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Genetic differentiation of two morphotypes of *Piper regnellii* (Miq.) C. DC. var. *regnellii*

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ABSTRACT. *Piper regnellii* (Miq.) C. DC. var. *regnellii*, popularly known as "pariparoba", is used for therapeutic purposes. However, the morphological similarity among *Piper* species can lead to misidentifications, so molecular markers have been used to validate the identification of species and their morphotypes. Among the molecular markers used in plants, the intergenic spacer region *trnL-trnF* has proven effective in identifying plant species. For this reason, this region was used to evaluate two morphotypes of *Piper regnellii* var. *regnellii*. Studies with the *trnL-trnF* region have shown this region as a good marker for establishing phylogenetic relationships, distinguishing species, and identifying new species. We concluded that the *trnL-trnF* sequenced region show one indel of difference between the two morphotypes. It would be interesting to analyze these two morphotypes with a more variable region than the one used here, aiming to show intraspecific differences.

Keywords: piperaceae; pariparoba; molecular identification; *trnL*; *trnF*.

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Introduction

Popularly known as pariparoba or caapeba, *Piper regnellii* is an herbaceous plant belonging to Piperaceae. This species is distributed in tropical and subtropical regions (Salatino & Silva, 1975; Cronquist & Takhtadzhian, 1981), frequently occurring in some states of Brazil, Paraguay and Argentina (Nakamura et al., 2006). Leaves of *Piper regnellii*, as well as other *Piper* leaves are being widely used in the form of crude extract in the anti-inflammatory, anti-tumor treatment, and in the evaluation of inhibitory activity (Fuertes, Pacco, Dextre & Cruz, 2018; Guo et al., 2019) becoming an important group to be studied.

The genus *Piper* stands out for its diverse therapeutic properties, used in folk medicine, helping to treat liver and stomach disorders. It is also used as an anti-inflammatory, anticancer and insect repellent (Salehi et al., 2019). Other interests that highlight the importance of this genus are its psychotropic and anxiolytic agents that are used by the pharmaceutical industry, and the great economic importance in the production of essential oils (Parmar et al., 1997; Nakamura et al., 2006). Among its species, *Piper methysticum*. Forst has been highlighted, popularly known as kawa-kawa, whose roots are used in the treatment of disorders of the central nervous system, especially in cases of depression and insomnia (Salehi et al., 2019).

In this sense, the correct identification of plants becomes essential when studying medicinal plants, since some species are very similar to each other, and thus, they are confused during harvest and final use, generating different results than expected. In *Piper* it has not been different, similarities between species result in identification problems, affecting the desired results exclusive of a certain species of this group (Salatino & Silva, 1975). Such errors can be serious since the properties differ even between the species (Salehi et al., 2019).

Another interesting aspect is the phenotypic plasticity that can be normally found in *Piper* species. For example, in the morphology of the leaf and the ear (Waller, 1986). Thus, analysis of the genetic diversity of plant species, as well as the analysis of the delimitation and phylogeny of the species, have been effectively supported by specific analyses of particular DNA sequences, and extremely used to establish phylogenetic relationships between species (Kobayashi, Fuse, & Tamura, 2019). In *P. regnellii* var. *regnellii*, there are at least two morphotypes that present morphological variation in ears and leaves, key characteristics for identification of Piperaceae species, both have been identified as being the same species.

In 2003 Hebert Cywinska, Ball and Dewaard proposed the DNA barcoding technique, a method that uses small fragments of DNA to identify organisms through nucleotide sequences. This technique has proven efficient in the identification of species, mainly solving the recurring problems to morphologically similar species (Hebert, Cywinska, Ball, & Dewaard, 2003). In the plant kingdom, the main sequences that stood out as barcodes were the matK and rbcL genes, which demonstrated high efficiency in distinguishing plants (CBOL Plant Working Group, 2009).

Another region that has been used for this purpose is the intergenic chloroplast *trnL-trnF* region (Li, Lu, & Barrington, 2008; Kobayashi et al., 2019). This intergenic region is located between the trnL and trnF genes in the chloroplast genome. This region has shown higher rates of mutation and variation when compared to other coding regions, such as, matK and rbcL (Borsch & Quandt, 2009). Studies indicate that the use of non-coding sequences of nuclear DNA and chloroplast DNA (cpDNA), has been useful to identify phylogenetic relationships between different plant species (Wall & Herbeck, 2003; Smith et al., 2006). Thus, among the nucleotide sequences frequently used to distinguish species, the *trnL-trnF* region has been used for differentiation, identification, validation and phylogenetic analysis (Li et al., 2008; Kobayashiet al., 2019). Therefore, the present study aimed to compare two different morphotypes of *Piper regnellii* (Miq.) C. DC. var. *regnellii*, using the partial sequence of the *trnL-trnF* intergenic region as a genetic marker.

