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Utility of DNA barcodes in the identification of melliferous plants associated with *Sabal yapa* honey from eastern Yucatan, Mexico

Utilidad de los códigos de barras de DNA en la identificación de plantas melíferas asociadas a la miel monofloral de *Sabal yapa* producida en el este de Yucatán, México

Utilidade dos códigos de barras de DNA na identificação de plantas melíferas associadas ao mel de *Sabal yapa* do leste de Yucatán, México

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ABSTRACT

Melliferous plants are natural source of nectar and pollen for honeybees and their pollen grains are present in honey. The melissopalynological study of *Sabal yapa* honey produced in Yucatan has shown that eighteen species contribute importantly to its honey composition. We use melliferous plants associated with *Sabal yapa* honey to test the potential of three barcodes (*rbcL*, ITS2, and *trnH-psbA*) in species identification to confirm the botanical origin and authentication of honey. Secondarily, we test the success rates of amplification and sequencing of each barcode. A total of 38 sequences were generated: 10 for *rbcL*, 13 for ITS2 and 15 for *trnH-psbA*. The success rate in PCR amplification was 94.73 % for *trnH-psbA* and 84.21 % for ITS2, while for *rbcL* was 52.63 %. Regarding sequencing success, 100 % of the products were amplified with *rbcL*. For *trnH-psbA* and ITS2, the sequencing success was 88.9 % and 87.5 %, respectively. ITS2 produced a species identification efficiency of 46.15 %, followed by *rbcL* (30 %). For *trnH-psbA*, the rate of species identification was 13.33 %. The sequences generated in this work will allow the construction of a reliable library of barcodes of melliferous plants, and with forward

increasing will be possible to have a solid base for metabarcoding studies in the honey of the Yucatan Peninsula.
Keywords: honey, *rbcL*, ITS2, *trnH-psbA*.

RESUMEN

Las plantas melíferas son fuente natural de néctar y polen para las abejas, por lo que sus granos de polen están presentes en la composición de la miel. El estudio melisopalinológico de la miel de *Sabal yapa* producida en Yucatán mostró que 18 especies contribuyen de manera importante en la composición de la miel. En este estudio utilizamos plantas melíferas asociadas a la miel de *Sabal yapa* para evaluar el potencial de tres códigos de barras (*rbcL*, ITS2 y *trnH-psbA*) en la identificación de especies para confirmar el origen botánico y la autenticidad de la miel. También evaluamos el éxito de amplificación y secuenciación de cada código de barras. En total se generaron 38 secuencias: 10 para *rbcL*, 13 para ITS2 y 15 para *trnH-psbA*. El éxito de amplificación para los marcadores fue: *trnH-psbA* (94.73 %), ITS2 (84.21 %) y *rbcL* (52.63 %). El éxito de secuenciación para los marcadores fue: *rbcL* (100 %), *trnH-psbA* (88.9 %) e ITS2 (87.5 %). La eficiencia en la identificación de especies para ITS2 y *rbcL* fue de 46.15 % y 30 %, respectivamente. La eficiencia de *trnH-psbA* para identificar especies fue de 13.33 %. Las secuencias generadas en este trabajo permitirán la construcción de una biblioteca de códigos de barras de plantas melíferas confiable y en la medida que se enriquezca, se podrá tener una base sólida para estudios de metabarcoding en la miel de la Península de Yucatán.
Palabras clave: miel, *rbcL*, ITS2, *trnH-psbA*.

RESUMO

As plantas melíferas são fonte natural de néctar e pólen para as abelhas, e seus grãos de pólen estão presentes na composição do mel. O estudo melissopalinológico do mel de *Sabal yapa* produzida em Yucatan mostrou que 18 espécies contribuem de forma importante para a composição do mel. Usamos plantas melíferas associadas ao mel de *Sabal yapa* para testar o potencial de três códigos de barras (*rbcL*, ITS2 e *trnH-psbA*) na identificação de espécies para confirmar a origem botânica e a autenticação do mel. Em segundo lugar, testamos as taxas de sucesso de amplificação e sequenciamento de cada código de barras. Foi generado um total de 38 sequências: 10 para *rbcL*, 13 para ITS2 e 15 para *trnH-psbA*. O sucesso da amplificação por PCR foi: *trnH-psbA* (94,73 %), ITS2 (84,21 %), *rbcL* (52,63 %). Com relação ao sucesso do sequenciamento, 100 % dos productos foram amplificados com *rbcL* (100 %). Para *trnH-psbA* e ITS2, o sucesso do sequenciamento foi de 88,9 % e 87,5 %, respectivamente. O ITS2 produziu uma eficiência de identificação de espécies de 46,15 % seguido por *rbcL* (30 %). A eficiência do *trnH-psbA* para identificar as espécies foi de 13.33 %. As sequências geradas neste trabalho permitirã construir uma biblioteca confiável de códigos de barras de plantas melíferas e, com o aumento futuro, será possível ter uma base sólida para estudos de metabarconding no mel da Península de Yucatán.
Palavras-chave: mel, *rbcL*, ITS2, *trnH-psbA*.

