Tropical Biomedicine 36(4): 938–957 (2019)

Genetic diversity and DNA barcoding of the black fly (Diptera: Simuliidae) vectors of parasites causing human onchocerciasis in Guatemala

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Received 30 June 2019; received in revised form 5 August 2019; accepted 8 August 2019

Abstract. Genetic variation based on mitochondrial cytochrome *c* oxidase I (COI) and II (COII) sequences was investigated for three black fly nominal species, *Simulium metallicum* Bellardi complex, *S. callidum* Dyar & Shannon, and *S. ochraceum* Walker complex, which are vectors of human onchocerciasis from Guatemala. High levels of genetic diversity were found in *S. metallicum* complex and *S. ochraceum* complex with maximum intraspecific genetic divergences of 11.39% and 4.25%, respectively. Levels of genetic diversity of these nominal species are consistent with species status for both of them as they are cytologically complexes of species. Phylogenetic analyses revealed that the *S. metallicum* complex from Guatemala divided into three distinct clades, two with members of this species from several Central and South American countries and another exclusively from Mexico. The *Simulium ochraceum* complex from Guatemala formed a clade with members of this species from Mexico and Costa Rica while those from Ecuador and Colombia formed another distinct clade. Very low diversity in *S. callidum* was found for both genes with maximum intraspecific genetic divergence of 0.68% for COI and 0.88% for COII. Low genetic diversity in *S. callidum* might be a consequence of the result being informative of only recent population history of the species.

INTRODUCTION

Black flies are important hematophagous insects. They transmit various disease agents such as arbovirus, protozoa and filarial nematode (Adler & McCreadie, 2019). Human onchocerciasis or River Blindness is among the most well-recognized diseases caused by the filarial nematode, *Onchocerca volvulus*, and is transmitted by at least 27 black fly species (Crosskey, 1990; Adler *et al.*, 2004). This disease occurs mainly in Africa where 27 countries have recorded the disease. In Central and South America, human onchocerciasis was endemic in six countries including Brazil, Colombia, Ecuador, Guatemala, Mexico and Venezuela (Adler & McCreadie, 2019) but recently the disease was declared to be eliminated from Colombia, Mexico, Ecuador and Guatemala (Rodríguez-Pérez *et al.*, 2015; Guevara *et al.*, 2018; WHO, 2019).

In Guatemala, three black fly species, *Simulium metallicum* Bellardi complex, *S. callidum* Dyar & Shannon and *S. ochraceum* Walker complex are the important vectors of the human onchocerciasis (Takaoka & Suzuki, 2015). *Simulium metallicum* complex is geographically widespread in Central and South America (Adler &

McCreadie, 2019). Chromosomal study found that the *S. metallicum* complex is comprised of 17 cytoforms and many within these are potentially full species (Adler *et al.*, 2017). Molecular characterization based on DNA barcoding sequences found considerable level of genetic variation (maximum intraspecific genetic distance of 5.26%) corresponding with the high cytogenetic diversity (Hernández-Triana *et al.*, 2015). However, specimens of the *S. metallicum* complex from Guatemala have not yet been molecularly investigated.

The *Simulium ochraceum* complex is also geographically widespread in Central and South America (Shelley *et al.*, 2010; Adler, 2019). Cytogenetic investigation revealed that this species is comprised of three cytoforms (A – C) (Hirai *et al.*, 1994) of which two (A and C) occur in Guatemala (Hirai *et al.*, 1994; Adler & McCreadie, 2019). These cytoforms are ecologically and behaviorally (i.e. biting preference) different (Hirai *et al.*, 1994). Cytoform A and C are also different based on isozyme patterns (Agatsuma, 1987). The analysis of COI sequences (called DNA barcoding) of the *S. ochraceum* complex revealed a high level of intraspecific genetic divergence with the maximum K2P distance of 6.13% (Hernández-Triana *et al.*, 2015) but again specimens from Guatemala were not included in previous studies.

Simulium callidum occurs mainly in Central America (Adler & McCreadie, 2019). There is no evidence of a cytological species complex in this species but molecular characterization based on DNA barcodes revealed that specimens from Belize are genetically very different from the others and raised suspicion that they are different species (Hernández-Triana *et al.*, 2015). Like those of the above two species, DNA barcoding sequences of specimens of this species from Guatemala have not yet been investigated.

DNA barcoding has been proven as an effective technique for black fly identification. Many studies from different geographic regions have revealed a high success rate for identification of the morphospecies (e.g. Rivera & Currie, 2009; Hernández-Triana *et al.*, 2012, 2014, 2015; Pramual & Adler, 2014; Ruiz-Arrondo *et al.*, 2018) or in some case, members of a species complex (Pramual & Adler, 2014). DNA barcoding has an important advantage over traditional taxonomy because it does not depend on developmental stages. This is very useful for black fly identification because many species are recognized as species complexes based on chromosomal analyses of the larva, but it is an adult female that transmits disease agents. Therefore, DNA barcode sequences could associate female adults and cytologically characterized larvae (Pramual & Wongpakam, 2014). In this study, although we are unable to obtain DNA from chromosomal characterized specimens, DNA barcoding sequences of the human and animal biting specimens will be very useful for further association with the cytoforms. In addition, although the three black fly species investigated in this study are from the Onchocerciasis foci in Guatemala, DNA barcoding sequences of these species have not yet been investigated.

MATERIALS AND METHODS

Specimen collection, DNA extraction, amplification and sequencing

Black fly specimens were collected when they bit humans or animals from five locations in Guatemala from April to June in 1992 (Table 1). Specimens were preserved in 80% ethanol and kept in a refrigerator at -20°C until use. DNA was extracted from whole adult specimens using E.Z.N.A.® Tissue DNA kit (Omega bio-tek, U.S.A.). Fragments of mitochondrial cytochrome oxidase subunit I (COI) and II (COII) were amplified using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) and TL2-J-3034 and TK-N-3785 (Simon *et al.*, 1994), respectively. Polymerase chain reaction (PCR) conditions and temperature profile for COI gene were as described in Vidergar *et al.* (2014) and for COII as described in Beckenbach and Borkent (2003). PCR products were purified using E.Z.N.A.® Gel

Table 1. Sampling site and number of the black fly specimens from Guatemala used in this study

Location	Latitude / Longitude	Elevation (m)	Date	Species (n)
Patulul, Suchitepéquez Department	14.263191 N / 91.083864 W	551	21 April 1992	*S. callidum* (5) *S. ochraceum* complex (5) *S. metallicum* complex (10)
Chicacao, Suchitepéquez Department	14.342342 N / 91.191731 W	925	28 April 1992	*S. callidum* (10) *S. ochraceum* complex (5) *S. metallicum* complex (10)
Yepocapa, Chimaltenango Department	14.304949 N / 90.565520 W	1,472	5 May 1992	*S. ochraceum* complex (3) *S. metallicum* complex (10)
Rincon, Chiquimula Department	14.454781 N / 90.525047 W	2,138	7 May 1992	*S. callidum* (3) *S. ochraceum* complex (10)
Escuintla, Escuintla Department	14.201702 N / 90.463321 W	771	5 June 1992	*S. metallicum* complex (10)

n, number of specimens

Extraction kit (Omega bio-tek, U.S.A.). Amplicons were directly sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) via a commercial service provider (eurofins, Japan).

Data analysis

A total of 80 black fly specimens representing three morphological taxa, *S. metallicum* complex, *S. callidum* and *S. ochraceum* complex were sequenced for COI and COII genes. However, amplification of the COII gene failed in five specimens therefore, in the present study we obtained a total of 155 sequences with 80 for COI (GenBank accession numbers: MN053562-MN053641) and 75 for COII (GenBank accession numbers: MN053642-MN053716). The COI sequences of these species were also retrieved from public databases (e.g. NCBI GenBank) (Table 2). In total, 201 COI sequences of the *S. metallicum* complex, 41 sequences for *S. callidum* and 59 sequences for the *S. ochraceum* complex were included in the genetic diversity analysis. Intraspecific and interspecific genetic divergences were calculated based on Kimura 2-parameter in MEGA X (Kumar *et al*., 2018).