Material and methods

Sample collection and DNA extraction

Samples were collected from the leaves of two morphotypes (35 and 45) of *Piper regnellii* var. *regnellii* (Miq.) C. DC. from the Horto Medicinal do Campus 2, *Universidade Paranaense* in Umuarama, state of Paraná, Brazil (23º46'09.1" S and 53º16'38.4" W) under the numbers: *Piper regnellii* var. *regnellii* (Miq) C. DC. number 262 (morphotype 35) and *Piper regnellii* var. *regnellii* (Miq) C. DC. 116 (morphotype 45).

A sterile stylet was used to cut the leaf fragments of each morphotype, then the fragments were washed with ultrapure water and stored at -20ºC.

We used the Thermo Fisher Scientific PureLink™ Genomic Plant DNA Purification kit for DNA extraction following the protocol specified by the manufacturer. Using the technique of horizontal agarose at a concentration of 1%, we checked whether the DNA extraction occurred as expected. DNA of the two morphotypes were immersed in elution buffer from the DNA extraction kit. Subsequently, samples were preserved in -20ºC resuspended with elution buffer.

PCR amplification and sequencing

Amplification of the *trnL-trnF* locus was performed through the polymerase chain reaction (PCR) with the primers described by Reid, Plunkett and Peters (2006): trn-c-F (5´-GGAAATCGGTAGACGCTACG-3´) and trn-f-R (5´-ATTTGAACTGGTGACACGAG-3´). PCR was performed in a thermocycler with an initial cycle of 94 ºC for 3 min; followed by 40 cycles of 94ºC for 30 s; 53ºC for 30 s and 72ºC for 3 min in a total volume of 25 µL, containing 1x rxn buffer Buffer, MgCl2 (50 mM), direct trn-cF primer (2.5 mM), reverse trn-fR primer (2.5 mM), dNTPs (2.5 mM of each dNTP), 0.1 µL Taq Platinum polymerase - Invitrogen (5U μL^{-1}) and 50 ng DNA. Amplicons obtained were analyzed by horizontal electrophoresis on 1% agarose gel, estimated by comparison with the λ Hind III standard. Amplicon purification occurred according to the protocol described by Rosenthal, Coutelle, and Craxton (1993).

Samples were sequenced using the Forward trn-c-F primer in an ABI sequencer AB 3500 platform from Ludwig Biotec - ACTGene Molecular Analysis in Alvorada, Rio Grande do Sul, Brazil. We followed the procedure for preparing the amplicons according to the company's instructions. Sequences obtained were deposited on the GenBank.

Phylogenetic analysis

Sequences were edited using the software UGENE v. 34. Subsequently, we proceeded with the search for homologous sequences through local alignment with the BlastN (Basic Local Alignment Search Tool) software algorithm available on GenBank - NCBI (National Center for Biotechnology Information) to confirm the obtained sequencing. Soon after, we aligned the sequences of the different morphotypes using the Muscle tool implemented in MEGA X.

After quality control, the sequence was prospected on Genbank and downloaded the top 10 obtained from the result. Then, we discarded the repeated sequences. The MEGA X software was used to calculate the genetic

distance according to the Kimura-3-parameter nucleotide replacement model. The maximum likelihood gene tree between species (with 1,000 bootstrap resamplings) was built from the best nucleotide replacement model indicated by testing the best model conducted at MEGA X.

Results and discussion

The morphological difference in leaf between the two *P. regnellii* var. *regnellii* is shown in Figure 1. Morphotype 35 (Figure 1a) has well-rounded cordiform leaves and its ear develops significantly at ripening. In contrast, morphotype 45 (Figure 1b) has more elongated cordiform leaves and its ear does not develop at ripening.