INTRODUCTION

DNA barcodes are small, standardized DNA fragments (500 to 800 base pairs bp) that are amplified and sequenced to identify organisms (Taberlet et al., 2012). This tool has helped to understand biological diversity and is currently applied to solve questions of systematics and phylogeny; as well as in the identification of parasites and vectors, the creation of forest and animal inventories, the detection of trafficking of endangered species, and the authentication of pharmaceutical and food products such as honey (Ajmal et al., 2014; Ferreira de Lima et al., 2018). Therefore, selecting universal DNA fragments as barcodes is a critical step for their implementation (Saravanan et al., 2019).

The Consortium for the Barcoding of Life (CBOL) has proposed *rbcL* as a core barcode in plants and several markers such as *trnH-psbA* or ITS2 as complementary barcodes to increase the species-level discrimination (Hollingsworth et al., 2009; Manivanan et al., 2018). The utility of these markers to corroborate honey authenticity has been evaluated by several authors (Bruni et al., 2015; Hawkins et al., 2015; Laha et al., 2017; Manivanan et al., 2018; Murthy et al., 2019; Prosser and Hebert 2017; Saravanan et al., 2019).

In tropical regions, where organism diversity is high and sampling difficult, barcode generation and implementation may be limited (Parmentier et al., 2013; Ferreira de Lima et al., 2018; Jones et al., 2021) as barcodes require reference sequences available on platforms such as GenBank or BOLD Systems for comparison and species identification (Hollingsworth et al., 2009; Jones et al., 2021). In Mexico, the generation of barcodes for melliferous plants is scarce (Hernández-Pineda, 2016). Only about 10,745 species including plants and animals have barcodes (103,190 records), of which 24.83 % correspond to vascular plants and 17 % to eudicotyledons (BOLD Systems, 2023a). Constructing reference libraries with reference sequences of well-verified specimens will allow correct identification by barcoding and more efficient analysis (Jones et al., 2021). For that reason, utilizing specimens available in herbaria is a key element in constructing solid databases (de Vere et al., 2012).

In the international markets where monofloral honey has a high value due to its organoleptic characteristics (Murthy et al., 2019) and honey may be subject to fraudulent practices (Prosser and Hebert 2017; Soares et al., 2017), DNA barcodes emerge as an innovative technique to authenticate honey and to determine its botanical origin (Soares et al., 2017). Since the Yucatan Peninsula is the most productive beekeeping region in Mexico (Servicio de Información Agroalimentaria y Pesquera, 2020) with 22 documented monofloral honey (Villanueva-Gutiérrez et al., 2009; Alfaro-Bates et al., 2010) and around 80 % the honey produced in this region is exported mainly to European countries (Cruz-Zamudio, 2017), the generation of DNA barcodes for melliferous plants of Yucatan is a critical step to identify potential barcodes to authenticate honey. We selected 18 species of plants associated with *Sabal yapa* monofloral honey to evaluate the success rates of amplification, sequencing, and identification of three barcodes (*rbcL*, ITS2, and *trnH-psbA*) and identify potential barcodes in melliferous plants.

MATERIALS AND METHODS

Selection of samples

Eighteen species were selected according to the melissopalynological analysis of *Sabal yapa* honey from Tizimin, Yucatan (Durán-Escalante et al., 2023) (Table 1). Seven of these species were the major contributors of pollen to *Sabal yapa* honey and are also the major plant contributors to honey production in the Yucatan Peninsula (Alfaro-Bates et al., 2010). Four species provided pollen categorized as minor or important minor pollen, and seven species are part of the group that we consider can help to give certainty to the pollen grains that could only be identified by palynology at the botanical family level (i.e., Malvaceae, Asteraceae) in *Sabal yapa* honey. The plants were gathered around the apiaries of a local beekeeper where this honey is harvested. Lately, the dried specimens were identified by expert taxonomists and deposited in the herbarium of the Universidad Autónoma de Yucatán (UADY). The specimens are available online in the portal of the Red de Herbarios del Noroeste de México (2023).

TABLE 1. List of species and vouchers used for DNA barcode generation and Process ID generated in BOLD Systems platform for studied species.

Family	Species	Voucher	Process ID
Arecaceae	*Sabal yapa* C. Wright ex Becc.*	UADY23569	UADY013-22
Asteraceae	*Bidens squarrosa* Kunth	UADY23544	UADY002-22
	Lasianthaea fruticosa (L.) K.M. Becker	UADY23549	UADY008-22
	Otopappus guatemalensis (Urb.) R.L. Hartm. & Stuessy	UADY23552	UADY010-22
	Synedrella nodiflora (L.) Gaertn.	UADY23515	UADY014-22
	Trixis inula Crantz.*	UADY23535	UADY016-22
	Viguiera dentata (Cav.) Spreng.*	UADY23495	UADY017-22
Burseraceae	*Bursera simaruba (L.) Sarg.* *	UADY23641	UADY003-22
Convolvulaceae	*Jacquemontia pentanthos* (Jacq.) G.Don.	UADY23499	UADY007-22
Euphorbiaceae	*Cnidoscolus aconitifolius* (Mill.) I.M. Johnst.	UADY23650	UADY005-22
Fabaceae	*Erythrostemon yucatanensis (*Greenm.) Gagnon & G.P. Lewis	UADY23563	UADY004-22
	Mimosa bahamensis Benth.	UADY23516	UADY009-22
	Piscidia piscipula (L.) Sarg.*	UADY23585	UADY012-22
Malvaceae	*Bakeridesia gaumeri* (Standl.) D.M.Bates	UADY23476	UADY018-23
	Bastardia viscosa (L.) Kunth	UADY23528	UADY001-22
	Hampea trilobata Standl.	UADY23774	UADY006-22
Nyctaginaceae	*Pisonia aculeata* L.	UADY23596	UADY011-22
Sapindaceae	*Thouinia paucidentata* Radlk.*	UADY23637	UADY015-22

*Main species that contribute to honey production in Yucatan.