Phylogenetic analyses were performed separately for members of the *S. metallicum* complex and for *S. callidum* + *S. ochraceum* complex because the latter two taxa belong to different subgenera. Phylogenetic analyses of *S. metallicum* complex were based on 40 COI sequences obtained in this study and 231 sequences representing seven species obtained from the GenBank (Table 2). Phylogenetic analyses of the *S. callidum* and *S. ochraceum* complex were based on 111 COI sequences representing 10 species retrieved from GenBank (Table 2) and 40 sequences obtained in this study. Genetic relationships between specimens were estimated using the maximum-likelihood (ML) method implemented in RAxML web server version (https://raxml-ng.vital-it.ch/#/) (Kozlov *et al*., 2019). Genetic relationships were also assessed using neighbor-joining (NJ) and Bayesian (BA) methods. The NJ tree was estimated in MEGA X. Branch support for NJ tree was calculated using the bootstrapping method with 1,000 replications. The BA analysis was performed in MrBayes 3.2.7a (Ronquist *et al*., 2012) with 2,000,000 generations and sampling frequency of 100 generation. In all phylogenetic analyses, *S. siamense* and *S. phurueaense* were used as outgroups.

Table 2. Lists of the COI sequences retrieved from GenBank used in this study

Species	n	GenBank accession numbers	Sources
subgenus *Aspathia* Enderlein			
S. metallicum complex	40	MN053602 - MN053641	This study
	2	KC015101 - KC015102	Hernández-Triana *et al.*, 2012
	16	KF840010 - KF840025	Hernández-Triana *et al.*, 2014
	143	KP252287, KP252291, KP252295, KP252296, KP252297, KP252300, KP252306, KP252311, KP252317, KP252322, KP252331, KP252333, KP252336, KP252343, KP252365, KP252370, KP252373, KP252374, KP252379, KP252381, KP252385, KP252386, KP252388, KP252391, KP252393, KP252406, KP252408, KP252411, KP252413, KP252415, KP252419, KP252423, KP252425, KP252427, KP252436, KP252443, KP252445, KP252448, KP252451, KP252456, KP252457, KP252458, KP252462, KP252464, KP252465, KP252469, KP252478, KP252481, KP252484, KP252495, KP252498, KP252515, KP252525, KP252527, KP252531, KP252532, KP252536, KP252537, KP252538, KP252541, KP252546, KP252551, KP252553, KP252556, KP252559, KP252560, KP252573, KP252577, KP252586, KP252589, KP252591, KP252596, KP252608, KP252611, KP252616, KP252627, KP252630, KP252637, KP252638, KP252641, KP252642, KP252644, KP252647, KP252656, KP252660, KP252671, KP252682, KP252692, KP252694, KP252704, KP252710, KP252717, KP252728, KP252732, KP252741, KP252742, KP252743, KP252745, KP252746, KP252749, KP252757, KP252764, KP252768, KP252775, KP252786, KP252797, KP252807, KP252808, KP252812, KP252813, KP252814, KP252815, KP252819, KP252820, KP252822, KP252823, KP252825, KP252835, KP252840, KP252842, KP252848, KP252857, KP252864, KP252867, KP252876, KP252877, KP252878, KP252883, KP252885, KP252887, KP252889, KP252891, KP252906, KP252911, KP252913, KP252915, KP252917, KP252921, KP252926, KP252928, KP252939, KP252943, KP252944	Hernández-Triana *et al.*, 2015
S. horacioi	10	KP252351, KP252442, KP252482, KP252501, KP252520, KP252572, KP252574, KP252615, KP252750, KP252934	Hernández-Triana *et al.*, 2015
S. hunteri	20	FJ524702 - FJ524721	Rivera & Currie, 2009
S. iriartei	8	KP252305, KP252347, KP252356, KP252358, KP252648, KP252690, KP252699, KP252933	Hernández-Triana *et al.*, 2015
S. jobbinsi	13	KP252299, KP252312, KP252348, KP252420, KP252468, KP252474, KP252479, KP252483, KP252513, KP252519, KP252568, KP252708, KP252778	Hernández-Triana *et al.*, 2015
S. piperi	11	FJ524752 - FJ524762	Rivera & Currie, 2009
S. puigi	8	KP252339, KP252429, KP252492, KP252673, KP252697, KP252705, KP252771, KP252780	Hernández-Triana *et al.*, 2015

Table 2 *continued...*

Species	n	GenBank accession numbers	Sources
subgenus *Psilopelmia* Enderlein			
S. callidum	17	MN053562 - MN053578	This study
	7	KF839967 - KF839973	Hernández-Triana *et al.*, 2014
	17	KP252288, KP252313, KP252319, KP252403, KP252488, KP252503, KP252523, KP252528, KP252533, KP252575, KP252652, KP252703, KP252727, KP252753, KP252777, KP252806, KP252834	Hernández-Triana *et al.*, 2015
S. ochraceum complex	23	MN053579 - MN053601	This study
	11	KF840028 - KF840038	Hernández-Triana *et al.*, 2014
	25	KP252303, KP252349, KP252369, KP252387, KP252396, KP252410, KP252499, KP252530, KP252552, KP252567, KP252607, KP252624, KP252683, KP252691, KP252721, KP252758, KP252769, KP252804, KP252811, KP252839, KP252858, KP252865, KP252866, KP252886, KP252892	Hernández-Triana *et al.*, 2015
S. travisi	8	KP252316, KP252354, KP252450, KP252599, KP252602, KP252653, KP252686, KP252687	Hernández-Triana *et al.*, 2015
S. nr. *pseudocallidum*	8	KP252290, KP252344, KP252545, KP252610, KP252620, KP252772, KP252948, KP252949	Hernández-Triana *et al.*, 2015
S. cormonsi	3	KU986863 - KU986865	Colorado-Garzon *et al.*, 2017
S. ignescens	2	KJ093479, KJ093484	Colorado *et al.* (unpublished data)
	22	KU986836 - KU986855, KU986859 - KU986861	Colorado-Garzon *et al.*, 2017
S. furcillatum	2	KJ093448 - KJ093449	Colorado *et al.* (unpublished data)
S. rubiginosum	1	KJ093480	Colorado *et al.* (unpublished data)
S. tolimaense	1	KJ093481	Colorado *et al.* (unpublished data)
S. tunja	4	KU986858, KU986862, KU986866, KU986867	Colorado-Garzon *et al.*, 2017

n, number of sequences.

Table 3. Range of intraspecific genetic divergences of three black fly species from Guatemala

Species/Gene	COI Guatemala (average) (n)	All (average) (n)	COII Guatemala (average) (n)
S. metallicum complex	0 – 11.39% (5.72%) (40)	0 – 11.56% (5.05%) (201)	0 – 10.72% (4.97%) (34)
S. ochraceum complex	0.17 – 4.25% (1.83%) (23)	0.17 – 13.09% (4.67%) (59)	0 – 11.16% (2.31%) (23)
S. callidum	0 – 0.68% (0.27%) (17)	0 – 10.37% (3.09%) (41)	0 – 0.88% (0.20%) (18)

n, number of sequences.

RESULTS

Genetic diversity

Simulium metallicum complex

A total of 40 sequences were obtained for the COI gene and 34 for COII gene from Guatemala. A high level of intraspecific genetic divergence (max. 11.39% for COI and 10.72% for COII) was found in this species. Similar levels of maximum intraspecific genetic divergence (max. 11.56%) were found when data were combined with COI sequences from other countries retrieved from NCBI GenBank (Table 3). There are no reports of COII sequences of *S. metallicum* complex in any public database.

Simulium ochraceum complex

We obtained 23 COI and 23 COII sequences of *S. ochraceum* complex from Guatemala. There is a considerable level of genetic variation within this species with maximum K2P genetic divergence of 4.25% for COI sequences. However, when comparing the genetic divergence with sequences from other countries obtained from NCBI GenBank, there is a strikingly high intraspecific genetic divergence with a maximum value of 13.09%. The intraspecific genetic divergence based on COII sequences is also very high with a maximum value of 11.16% (Table 3).