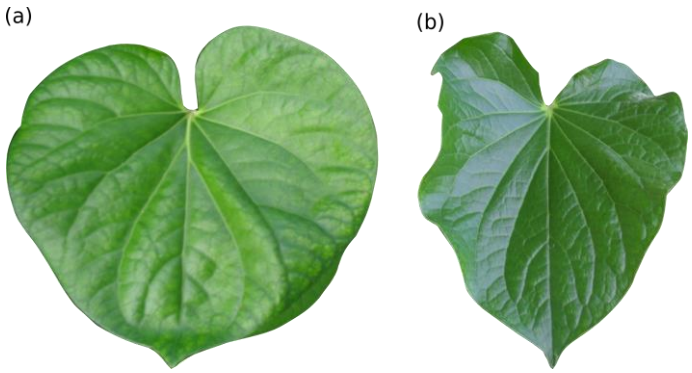

Figure 1. Leaf morphology of *Piper regnellii* var *regnellii*. Morphotype 35 (a) and 45 (b) - Horto Medicinal - UNIPAR – Umuarama, Paraná State, Brazil.

The final alignment of the sequences obtained showed 495 bp. The accession number of the sequences deposited for morphotype 35 were MT774363, MT774364 and MT774367 whereas, for morphotype 45 were MT774362, MT774365 and MT774366. This is the first contribution of this molecular marker for this species. The comparison between the sequences of the two *P. regnellii* morphotypes with *Piper* sequences obtained from GenBank showed 40 variable sites (Table 1; numbering follows the alignment of *Piper* used in this study).

Table 1. Polymorphic sites and gaps of the species based on the *trnL-trnF* intergenic sequence.

	8 6 5 A	8 6 9 G	9 1 4 C	9 2 8 T	1 9 1 A	9 4 5 G	9 5 0 C	9 5 0 7 T	1 0 6 G	1 0 0 1 4 6 9 T	1 0 0 1 4 7 4 G	1 0 0 1 5 5 7 A	1 0 0 1 5 5 9 C	1 0 0 1 6 7 1 C	1 0 0 1 7 8 7 G	1 0 0 1 7 8 7 A	1 0 0 1 8 8 9 A	1 0 0 1 9 0 1 A	1 0 1 1 4 5 4 T	1 0 1 2 5 3 2 C	1 1 1 3 4 4 1 C	1 1 1 4 4 4 3 C	1 1 1 4 5 6 4 T	1 1 1 5 7 3 4 T	1 1 1 7 7 4 1 A	1 1 1 8 9 1 1 A	1 1 1 9 9 2 8 A	1 1 2 0 1 2 8 A	1 1 2 2 1 1 2 A	1 1 2 2 8 8 9 T	1 1 2 2 8 9 4 C	1 1 2 3 9 4 0 T	1 1 3 2 4 0 5 A	1 1 3 3 4 5 7 A	1 1 3 3 4 4 8 G
MT774363.1 *P. regnellii* (morf. 35)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MT774364.1 *P. regnellii* (morf. 35)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MT774367.1 *P. regnellii* (morf. 35)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MT774362.1 *P. regnellii* (morf. 45)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MT774365.1 *P. regnellii* (morf. 45)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MT774366.1 *P. regnellii* (morf. 45)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EF422823.1 *Piper betle*	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	-	-	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MH476670.1 *Piper majusculum*	.	.	.	.	.	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	C	-	.	.	.	.	C	.	.	G	.	.	.
MH476669.1 *Piper majusculum*	.	.	.	.	.	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	C	-	.	.	.	.	C	.	.	G	.	.	.
MH476649.1 *Piper febrifugum*	.	.	.	.	.	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	C	-	.	.	.	.	C	.	.	G	.	.	.
MH476688.1 *Piper quinqueangulatum*	.	.	.	G	G	.	.	.	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	.	-	T	.	.	.	C	.	.	G	C	.	.	T	.	.	.
MH476694.1 *Piper rampilum*	.	.	.	.	G	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	.	.	T	.	.	.	C	.	.	G	.	.	.	.	.	.	.
MH476693.1 *Piper rampilum*	.	.	.	.	G	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	.	-	T	.	.	.	C	.	.	G	.	.	.	.	.	.	.
MH476690.1 *Piper quinqueangulatum*	.	.	.	.	G	.	.	C	A	.	T	.	T	.	A	G	.	G	G	.	T	T	.	.	-	T	.	.	.	C	.	.	G	C	.	.	.	.	.	.
MH476637.1 *Piper celebicum*	.	.	.	.	.	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	C	-	-	T	.	.	.	C	.	.	G	.	.	.	.	.	.	.
EU519818.1 *Piper yucatanense*	.	.	.	.	.	.	.	C	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
EU519817.1 *Piper guazacapanense*	.	A	.	.	.	.	.	C	.	.	.	G	T	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.
EU519809.1 *Piper aduncum*	.	.	.	.	.	.	A	C	.	.	.	.	T	T	.	.	.	.	.	A	.	.	.	-	-	.	.	.	.	C	.	G	.	.	.	.	.	T	-
EU519819.1 *Piper humistratum*	.	.	T	.	.	A	.	C	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	-	-	-	.	.	.	C	.	.	.	.	.	C	.	.	.	.
EU519811.1 *Piper hispidum*	G	.	.	.	.	.	A	C	.	.	.	.	T	T	.	.	.	.	.	A	.	.	.	-	-	.	.	.	.	C	.	G	.	.	.	.	.	.	C
EU519820.1 *Piper aequale*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	.	.	.	.	T	-
MH476727.1 *Piper* sp. RA863	.	.	.	.	G	.	.	C	A	.	T	.	T	.	.	G	G	T	G	.	T	T	.	-	-	.	.	.	G	C	.	.	G	.	T	.	.	.	.
MH476659.1 *Piper kurzii*	.	.	.	.	G	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	C	-	T	T	C	.	C	C	.	G	.	.	.	.	.	.
MH476724.1 *Piper* sp. RA758	.	.	.	.	.	.	.	C	A	C	T	.	T	.	.	G	.	G	G	.	T	T	.	-	-	T	.	.	.	C	.	.	G	.	.	.	.	.	.
MH476672.1 *Piper blumei*	.	.	.	.	G	.	.	C	A	.	T	.	T	.	.	G	.	G	G	.	T	T	.	T	-	T	.	.	.	C	.	.	G	.	.	.	.	.	.