DNA isolation and Polymerase Chain Reaction (PCR)

Total genomic DNA extraction was performed from 20 mg of leaf tissue (freshly taken and transferred to the laboratory in a bag with silica gel at room temperature) using the DNeasy Plant mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Yields and quality of DNA were verified by agarose gel electrophoresis (0.75 %) and spectrophotometer (Jenway 737501 Genova Nano Micro-spectrophotometer, United Kingdom). DNA barcodes were amplified by PCR using universal primers for *rbcL* (Kress and Erickson, 2007; Fazekas et al., 2008), ITS2 (Chiou et al., 2007) and *trnH-psbA* (Sang et al., 1997; Tate and Simpson, 2003). The primer sequences (5' to 3') were *rbcL*a_F (ATG TCA CCA CAA ACA GAG ACT AAA GC) and *rbcL*ajf634 (GAA ACG GTC TCT CAA ACG CAT); ITS-S2 F (ATG CGA TAC TTG GTG TGA AT) and ITS2 R (GAC GCT TCT CCA GAC TAC AAT); *trnH-psbA* F (GTT ATG CAT GAA CGT AAT GCT C) and *trnH-psbA* R (CGC GCA TGG TGG ATT CAC AAT CC).

All PCR reactions were performed on a MultiGene TC020-24 thermal cycler (Labnet International, China) using a reaction mixture of a total volume of 25 or 50 ml containing 12.5 or 25 ml of GoTaq® Green Master Mix 2X, 1.5 ml forward and reverse primer each (10 mM), 0.5 ml of BSA (0.04 %), and template DNA (< 250 ng). The reaction volume was topped up to 25 or 50 ml using nuclease - free water. The PCR conditions for amplification of *rbcL* were initial denaturation at 94 °C 4 min, followed by 35 cycles of 94 °C 30 s, 53 °C 30 s, and 72 °C 60 s; extension at 72 °C 10 min, and hold 4 °C. The PCR conditions for amplification of ITS2 were initial denaturation at 94 °C 3 min, followed by 35 cycles of 95 °C 30 s, 48 °C 30 s, and 72 °C 30 s; extension at 72 °C 7 min, and hold 4 °C. Finally, the PCR conditions for amplification of *trnH-psbA* were initial denaturation at 95 °C 2 min and 30 s, followed by 35 cycles at 95 °C 30 s, 49 °C 30 s, 64 °C 60 s; extension at 72 °C 7 min, and hold 4 °C. PCR products were visualized on 0.75 % agarose gels stained with SYBR Safe DNA under ultraviolet light. Amplified products were sent to Macrogen (Seoul, South Korea) for purifying and DNA sequencing using the same primers as in the amplification.

DNA sequence editing, nucleotide Basic Local Alignment Seach Tool (BLAST) and dendrogram construction

The assembly of the sequences was performed in Sequencher 4.1.4 (Genes Codes Corporation, 2002) removing low-quality segments at two ends of the sequences by manual editing. Efficiencies of the three markers for species identification were evaluated using the nucleotide Basic Local Alignment Seach Tool (BLAST), which employs a local alignment and searches for segments with the highest score (McGinnis and Madden, 2004; Newell et al., 2013) in the NCBI platform (National Center for Biotechnology Information, 2023). The default database selected is Nucleotide Collection (nr/nt), and highly similar sequences (megablast) as program selection. The query sequence was considered correctly assigned when a) the percentage of similarity corresponded to ≥ 99 % with the reference sequences in the database and b) the analyzed sequence matched the species name. When the percentage of identity was < 99 %, the identification was considered incomplete because of the absence of specific sequences in the GenBank (Aghayeva et al., 2021).

The sequences edited were aligned in MEGA 11 (Tamura et al., 2021) with MUSCLE (Edgar, 2004) for multiple alignment including sequences available on the GenBank platform for the genus or species (downloaded on September 22, 2022). A phenetic dendrogram was elaborated for each marker with the multiple alignment matrix to corroborate visually the BLAST results obtained. This strategy uses a global alignment where the alignment strain along the length of both sequences (Ignacimuthu, 2005) and allows the recognition of clusters of very similar sequences due to their high level of similarity (Peña, 2011). Trees were constructed by Neighbor-Joining (NJ) method (Saitou and Nei, 1987). The marker was considered an efficient barcode for species identification when the query sequence clustered into a group with the same species or genus sequences. Analyses were conducted in MEGA 11 (Tamura et al., 2021) using the Maximum Composite Likelihood method (Tamura et al., 2004) and a Bootstrap test (1000 replicates) (Felsenstein, 1985). Finally, all information related to the specimens (sequences, taxonomic description, voucher number assigned by the herbarium, collection date, and georeferencing) was uploaded to GenBank and BOLD Systems v4 platforms (BOLD Systems, 2023b) as part of the project "UADY DNA Barcoding of melliferous plants in the Yucatan Peninsula (Tizimin)".