Simulium callidum

COI and COII genes were sequenced in 17 and 18 specimens of *S. callidum* from Guatemala, respectively. Low intraspecific genetic divergence was found for both genes with maximum values of 0.68% and 0.88% for COI and COII, respectively. However, when comparing COI sequences of *S. callidum* from Guatemala with those retrieved from GenBank (which are derived from various countries) revealed very high genetic divergence (max. 10.37%) (Table 3).

Phylogenetic relationships

Simulium metallicum complex and members of subgenus *Aspathia* Enderlein

All phylogenetic analyses revealed similar tree topologies thus, only the NJ tree is shown (Fig. 1). Specimens of the *S. metallicum* complex divided into two large clades (I and II). Clade I comprised specimens of the *S. metallicum* complex from various Central and South America countries. Members of this species in clade I further divided into three subclades each with strong support. Subclade I-1 is the largest group consisting of the specimens from Costa Rica, Venezuela, Belize, Colombia, Ecuador, Mexico and Guatemala. Subclade I-2 is a small group represented by only three sequences from Mexico. Subclade I-3 consists of the specimens from Mexico, Guatemala, and Belize. Clade II comprised *S. metallicum* complex from Mexico and Guatemala and is sister-group to the clade of *S. horacioi* Okazawa & Onishi complex + *S. puigi* Vargas, Martínez Palacios & Díaz Nájera.

Simulium callidum and the *S. ochraceum* complex

The NJ tree (Fig. 2) revealed that both species are not monophyletic. The clade of *Simulium ochraceum* complex is paraphyletic because it included *Simulium tunja* Coscarón, *S. ignescens* Roubaud, *S. furcillatum* Wygodzinsky & Coscarón, *S.*

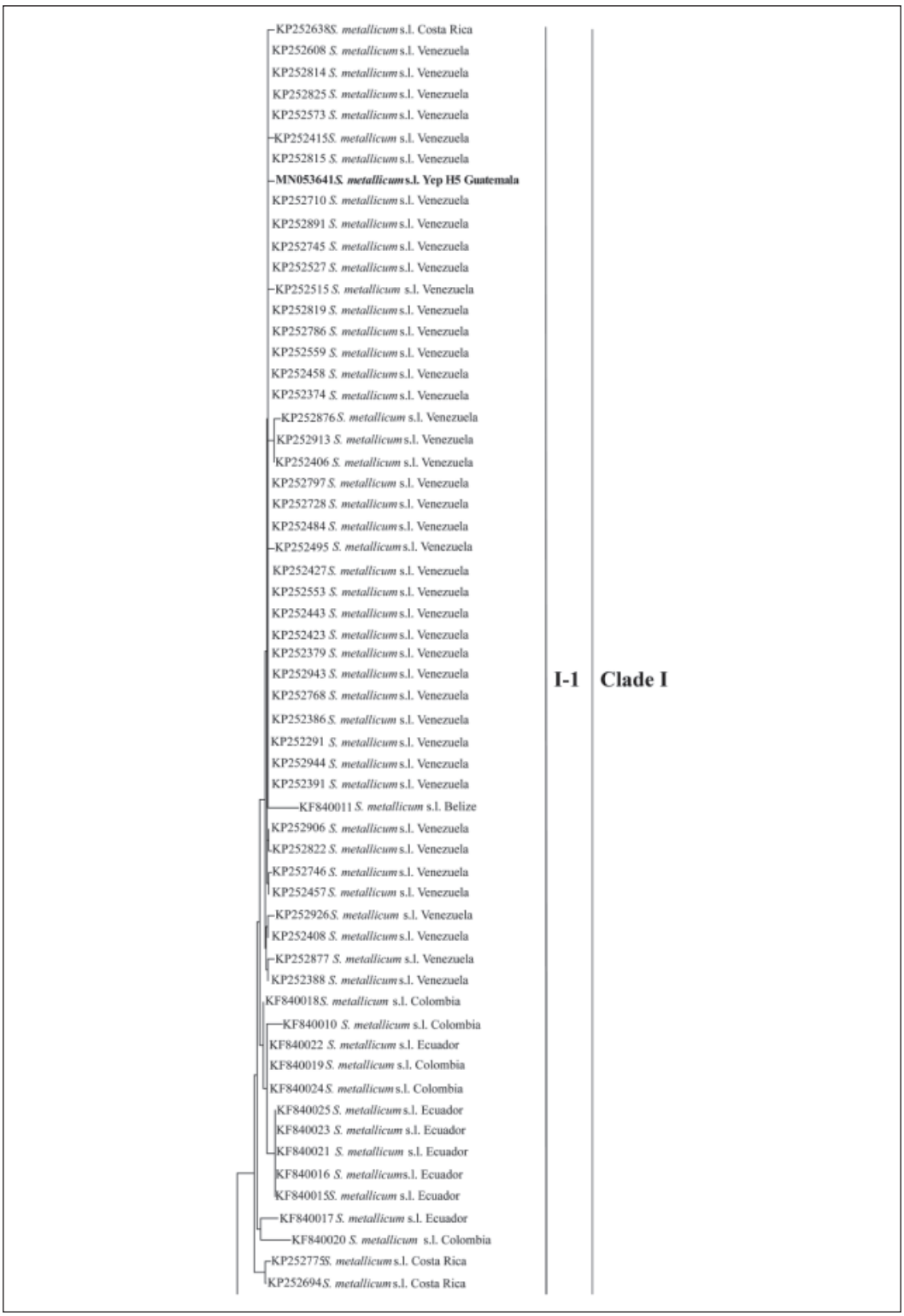

Figure 1. Neighbor-joining tree constructed based on mitochondrial COI sequences for 271 sequences of *Simulium metallicum* complex and members of the subgenus *Aspathia*. Bootstrap values for neighbor joining, maximum likelihood and posterior probability of Bayesian analysis are shown above or near the branches. Bold characters indicate specimens obtained in this study.