Only site 1144 presented variation between morphotypes 35 and 45. Samples of morphotype 35 presented T, while samples of morphotype 45 possess an indel. Only *Piper ramipilum* also presented T1144, while all the other samples showed an indel.

Values of genetic distance between all evaluated sequences were between 0.0% and 20.8% (Table 1). The two morphotypes did not show genetic distance (0.0%), showing that in the sequenced region, there are not enough differences to distinguish them at the species level. However, as previously mentioned, an indel distinguished the two morphotypes (Table 2), considering that indels are not considered in this type of analysis.

The average intraspecific distance for species with two or more sequences was 3% and the average interspecific distance between the evaluated *Piper* is listed in Table 1, indicating the *trnL-trnF* region as a useful molecular marker for *Piper*.

Table 2. Distance values corrected by the Kimura-3-parameter model for the *trnL-trnF* region between different *Piper* spp.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1																								
2	0.000																							
3	0.000	0.000																						
4	0.000	0.000	0.000																					
5	0.000	0.000	0.000	0.000																				
6	0.000	0.000	0.000	0.000	0.000																			
7	0.076	0.006	0.006	0.130	0.011	0.136																		
8	0.030	0.025	0.025	0.029	0.024	0.032	0.198																	
9	0.031	0.025	0.025	0.029	0.025	0.032	0.201	0.000																
10	0.032	0.025	0.025	0.032	0.026	0.034	0.200	0.002	0.003															
11	0.033	0.029	0.029	0.032	0.029	0.034	0.206	0.009	0.009	0.012														
12	0.034	0.027	0.027	0.033	0.032	0.035	0.205	0.008	0.008	0.010	0.006													
13	0.034	0.027	0.026	0.033	0.031	0.035	0.207	0.008	0.009	0.011	0.007	0.000												
14	0.036	0.033	0.033	0.036	0.032	0.038	0.206	0.010	0.011	0.013	0.007	0.007	0.007											
15	0.032	0.027	0.027	0.032	0.026	0.034	0.205	0.003	0.003	0.007	0.009	0.008	0.009	0.011										
16	0.010	0.008	0.010	0.010	0.010	0.010	0.004	0.020	0.021	0.020	0.031	0.024	0.025	0.031	0.025									
17	0.012	0.012	0.012	0.012	0.012	0.012	0.006	0.022	0.023	0.022	0.033	0.026	0.027	0.033	0.027	0.006								
18	0.016	0.016	0.016	0.016	0.016	0.016	0.012	0.027	0.027	0.027	0.037	0.033	0.031	0.037	0.031	0.012	0.016							
19	0.012	0.012	0.012	0.012	0.012	0.012	0.008	0.022	0.023	0.023	0.033	0.029	0.027	0.033	0.025	0.008	0.012	0.016						
20	0.022	0.018	0.023	0.022	0.024	0.023	0.020	0.035	0.033	0.033	0.045	0.041	0.037	0.045	0.037	0.016	0.024	0.006	0.020					
21	0.002	0.002	0.002	0.002	0.002	0.002	0.010	0.025	0.025	0.025	0.031	0.031	0.029	0.035	0.029	0.010	0.014	0.014	0.014	0.020				
22	0.034	0.031	0.031	0.033	0.032	0.035	0.206	0.008	0.008	0.011	0.009	0.008	0.009	0.011	0.011	0.029	0.031	0.037	0.033	0.045	0.035			
23	0.037	0.035	0.035	0.036	0.034	0.038	0.208	0.008	0.008	0.011	0.008	0.007	0.008	0.010	0.009	0.031	0.033	0.037	0.033	0.043	0.035	0.009		
24	0.032	0.027	0.027	0.032	0.028	0.034	0.205	0.006	0.006	0.009	0.007	0.006	0.007	0.009	0.007	0.025	0.027	0.031	0.027	0.037	0.029	0.009	0.007	
25	0.035	0.027	0.027	0.033	0.031	0.035	0.206	0.008	0.008	0.010	0.006	0.000	0.000	0.007	0.008	0.025	0.027	0.031	0.027	0.037	0.029	0.008	0.007	0.006

