, and maximum parsimony bootstrap support values (where 50 %) appear below the nodes. In the parsimonious analysis, 264 equally parsimonious trees with a length of 355 steps, and a consistency index of 0.87, homoplasy index of 0.13 and a retention index of 0.81 were obtained.

Discussion

Persea is one of the most complex genera of the Lauraceae. Previous phylogenetic analyses of the *matK* gene (Chanderbali et al., 2001; Rohwer, 2000; Rohwer et al., 2009) have shown that the *Persea* group is a monophyletic group deeply nested within the Lauraceae, close to the *Litsea* and *Ocotea* complexes. In previous analyses, such as the *trnL-trnF/trnH-psbA* phylogenetic tree of Chanderbali et al.

(2001), both subgenera of *Persea* are grouped in the same clade, related to *Machilus thunbergii* and *Alseodaphne semecarpifolia*. In the ITS phylogeny of Chanderbali et al. (2001), the three species of subgenus *Eriodaphne* formed a small clade (97 % BS), with *Persea americana* as its immediate sister group and several other, mainly Asian species of the *Persea* group as sister group to both. However, the small number of specimens analyzed of the two subgenera did not allow resolving the relationships within the *Persea* group.

Rohwer et al. (2009) used ITS sequences of several genera of the family. They found that the species of the subgenera *Persea* and *Eriodaphne* grouped separately from each other and from *Machilus* species. In our study, although *matK* gene showed a low degree of divergence in the sequences analyzed, BI and MP phylogenies could set the subgenus *Eriodaphne* in an independent clade, separated from species of the subgenus *Persea* and the other genera analyzed. Rohwer (2000) also found low levels of divergence within sequences of *matK* in Lauraceae (9.7 %) and less than 1 % within the genus *Persea*.

Although the *trnH-psbA* spacer region and the *rbcL* gene are more variable than *matK* (Table 3), these genes were not selected to investigate the position of *Persea* within the Lauraceae, because the *trnH-psbA* intergenic spacer has two areas subjected to frequent inversions that are not analyzed in this study and the phylogenetic trees of the *rbcL* (not shown) had the same topologies as the trees of *matK*.

The trees obtained from the analysis of chloroplast sequences and the eight concatenated ones are almost the same, due to the 55 PI sites of the chloroplast sequences, making them the most useful for the phylogenetic reconstruction of the clades, especially for the subgenus *Persea*. The mitochondrial and *18S rRNA* genes only contributed to the separation of two accessions of *Persea schiedeana* (CH-H-5 and CH-Gu-1), although with moderate support.

In the subgenus *Eriodaphne* all species considered were resolved completely, but in the subgenus *Persea* the analysis failed to separate *Persea americana* from all the species, especially from *Persea schiedeana*, which has also been found in a study of avocado germplasm and additional species of subgenus *Persea* with ISSR markers (Reyes-Alemán et al., 2016). The genetic variability level of the avocado, despite its cross-pollination system, is not considered to be exceptionally high compared with estimates that have been made with temperate fruit species (Chen, Morrel, de la Cruz, & Clegg, 2008), which seems to be what was found in part in the present study.

Persea parvifolia L. O. Williams (*Persea pallescens* [Mez] Loera-Hernández), a shrub with thin shoots, small narrow obovate to elliptic leaves and small fruits (Figure 4), which was first described by L.O Williams (1977) and not considered by van der Werff (2002) as a subgenus *Persea* species, is one of the most ancestral species in the subgenus *Persea* clade, so it could be considered as a good candidate for the species that gave rise to the avocado; however, it was unresolved with the other two individuals of *P. americana* that also have a conserved sequence,

so they could be primitive forms of those races. More individuals of this species are needed for a further analysis as well as other *P. americana* and other sources of *P. parvifolia* to support this.

Figure 4
Branch and fruit of *Persea parvifolia* L. O. Williams (*Persea pallescens* [Mez] Loera-Hernández).