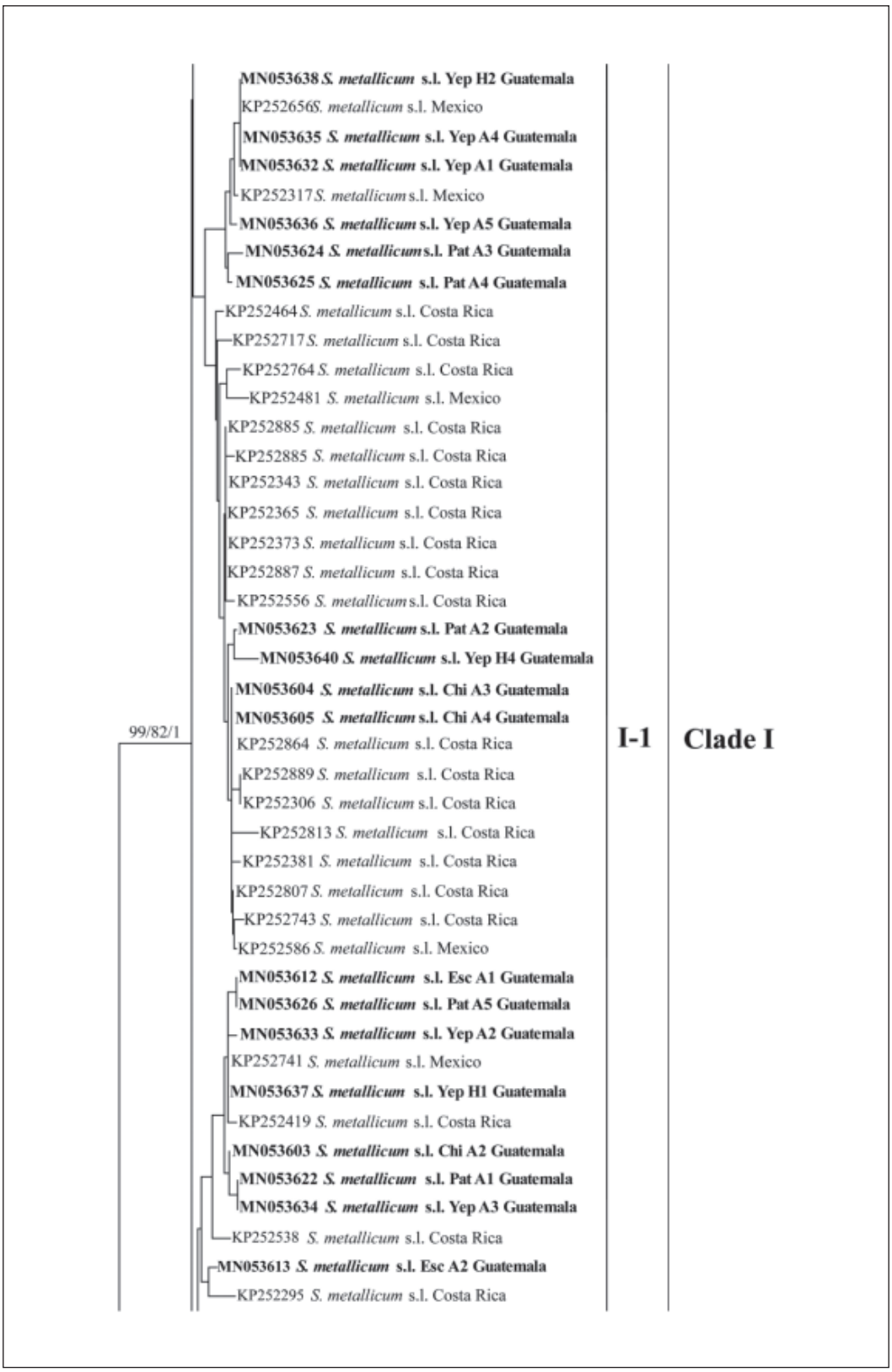

Figure 1 *cont.*

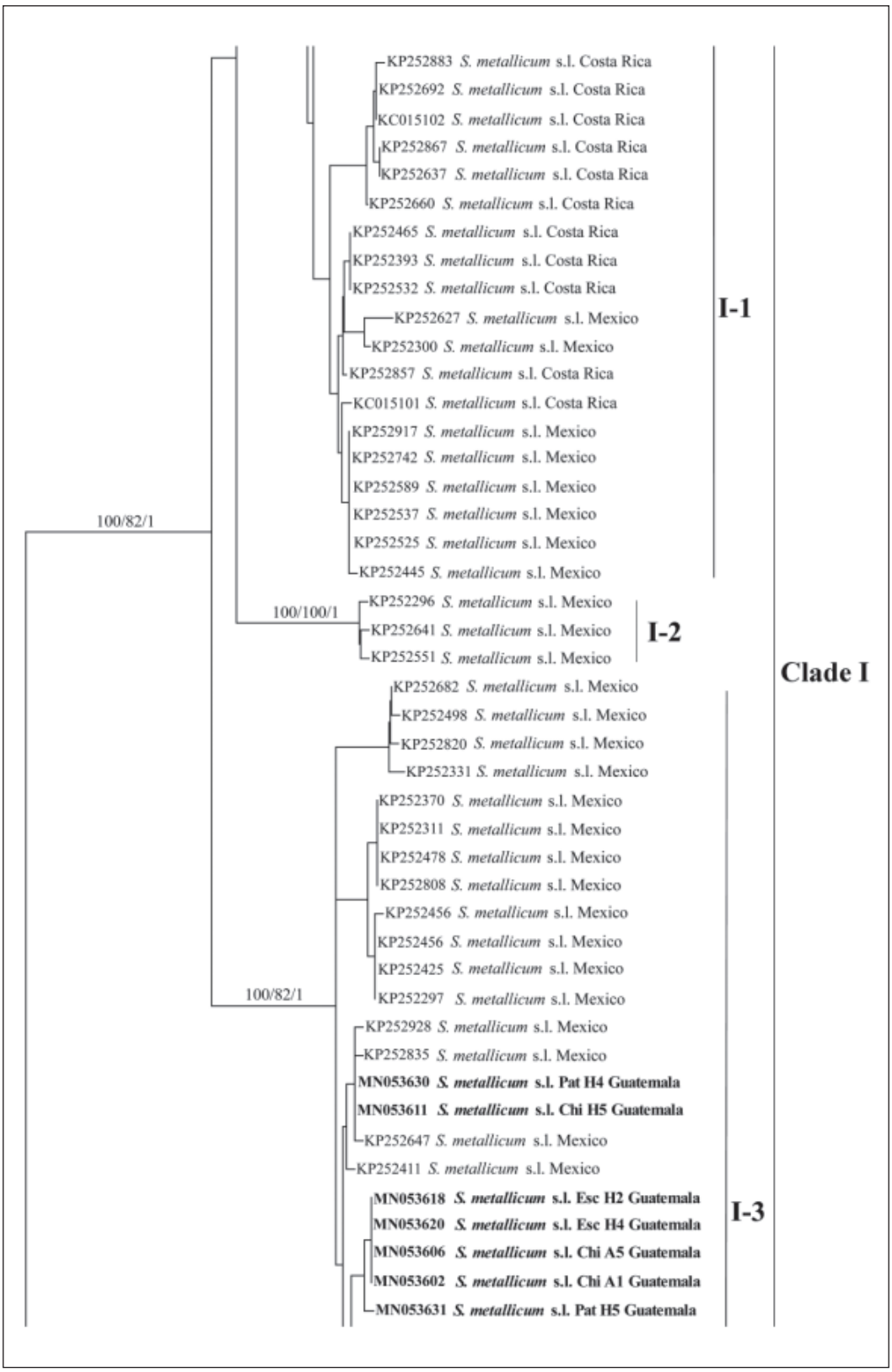

Figure 1 *cont.*

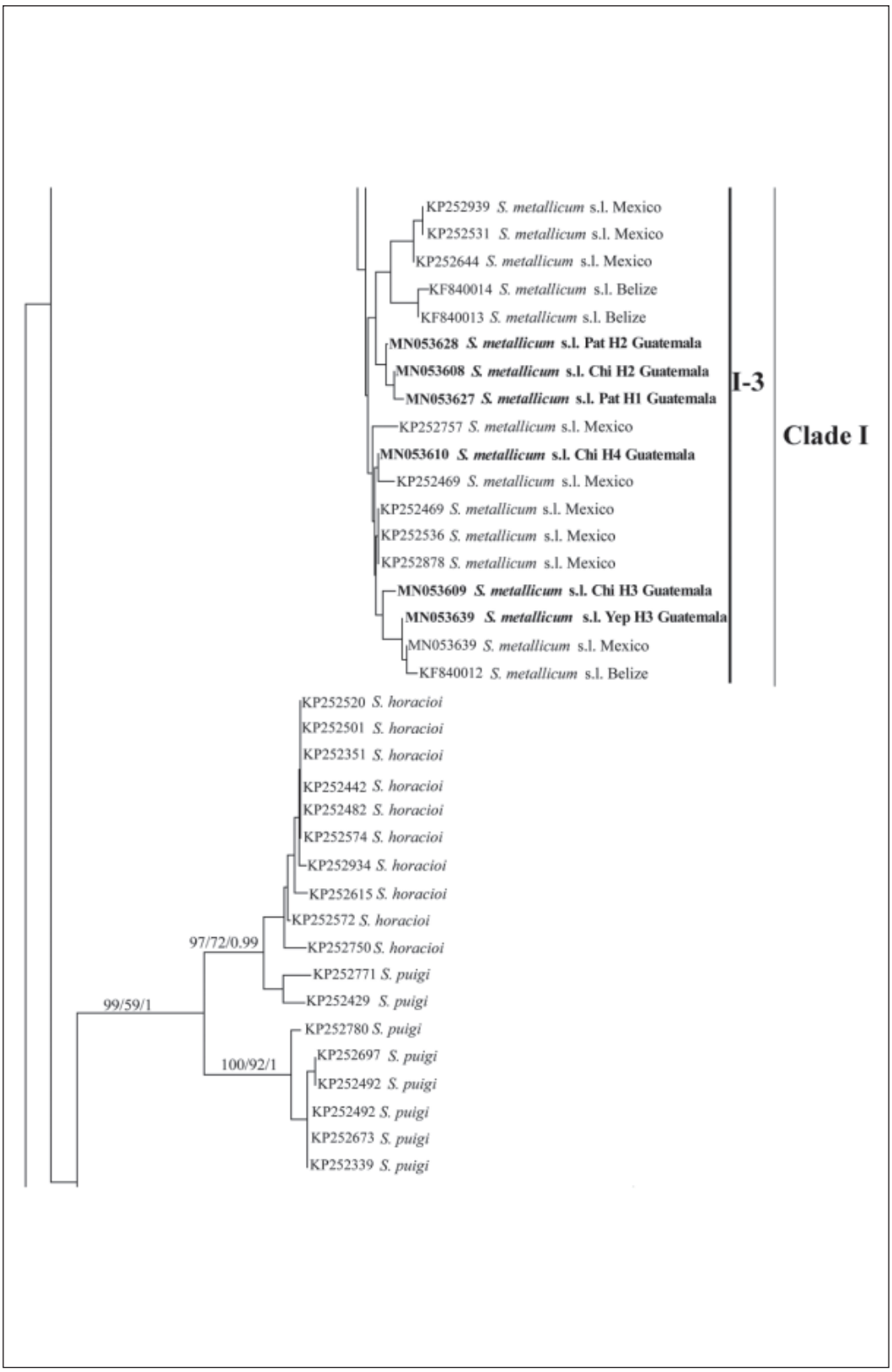

Figure 1 *cont.*

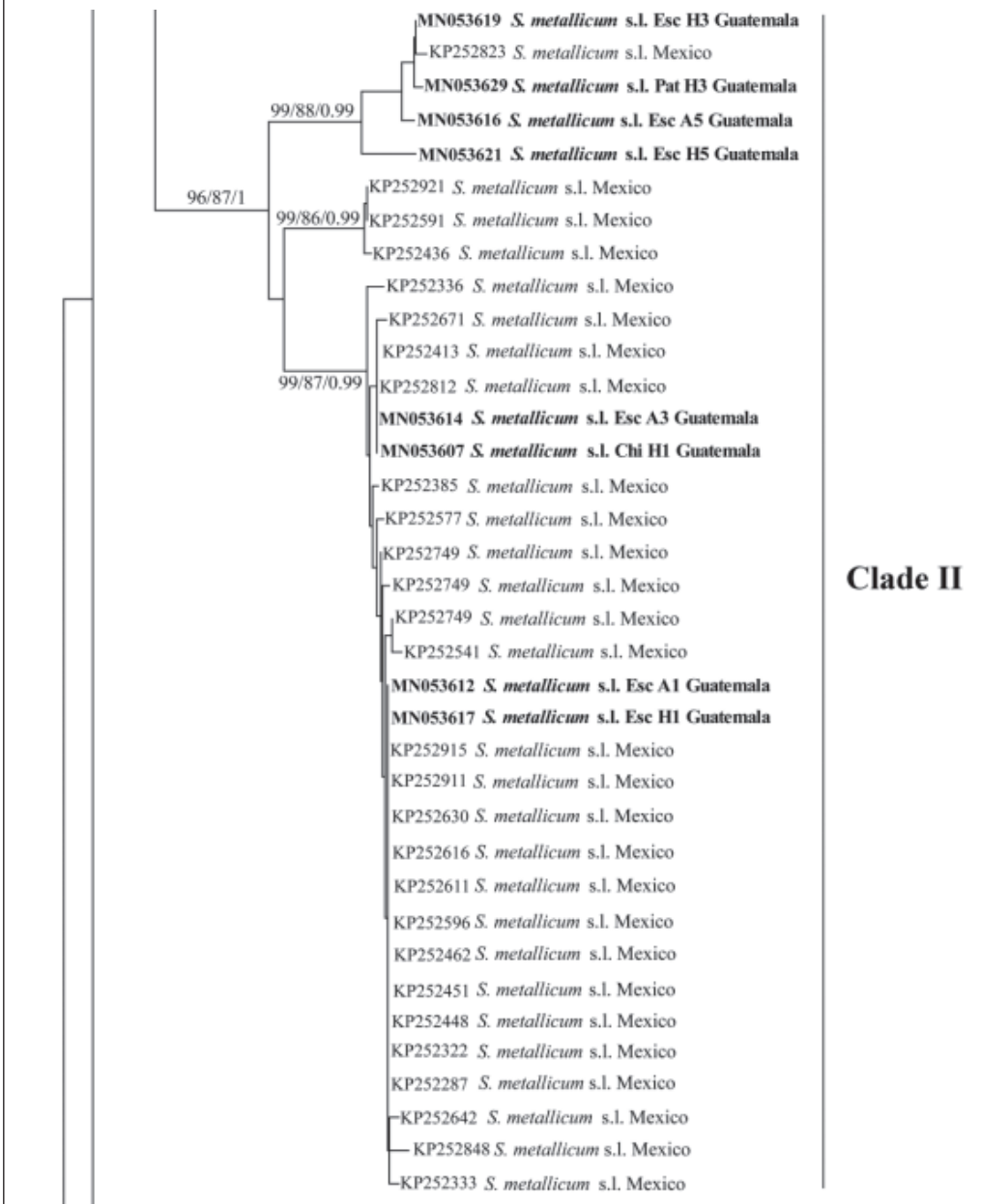

Figure 1 *cont.*