1 - MT774363.1 *Piper regnellii* (morf. 35); **2 -** MT774364.1 *Piper regnellii* (morf. 35); **3 -** MT774367.1 *Piper regnellii* (morf. 35); **4 -** MT774362.1 *Piper regnellii* (morf. 45); **5 -** MT774365.1 *Piper regnellii* (morf. 45); **6 -** MT774366.1 *Piper regnellii* (morf. 45); **7 -** EF422823.1 *Piper betle*; **8 -** MH476670.1 *Piper majusculum*; **9 -** MH476669.1 *Piper majusculum*; **10 -** MH476649.1 *Piper febrifugum*; **11 -** MH476688.1 *Piper quinqueangulatum*; **12 -** MH476694.1 *Piper ramipilum*; **13 -** MH476693.1 *Piper ramipilum*; **14 -** MH476690.1 *Piper quinqueangulatum*; **15 -** MH476637.1 *Piper celebicum*; **16 -** EU519818.1 *Piper yucatanense*; **17 -** EU519817.1 *Piper guazacapanense*; **18 -** EU519809.1 *Piper aduncum*; **19 -** EU519819.1 *Piper humistratum*; **20 -** EU519811.1 *Piper hispidum*; **21 -** EU519820.1 *Piper aequale*; **22 -** MH476727.1 *Piper sp*. RA863; **23 -** MH476659.1 *Piper kurzii*; **24 –** MH476724.1 *Piper sp*. RA758; **25 –** MH476672.1 *Piper blumei*.

The maximum likelihood gene tree is illustrated in Figure 2. The distribution of the sequences that best aligned with sequences of *P. regnellii,* also with high scores of similarities according to BlastN results. Some species did not show enough interspecific variation to separate them into different clades. This was also seen for the two morphotypes in question, again showing the similarities between them.

The results also showed that the *trnL-trnF* region varied between *Piper* species, reinforcing other studies on this region, which is quite diverse in size and composition, so it is widely used for the reconstruction of phylogenetic patterns in plants (Hilu & Liang, 1997). According to Machado, Oliveira, Fabrin, Prioli, and Prioli (2016), the *trnL-trnF* spacer in *Salvinia* species proved to be efficient in distinguishing close species. Ng, Sandoval and Murphy (2016) studied this same region in *Lophophora* and reported this region behaved as a good molecular marker for differentiation of species and genera, supporting our findings. Roslim and Herman (2017) also studied the use of the *rps16* intron sequence and the *trnL-trnF* region for the identification of *Gluta*

renghas. These authors were able to sequence 527 bp of the *trnL-trnF*, and according to the BlastN algorithm, *Gluta sp*. was identified as *Gluta renghas*.

Other studies have used the *trnL-trnF* region, presenting it as a good molecular marker for phylogenetic relationships (Buerki et al., 2012), species distinction (Ng et al., 2016), and characterization of new species (Yurtseva, Kuznetsova, & Mavrodiev, 2016). The identification of an organism can be performed through morphological characteristics and/or through the analysis of DNA sequences. Therefore, it can be a useful tool due to the environmental influence on phenotype, being difficult, in some cases, the correct species identification using only morphology.

Our study shows that the molecular analysis of the two morphotypes studied, indicated they belong to the *Piper* genus, shows morphotypes 35 and 45 of *Piper regnelli* can be distinguished by an indel at site 1144, although there is no distinction in phylogeny. Another contribution is the novel sequences of *trnL-trnF* of *P. regnellii*.