RESULTS

The universality of primer sequences

DNA extracted from leaf tissue samples was amplified for the three target regions using a single pair of universal primers for each locus. A total of 38 sequences were obtained: 10 for *rbcL*, 13 for ITS2, and 15 for *trnH-psbA*. The *rbcL* fragment showed an amplification success rate of 55.55 %, ITS2 83.33 % and *trnH-psbA* 94.44 %. Of the nine botanical families studied, *rbcL* and ITS2 amplified for all except Convolvulaceae. The *trnH-psbA* fragment amplified for all families and species except for *Bakeridesia gaumeri* (AB12), a member of the Malvaceae family.

Regarding DNA sequencing, the success rate of sequencing was 100 % for *rbcL*, followed by *trnH-psbA* (88.9 %) and ITS2 (87.5 %). In all cases, amplification products of the expected size were obtained. The average length of the *rbcL* sequences was 631 bases, ITS2 of 473 bases, and *trnH-psbA* of 509 bases. The latter fragment exhibited a variation in the length of the sequences between species. Although *Sabal yapa* and *Cnidoscolus aconitifolius* amplified for ITS2, overlapping peaks were found in sequencing results and could not be used for further analysis. The same occurred with *Mimosa bahamensis* with the *trnH-psbA* fragment, where the electropherograms were unclear.

Identification efficiency

The *rbcL*, ITS2 and *trnH-psbA* markers are suitable for plant identification but at different levels. Using the BLAST method to identify species with given samples, the success of species identification with ITS2 was 46.15 %, followed by *rbcL* (30 %) and *trnH-psbA* (13.33 %). From 10 *rbcL* sequences, three could be assigned to the species level, three to

the genus level, and four to the family level (Table 2). Six out of the 13 ITS2 sequences could be correctly assigned to the species level, five at the genus level, and two at the family level (Table 3). Finally, from the 15 *trnH-psbA* sequences, only two could be correctly assigned to the species level. Of the rest, eight were assigned to the genus level and five to the family level (Table 4).

TABLE 2. BLAST results for the *rbcL* region in plant samples collected in this study.

Species	Code	Species match	ID (%)	E-value
Bastardia viscosa	AB13	*Abutilon hulseanum*	99.84	0.0
		Abutilon eremitopetalum	99.83	0.0
Bursera simaruba	BU10	*Bursera simaruba*	99.82	0.0
Cnidoscolus aconitifolius	CHA16	*Cnidoscolus aconitifolius*	99.68	0.0
		Manihot carthagenensis	99.20	0.0
Erythrostemon yucatanensis	CYU16	*Erythrostemon hughesii*	100	0.0
		Cenostigma pyramidale	99.52	0.0
Hampea trilobata	HAM7	*Hampea appendiculata*	99.82	0.0
		Gossypium raimondii	99.04	0.0
		Gossypium schwendimanii	99.04	0.0
Pisonia aculeata	PIS8	*Pisonia aculeata*	99.20	0.0
		Guapira noxia	99.20	0.0
Sabal yapa	SA1A	*Acoelorraphe wrightii*	100	0.0
Synedrella nodiflora	SYN30	*Synedrella nodiflora*	100	0.0
Thouinia paucidentata	THD5	*Thouinia portoricensis*	99.67	0.0
Viguiera dentata	VD2	*Iostephane heterophylla*	99.36	0.0
		Aldama excelsa	99.20	0.0

TABLE 3. BLAST results for the ITS2 region in plant samples collected in this study.

Species	Code	Species match	ID (%)	E-value
Bakeridesia gaumeri	AB12	*Bakeridesia gaumeri*	100	6e-166
		Bakeridesia gloriosa	99.15	9e-179
Bastardia viscosa	AB13	*Abutilon abutiloides*	94.71	3e-153
		Abutilon theophrasti	93.86	0.0
Bidens squarrosa	BSQ31	*Bidens squarrosa*	99.39	7e-165
		Bidens boquetiensis	99.36	7e-156
Bursera simaruba	BU10	*Bursera simaruba*	99.13	4e-173
Hampea trilobata	HAM7	*Hampea trilobata*	98.01	1e-173
Lasianthaea fruticosa	LASF28	*Lasianthaea helianthoides*	98.80	6e-166
Otopappus guatemalensis	OTP29	*Wollastonia biflora*	92.83	0.0
Pisonia aculeata	PIS8	*Pisonia aculeata*	99.72	2e-180
Piscidia piscipula	PISC4	*Piscidia piscipula*	99.70	2e-170
Synedrella nodiflora	SYN30	*Synedrella nodiflora*	99.13	3e-173
Thouinia paucidentata	THD5	*Thouinia paucidentata*	99.12	4e-172
Trixis inula	TRI1	*Trixis californica*	98.55	2e-169
		Trixis cacalioides	98.21	1e-162
		Trixis inula	98.20	4e-162
Viguiera dentata	VD2	*Viguiera dentata*	98.38	9e-149

TABLE 4. BLAST results for the *trnH-psbA* region in plant samples collected in this study.