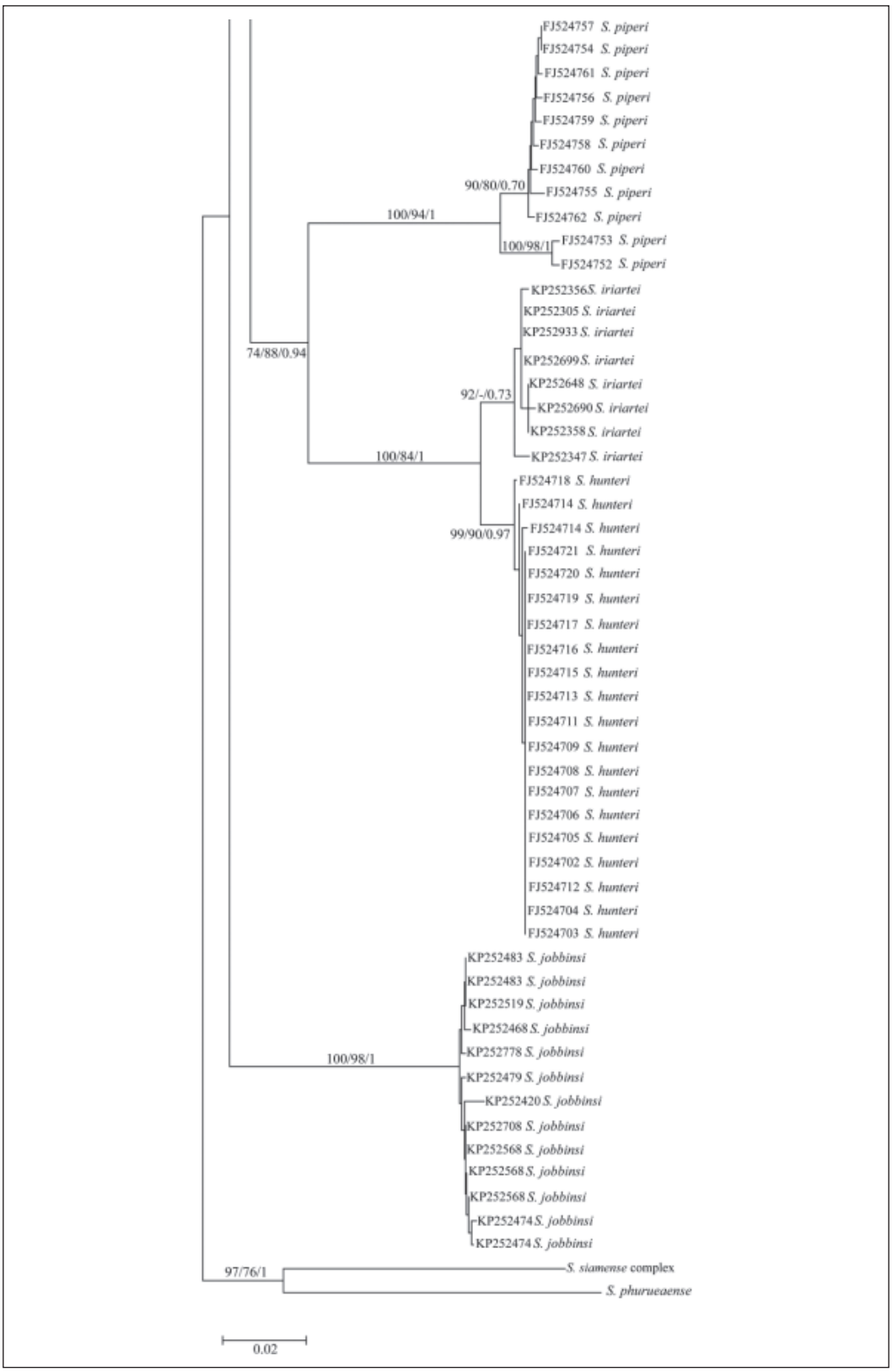

Figure 1 *cont.*

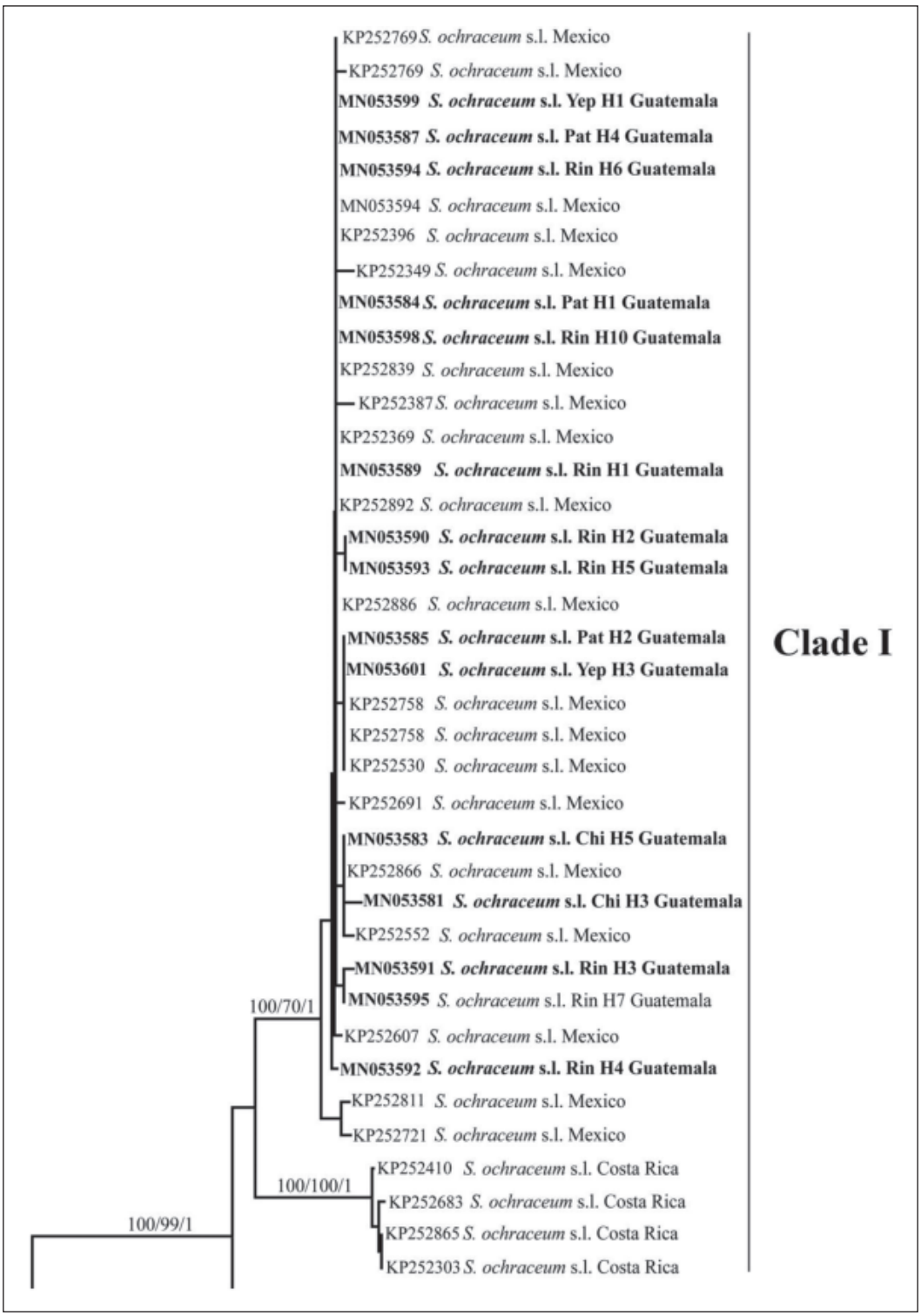

Figure 2. Neighbor-joining tree constructed based on mitochondrial COI sequences for 151 sequences of *Simulium ochraceum* complex, *S. callidum* and members of the *S. bicoloratum* species group of the subgenus *Psilopelmia*. Bootstrap values for neighbor joining, maximum likelihood and posterior probability of Bayesian analysis are shown above or near the branches. Bold characters indicate specimens obtained in this study.