It has been indicated that although *P. nubigena*, *P. steyermarkii* and *P. floccosa* could be separated from *P. americana* by restriction fragment length polymorphism (RFLP), they are considered to be only variants of *P. americana* (Furnier, Cummings, & Clegg, 1990); however, the results show that some of these species cluster together, which is the case of *P. nubigena*, *P. steyemarkii*, and *P. tolimanensis*, species considered to contribute to the ancestry of *P. americana* var. guatemalensis (Schieber & Bergh, 1987); nevertheless, this does not seem to correspond to our findings.

With respect to *P. americana*, a well-supported clade that includes five accessions of the Mexican race (*P. americana* var. drymifolia) were grouped together with two of the West Indian one (*P. americana* var. americana) indicates that they are closely related. It can be assumed that the last two accessions are not completely pure and that they may have genetic characteristics of the Mexican race. Conversely, an apparent conflict between phenotypic and genotypic data can help adjust pedigree information (Ashworth & Clegg, 2003), and be used to reclassify accessions in the germplasm bank as possible hybrids. This last point also applies to another clade that grouped accessions of the Guatemalan race, possibly hybrid, one *P. americana* var. costaricensis, and a *P. schiedeana* from Honduras, the last of which was also reported using DFP and SSR markers which did not find unique DNA patterns which could characterize the three races of *P. americana* and the three accessions of *P. schiedeana* (Mhameed et al., 1997). This is also in accordance for the subclade that grouped two *P. schiedeana*, one from Honduras and the other from Guatemala. In the other subclade, two accessions of Costa Rica were together an unclassified one ('Freddy 4') and a *P. americana*

var. americana (CH-CR-28), which is probably the West Indian Race subclade.

The complex legacy of ancient and recent avocado improvement has left a profusion of genotypes of uncertain affinities and with diffuse racial boundaries (Ashworth & Clegg, 2003), where other factors may have a role, including the possibility of remote hybridization events (Bufler & Ben-Ya'acov, 1992) or a more recent date for racial differentiation than previously thought (Ashworth & Clegg, 2003).

It must be considered that although the analyses of the eight concatenated sequences separate both subgenera of *Persea*, the variation of the eight sequences is low, 4.16 % of VS and 0.86 % of Pi sites (Table 3). This was reported for *trnH-psbA* (Chanderbali et al., 2001) and *matK* (Rohwer, 2000) in the family Lauraceae, but not for the other sequences. Therefore, it is necessary to find sequences showing a greater variation that allow a better resolution of the phylogenetic relationships within subgenus *Persea*. A suitable candidate may be the nuclear ITS region, which has 33 % parsimony-informative sites for many Lauraceae accessions (Rohwer et al., 2009), but in our experience it has the disadvantage of being difficult to amplify and sequence in some accessions of *Persea*, and to align because of too many indels. Liu, Chen, Song, Zhang, and Chen (2012) found that the ITS2 region produced a low success rate in direct PCR amplification and sequencing in Lauraceae species and it is also unsuitable to be the DNA barcode of the family.

Based on the hypothesis of a monophyletic origin of the genus *Persea*, our results partially suggest that this genus is not a monophyletic group; therefore, one could think that the subgenera *Persea* and *Eriodaphne* should be recognized as independent genera, confirming the analysis of Rohwer et al. (2009), where *Persea* does not appear to be monophyletic, because the subgenus *Persea* seems to be more closely related to *Phoebe* and *Alseodaphne* than to the subgenus *Eriodaphne*.

Conclusions

The eight concatenated sequences separated both subgenera (*Persea* and *Eriodaphne*) into two different clades, where 14 fixed mutations were found in the studied species of the subgenus *Eriodaphne*, supporting the hypothesis of independent genera. In the subgenus *Persea*, the concatenated sequences used failed to separate *Persea americana* from all the species, especially from *Persea schiedeana*, the most distinct species in the subgenus. The chloroplast intergenic spacer *trnH-psbA* sequence held the highest variation and informative sites, while the mitochondrial and nuclear rDNA sequences studied were not informative.

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