Species	Code	Species match	ID (%)	E-value
Bastardia viscosa	AB13	*Hibiscus micranthus*	97.87	1e-59
		Hibiscus meyeri	97.87	1e-59
		Abutilon auritum	97.37	2e-63
Bursera simaruba	BU10	*Bursera simaruba*	98.71	0.0
		Bursera longipes	98.28	0.0
		Bursera krusei	98.28	0.0
Cnidoscolus aconitifolius	CHA16	*Cnidoscolus aconitifolius*	100	0.0
		Cnidoscolus megacanthus	100	0.0
Erythrostemon yucatanensis	CYU16	*Erythrostemon hughesii*	96.52	0.0
Hampea trilobata	HAM7	*Hampea appendiculata*	98.18	0.0
Jacquemontia pentanthos	JPE17	*Jacquemontia cayensis*	100	3e-64
		Jacquemontia verticillata	100	2e-61
		Jacquemontia havanensis	100	5e-57
		Jacquemontia nipensis	100	5e-57
		Jacquemontia curtisii	100	4e-53
		Jacquemontia reclinata	100	4e-53
		Jacquemontia pentanthos	99.78	0.0
Lasianthaea fruticosa	LASF28	*Lasianthaea macrocephala*	99.63	2e-135
Otopappus guatemalensis	OTP29	*Baltimora recta*	97.83	2e-154
Pisonia aculeata	PIS8	*Pisonia aculeata*	98.30	0.0
Piscidia piscipula	PISC4	*Connarus paniculatus*	96.60	3e-88
Sabal yapa	SA1A	*Acoelorraphe wrightii*	98.87	0.0
Synedrella nodiflora	SYN30	*Synedrella nodiflora*	100.00	0.0
Thouinia paucidentata	THD5	*Thouinia brachybotrya*	98.63	0.0
		Thouinia portoricensis	95.49	2e-164
Trixis inula	TRI1	*Trixis inula*	99.78	0.0
Viguiera dentata	VD2	*Iostephane heterophylla*	96.42	0.0

When distance methods were used for species identification, Neighbor-Joining trees showed our sequences cluster correctly with GenBank sequences for the same species or genus (in a few cases with the same family members). The *rbcL* sequences were correctly grouped by genus except for *Bastardia viscosa*, which was grouped with other Malvaceae

species because it does not have reference sequences in GenBank (Figure 1). The tree size for ITS2 is larger than for the other two markers due to the availability of reference sequences for the organisms studied (Figure 2). Although the species identification efficiency of *trnH-psbA* using BLAST was low, species formed well-defined groups in trees. Since *Otoppapus guatemalensis* has no reference sequences for *trnH-psbA* in GenBank, the sequence clustered with other species of Asteraceae but on a different branch within the tree (Figure 3).