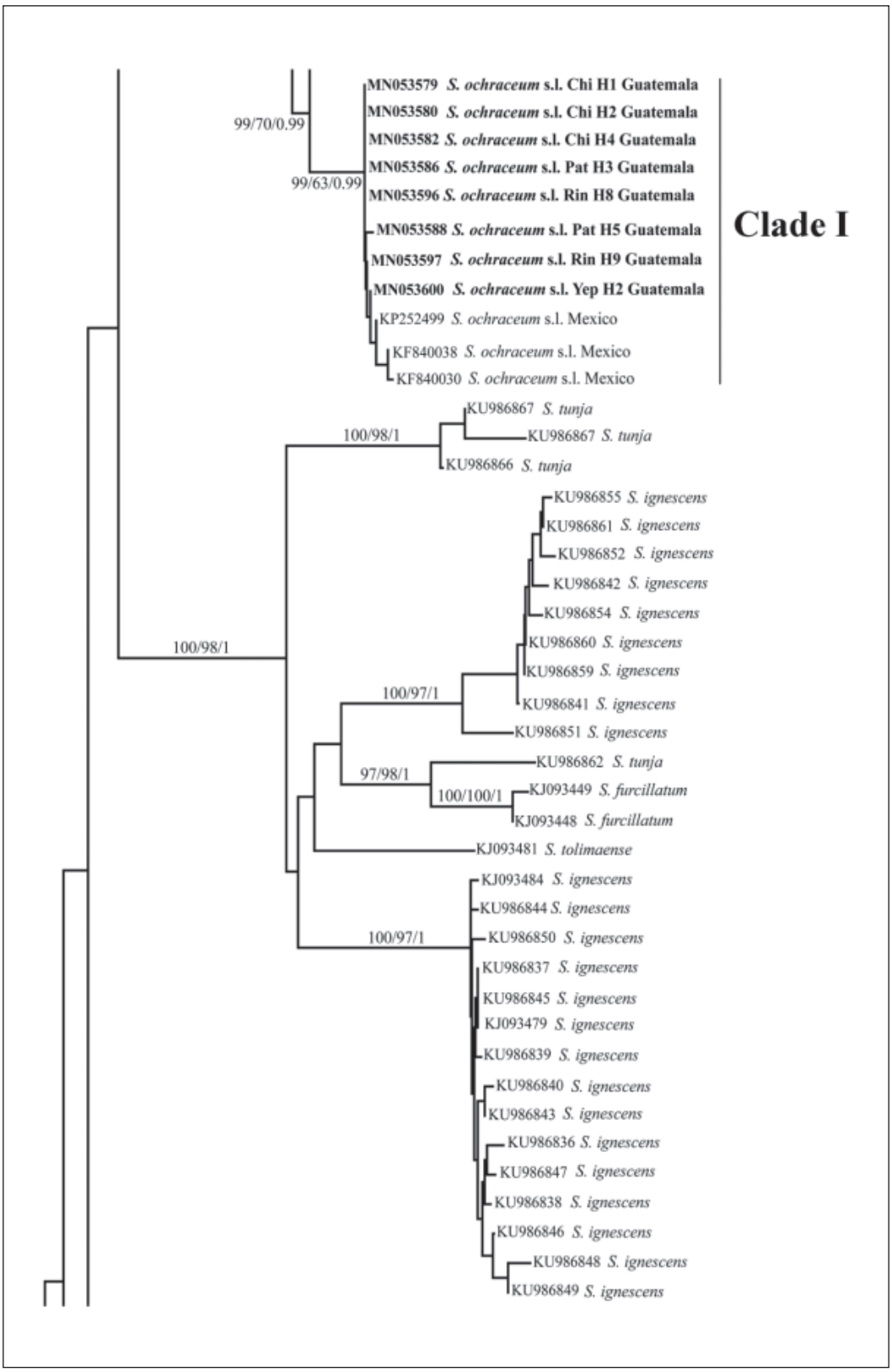

Figure 2 *cont.*

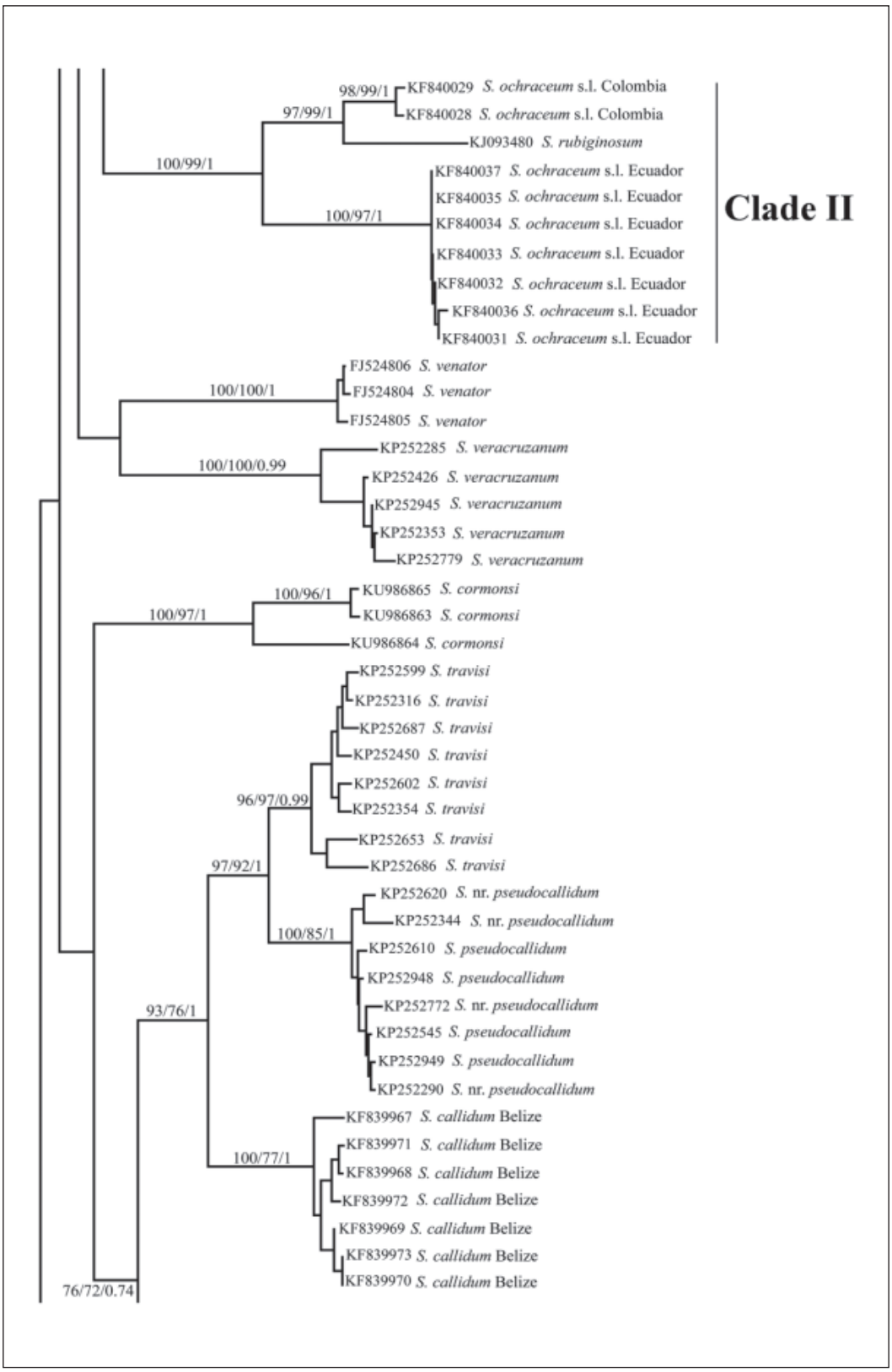

Figure 2 *cont.*

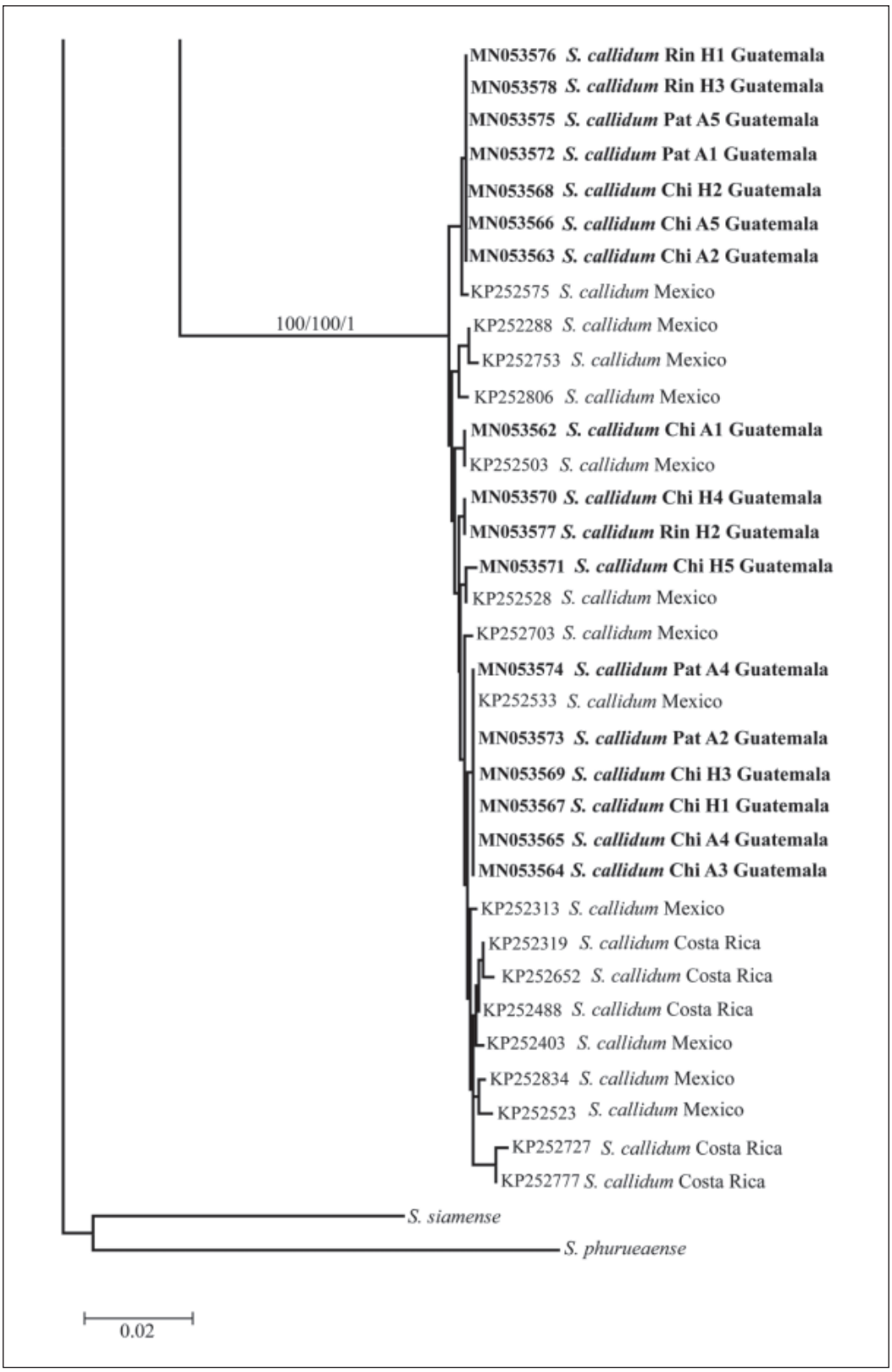

Figure 2 *cont.*

tolimaense Coscarón and *S. rubiginosum* Enderlein. Specimens of *S. ochraceum* complex divided into two distinct clades with most belong to clade I. All specimens of this species from Guatemala, Costa Rica and Mexico belong to this clade. Clade I of *S. ochraceum* complex was sister group to the clade of *S. tunja*, *S. ignescens*, *S. furcillatum* and *S. tolimaense*. Specimens of *S. ochraceum* complex from Colombia and Ecuador formed clade II and included *S. rubiginosum*.

The *S. callidum* clade is paraphyletic because its clade was included in *S. travisi* Vargas, Vargas & Ramírez-Pérez and *S. pseudocallidum* Díaz Nájera. Specimens of *S. callidum* were divided into two clades. Members of this species from Guatemala studied here formed a clade with strong support with conspecific specimens from Mexico and Costa Rica. *Simulium callidum* from Belize formed another clade with strong support. This clade is sister-group to the clade that consists of *S. travisi* and *S. pseudocallidum*.

DISCUSSION

Genetic diversity of the *S. metallicum* complex

DNA barcoding sequences of the *S. metallicum* complex have been reported previously (Hernández-Triana *et al*., 2015) but did not include members of this complex from Guatemala. Thus, our results are the first report of COI sequences from the *S. metallicum* complex from Guatemala, one of the important Onchocerciasis foci (Takaoka & Suzuki, 2015). Maximum intraspecific genetic divergence for the *S. metallicum* complex from Guatemala (11.39%) is much higher than conspecific specimens from other countries (5.26%) (Hernández-Triana *et al*., 2015). High intraspecific genetic divergence in *S. metallicum* complex from Guatemala corresponds with the DNA barcode tree. The NJ tree revealed that the *S. metallicum* complex divided into two main clades (I and II) within which clade I further divided into three subclades. Most sequences of the *S. metallicum* complex belong to clade I with the exception of 28 sequences from Mexico and eight sequences from Guatemala that form clade II which is sister group to *S. horacioi* + *S. puigi* clade. Therefore, *S. metallicum* complex is not a monophyletic species. Cytological studies indicated that *S. metallicum* is comprised of 17 cytoforms (Conn *et al*., 1989; Arteaga & Muñoz de Hoyos, 1999; Adler *et al*., 2017). Among these, four (A, B, H, I) occur in Guatemala (Conn *et al*., 1989). We cannot associate the three clades of *S. metallicum* complex from Guatemala into cytoforms, but at least our results support cytogenetic information that found at least four cytoforms in Guatemala. Cytoform A and B are retained as members of the *S. metallicum* complex but cytoform H and I are assigned as members of the *S. horacioi* complex in which the name *S. horacioi* is given as the formal name for cytoform H (Adler *et al*., 2017). Our results found that some specimens of the *S. metallicum* complex from Guatemala studied here are genetically closely related to *S. horacioi*, supporting the above assignment although cytoforms of our specimens could not be identified.

Genetic diversity of the *S. ochraceum* complex and *S. callidum*

Genetic diversity based on COI sequences for *S. ochraceum* complex is high with maximum value of 13.09%. This is due to the separation of specimens of this nominal species into two distinct clades. The main clade that included specimens from Mexico, Costa Rica and our specimens from Guatemala most likely represents the true *S. ochraceum* because the type locality of this species is in Mexico. Therefore, specimens from *S. ochraceum* complex from Colombia and Ecuador could be different species.

Two cryptic species of the *S. ochraceum* complex were recognized in Guatemala; *S. ochraceum* A and C (Hirai *et al*., 1994). Phylogenetic analysis based on the COI sequences revealed that specimens from Guatemala are divided into two groups with