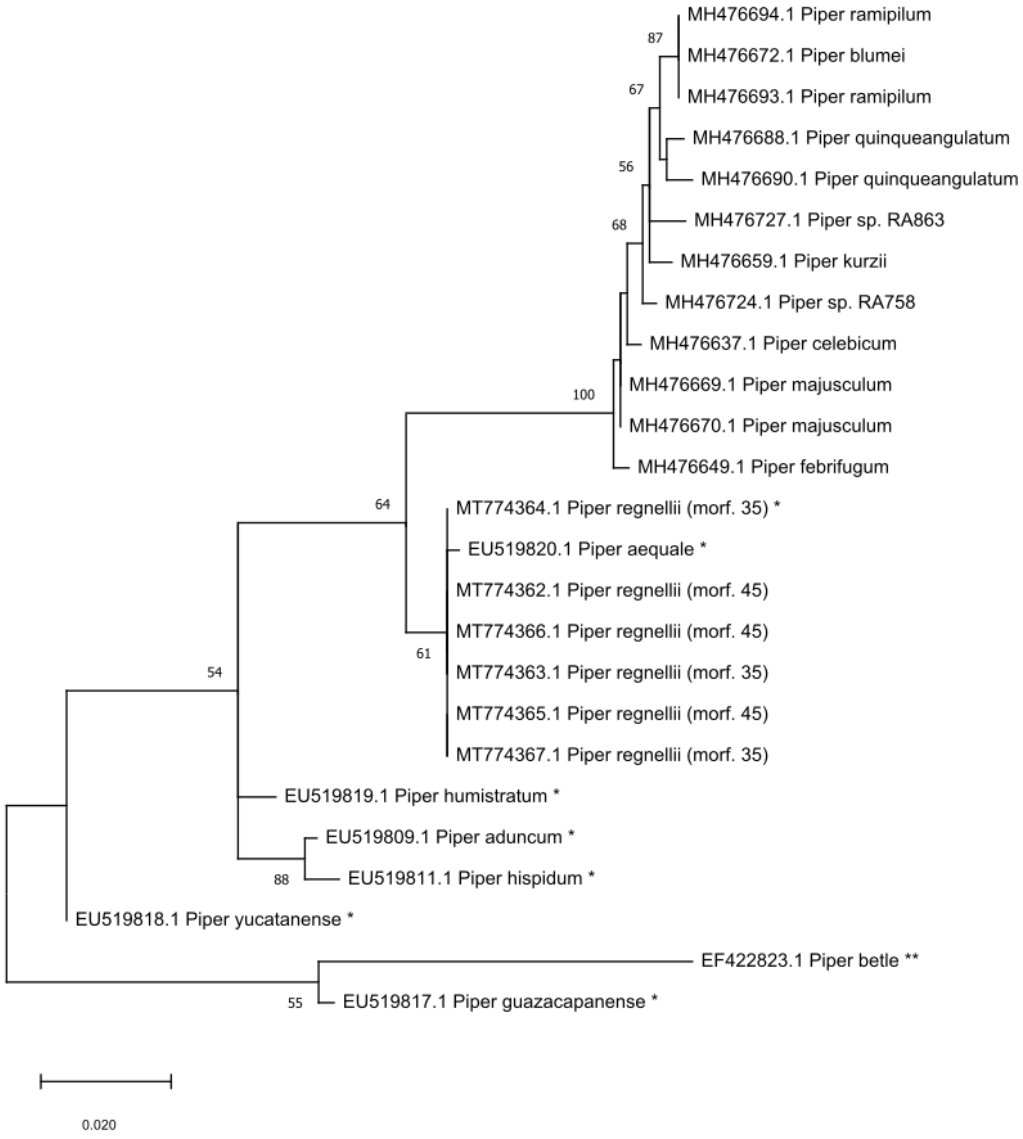

Figure 2. Maximum likelihood gene tree of the *trnL-trnF* and trnL marker of *Piper* species (1,000 bootstrap resampling) and the Tamura-3-parameter model.

Conclusion

The use of the *trnL-trnF* molecular marker proved to be effective in identifying the evaluated *Piper*, also was effective in the distinction between the sequence of morphotypes 35 and 45 of *Piper regnellii* (Miq.) C. DC. var. *regnellii* by the presence of one indel. It might be useful, in future studies on *P. regnellii*, to test a region presenting greater variation, trying to find more differences when comparing these different morphotypes.

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