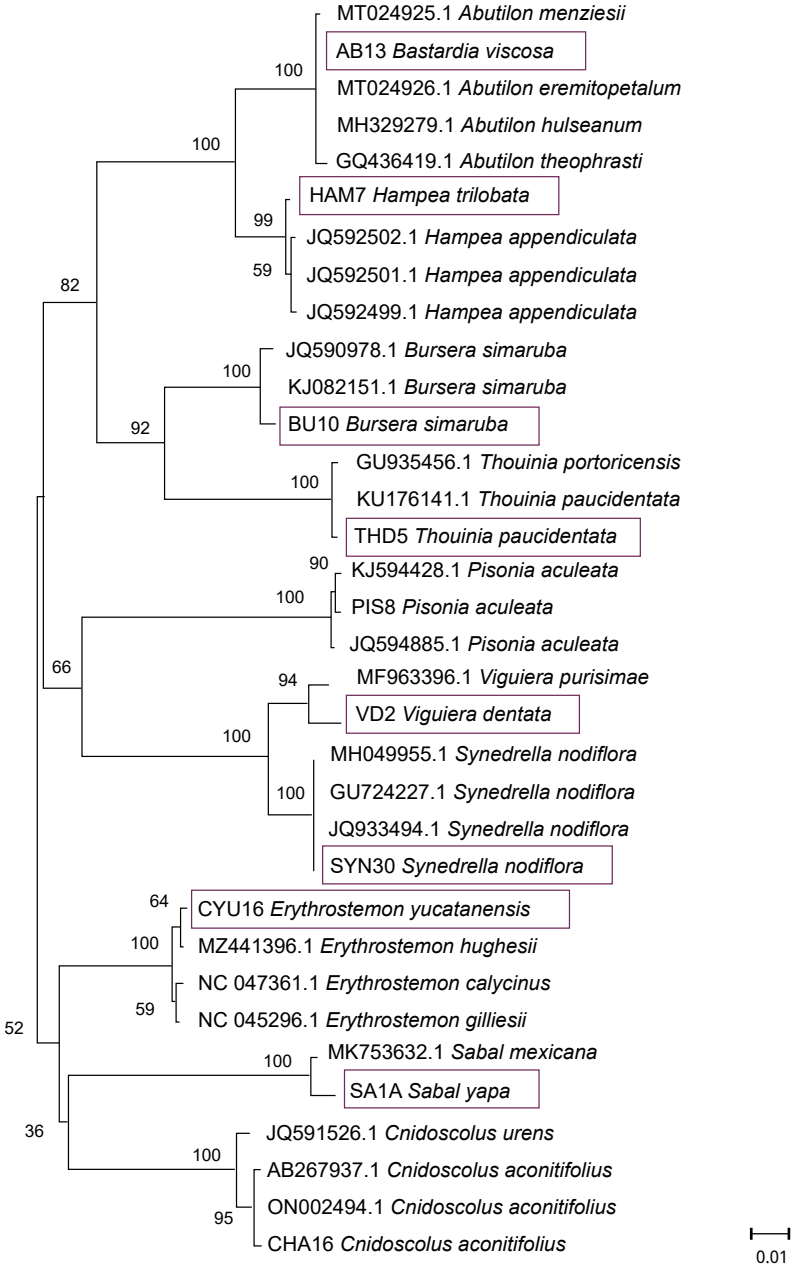

FIGURE 1. Neighbor-Joining (NJ) tree, based on *rbcL* sequences. Above the branches, the Bootstrap value is shown. The blue rectangles indicate the location of our samples in the corresponding clusters. Matrix variability was 23.01 %.

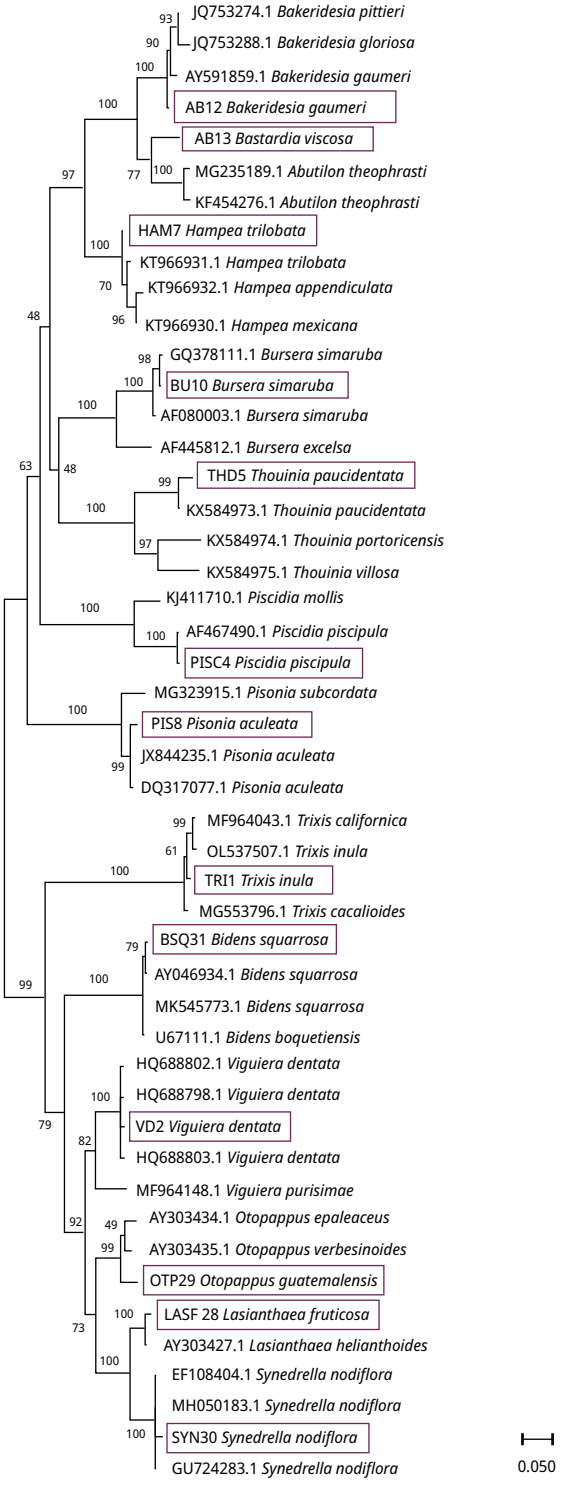

FIGURE 2. Neighbor-Joining (NJ) tree, based on ITS2 sequences. Above the branches, the Bootstrap value is shown. The blue rectangles indicate the location of our samples in the corresponding clusters. Matrix variability was 64.91 %.