the maximum sequences divergence of 4.25%. However, lacking the DNA barcoding sequences of the chromosome characterized specimens (i.e. larva for cryptic species identification) preclude us from associating these two groups with cryptic species.

Although the DNA barcode tree revealed that specimens from Guatemala divided into two clades, the level of genetic diversity of the *S. ochraceum* complex found within specimens from Guatemala is lower, compared with those previously reported based on specimens from Colombia, Costa Rica, Ecuador and Mexico (Hernández-Triana *et al.*, 2015). Thus, it is not possible to conclude here if these two clades represent two cryptic species of *S. ochraceum*. Further investigation using DNA barcodes based on chromosomal identified larval specimens will be useful to link with adult specimens.

Simulium callidum was suspected to be a species complex based on the deep split of the specimens from Belize in the phylogenetic analysis of Hernández-Triana *et al.* (2015). Specimens from Guatemala studied here belonged to the large clade that included this species and those from many other countries including Mexico where the type locality of this species is located. The results suggests that our specimens are the true *S. callidum* and those from Belize need further taxonomic examination because they are likely to be different species.

Very low genetic diversity of this species was found in the specimens from Guatemala with maximum K2P genetic divergence of 0.68%. Genetic diversity of *S. callidum* is much lower, compared to the species of the same species group, *S. ochraceum* complex that has maximum K2P genetic distance of 4.25%. Differentiations in levels of genetic diversity could be related to the differentiation in ecology or demographic history. In the Oriental black flies, closely related species that utilize different ecological ranges possess different level of genetic diversity. Species occupying wider ecological conditions possessed greater diversity. Species that have longer history could also possess greater genetic diversity (Pramual & Kuvangkadilok, 2012).

Ecological observations of both immature and adult stages found similar ecological conditions (i.e. ranges of elevation, stream width, depth, current velocity, water temperature, and types of larval substrates) for *S. ochraceum* and *S. callidum* in Guatemala (Dalmat, 1954). In fact, the ecological condition of the larval habitat is wider for *S. callidum* with regard to the elevation (Dalmat, 1954). Because similar habitat is utilized by both species, this factor is unlikely to explain much lower genetic diversity in *S. callidum*. We hypothesize that low diversity in *S. callidum* could be a result of recent demographic history of this species. However, small sample sizes preclude us from in depth analysis and thus require further investigation.

In conclusion, our results revealed that the COI and COII gene sequences showing similar levels of genetic variations but the former is much more available in the public database thus it is more useful. We found a high level of molecular genetic variations in the *S. metallicum* complex and the *S. ochraceum* complex but very low for *S. callidum* from Guatemalan specimens. The results are consistent with high diversity at chromosomal level of first two species. Divergent lineages detected on the basis of COI phylogenetic analyses also correspond with numbers of cytoforms of these species reported in the country. Sequences of three important black fly species vectors of the human onchocerciasis in this study will be useful for further association with the chromosomal characterize larvae. This will enable an investigation of the molecular genetic differentiations among cryptic species or cytoforms of the species complex.

Acknowledgements. This work was supported by Mahasarakham University. The authors are indebted to the staffs of the institute Servicio Nacional de Erradicacion de la Malaria (SNEM), Ministerio de Salud Publica, Guatemala, for their invaluable cooperation.

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