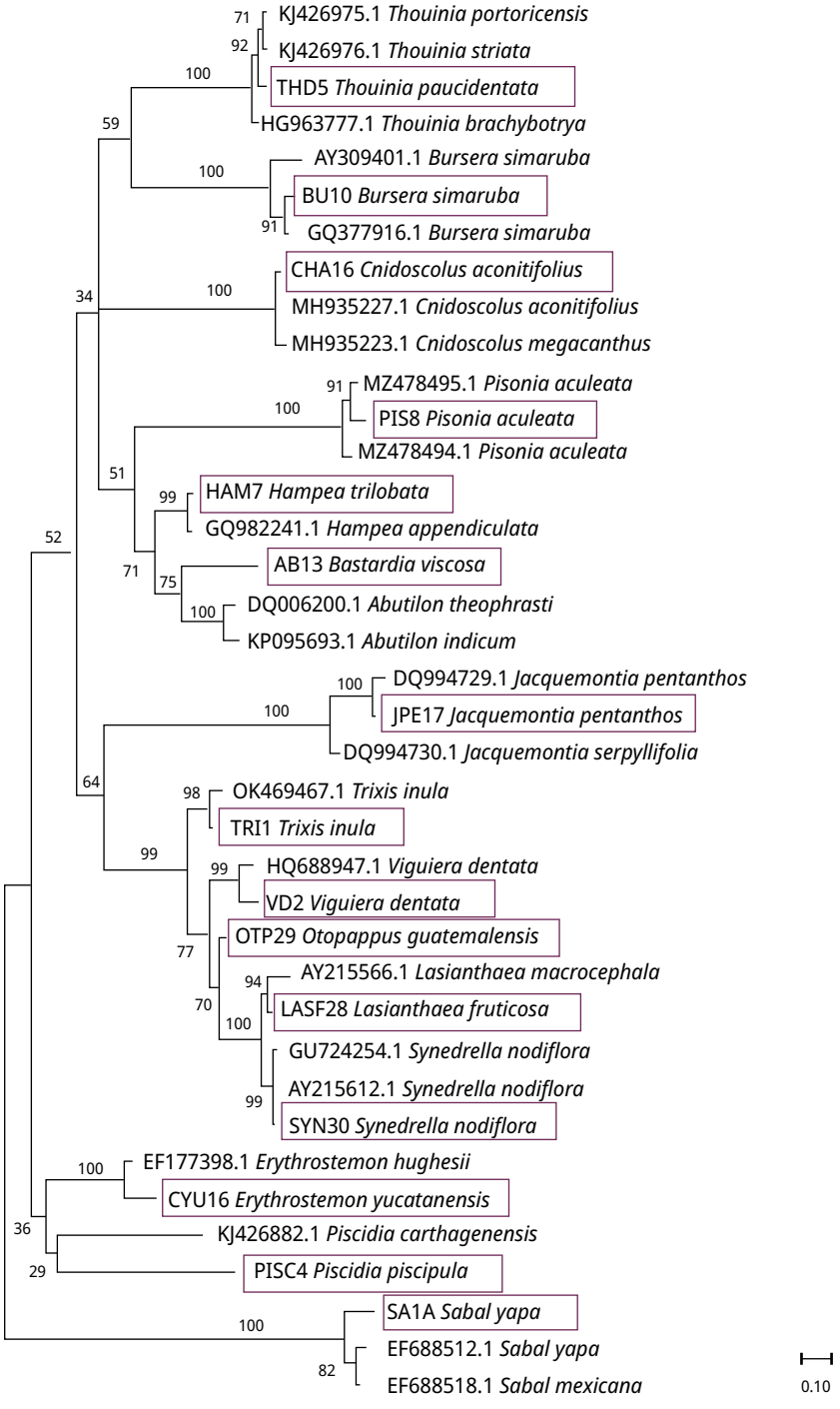

FIGURE 3. Neighbor-Joining (NJ) tree, based on *trnH-psbA* sequences. Above the branches, the Bootstrap value is shown. The blue rectangles indicate the location of our samples in the corresponding clusters. Matrix variability was 75 %.

DISCUSSION

Differences between amplification and sequencing success were observed between the three regions using a single pair of universal primers. Four plant species belonging to the Asteraceae family (*Bidens squarrosa*, *Lasianthaea fruticosa*, *Otopappus guatemalensis*, and *Trixis inula*) did not amplify using the universal *rbcL* (*rbcL*a-F/ *rbcL*aif634) primers. This has also been observed in other Asteraceae species using *rbcL*a/*rbcL*a R and *rbcL*1F/*rbcL*-724R primers (Bafeel et al. 2011) probably due to a primer mismatch at the 3' end of the primer sequence (Bru et al., 2008). For this reason, the combination of several reverse primers is required to amplify successfully in different groups of plants (de Vere et al., 2012). In contrast to *rbcL*, amplification rates for ITS2 and *trnH-psbA* were higher and agree with those obtained by several authors where ITS2 amplifies between 84.39 % and 93.8 % (Gong et al., 2018; Chen et al., 2010, Gao et al., 2010) and *trnH-psbA* 72.2 % and 92.8 % (Chen et al., 2010; Gao et al., 2010; Kang et al., 2017).

Hollingsworth et al. (2009) have reported obtaining high-quality bidirectional *rbcL* sequences like those we got in our study (100 %). In some species, secondary metabolites can interfere with the DNA extraction causing PCR amplification and sequencing failures (Bafeel et al., 2011; Kang et al., 2017). That was probably the case for *Mimosa bahamensis* (*trnH-psbA*), *Cnidoscolus aconitifolius* (ITS2), and *Sabal yapa* (ITS2), where purity indicators A260/280 and A260/230 were lower than 1.8.

The discriminatory capacity of each marker relies on nucleotide substitution rates and the availability of sequences in public databases (Aghayeva et al., 2021; Jones et al., 2021). On one hand, it is essential for a marker to exhibit species-level differentiation at sequence level, to discriminate biological entities effectively (Casiraghi et al., 2010). Moreover, the presence or absence of sequences from specific interest groups in the reference databases can significantly impact the efficiency of barcodes (Rey Bentos and Capdevielle Sosa, 2020; Taberlet et al., 2007). Therefore, the likelihood of accurately assigning a query sequence also depends on the completeness of sequences available for the target taxa in public databases (BOLD Systems, 2023c).

The *rbcL* marker has a conservative rate of nucleotide substitution, which makes this marker very useful for plant identification at the genus or family level (Martínez, 1997). Despite its limited ability to identify closely related species (Bruni et al., 2015; Saravanan et al., 2019), it is chosen as a core barcode to maintain a certain degree of compatibility with the genetic repositories as established by the CBOL (Galimberti et al., 2014) and has many reference sequences for many species. The internal transcribed spacer 2 (ITS2) is a highly variable region (Newmaster et al., 2013; Duan et al., 2019), suitable for genera and species identification because, in general, the spacers evolve faster than coding regions (Poczai and Hyvönen, 2010). This barcode has many sequences in public databases and is the locus more used in pollen DNA barcoding studies (Bell et al., 2016, Milla et al., 2022). In this study, ITS2 had the highest species identification efficiency (46.15 %) and it is consistent with the obtained from other authors in diverse barcoding plant identification projects (Chen et al., 2010; Gao et al., 2010; Liu et al.,2019; Newmaster et al., 2013).

Some authors have highlighted that *trnH-psbA* can correctly identify plants at the genus level (between 90 % and 98 %) better than *rbcL* (Loera-Sánchez et al., 2020; Parmentier et al., 2013) as we observed in our study, where *trnH-psbA* correctly assigned

eight sequences at the genus level against three *rbcL* sequences. At the species level, *trnH-psbA* by itself (Parmentier et al., 2013; Loera-Sánchez et al., 2020) or as a compliment to ITS2 (Liu et al., 2019; Pang et al., 2012) or *rbcL* (Galimberti et al., 2014; Parmentier et al., 2013) shows high efficiency in the identification of plants but in our case, we observed the opposite. For example, *trnH-psbA* could not differentiate correctly between *Cnidoscolus aconitifolius* and *C. megacanthus* and *Jacquemontia pentanthos vs.* other six species. Second, we obtained only 13.33 % correct assignment to species level with this marker. This is partly because 16 of the18 species, have few or no reference sequences for this region in public databases to allow comparison. The scarce availability of this region in the databases is associated with problems obtaining bidirectional sequences due to high mononucleotide repeats (Bruni et al., 2015; Hollingsworth et al., 2009). Despite its limitations for species identification in our study, it seems to be a suitable genus-level marker used as DNA barcode to characterize honey from a limited geographic area with well-known flora (Bruni et al., 2015) such as *Sabal yapa* honey produced in Tizimin, Mexico.

BLAST and dendrograms are different strategies to explore and evaluate the usefulness of barcodes for species identification (Bolson et al., 2015). In a heuristic search, BLAST attempts to find the best overall results after comparing a query sequence with an entire public nucleotide database (McGinnis and Madden, 2004). Nevertheless, BLAST has a bias for hits that contain smaller or no gaps over hits with long gaps, so alignments can fail to identify species correctly (Dereeper et al., 2010). BLAST analyses with ITS2 failed for example to correctly assign *Trixis inula* with its reference sequence (98.20 %) and showed a high percentage of similarity with *T. californica* (98.55 %) and *T. calicoides* (98.21 %) but in the dendrograms, the query sequence clustered correctly with the reference species, demonstrating that distance methods are adequate for corroborating BLAST results.

Lastly, we underscore the significance of producing accurately identified plant barcodes, supported by herbarium vouchers for validation purposes. In this study, we identified a reference sequence labeled in GenBank as Asteraceae sp. (Accession HG964019.1), which exhibited a 100 % match with the query sequence TRI1 (*Trixis inula*) for *trnH-psbA* region.

CONCLUSIONS

In this work, we generated barcodes for the most important melliferous plants associated with *Sabal yapa* honey production of Eastern Yucatan with well-verified specimens. None of the three markers evaluated could independently identify all the studied species successfully. Nonetheless, the results from this preliminary study suggest that the ITS2 marker demonstrated favorable amplification and sequencing rates using a single primer pair, leading to more accurate species identification. This increased accuracy is attributed, in part, to the availability of reference sequences in GenBank. This first approach suggests the potential of ITS2 as a candidate barcode for honey flora. Furthermore, we recommend continued efforts in generating the *trnH-psbA* barcode for melliferous species to assess its utility as a honey barcode in the Yucatan Peninsula. Additionally, it is valuable to use other commercially significant monofloral honey as models to replicate this study, broadening the coverage of melliferous species. Generating reference sequences from well-verified

specimens will provide a comprehensive representation of these species in public databases, and these sequences can be employed with utmost confidence for future analyses (e.g., metabarcoding, food authentication) specifically targeting the species of interest.

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