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Abundance, distribution mapping and DNA barcoding of *Procontarinia robusta* (Diptera: Cecidomyiidae), a mango gall midge in Bali, Indonesia

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Abstract. *Susila IW, Sumiartha IK, Supartha IW, Yudha IKW, Utama IWEK, Yasa IWS, Wiradana PA. 2022. Abundance, distribution mapping and DNA barcoding of* Procontarinia robusta *(Diptera: Cecidomyiidae), a mango gall midge in Bali, Indonesia. Biodiversitas 23: 6428-6436.* Gall midge (*Procontarinia robusta*) is an important pest of mango plants in various countries in the world, including Indonesia. This pest causes very serious damage to mango leaves which until now has not been reported. This study aims to map the distribution and abundance of the pest population and identify the pest species using the DNA barcode method on mango tree plantations in Bali, Indonesia. The survey method was used to collect data from various districts and cities in Bali Province, Indonesia. Mitochondrial COI primers were used to identify DNA barcodes. The results showed that the highest population abundance of *P. robusta* was found in Denpasar City. These pests have spread evenly throughout the Province of Bali, from the lowlands to the middle and highlands. Through a molecular approach, the insect pest that causes mango leaf gall in Bali Province is identified as *P. robusta* as the first report that can be used by researchers, related agencies, and farmers to be alert and ready with strategies and control tactics in the future. Further research is needed related to monitoring using sex pheromones or plant volatiles and the search for natural enemies for monitoring purposes and initiation towards biological control.

Keywords: DNA barcoding, gall midge, mango, monitoring, *Procontarinia robusta*

INTRODUCTION

Mango commodities are the main source of income for the horticultural industry in Indonesia (Akrong et al. 2020; Wardhan et al. 2022). According to BPS and Directorate General of Horticulture (2019), mango production in Indonesia increased by 19.1% in 2019. The largest mango-producing area in Indonesia is East Java Province. Mango trees are also widely planted in other Asian continents as fruit sources, ornamental plants and shade trees that are useful for protecting soil from the threat of erosion.

Mango production in Bali Province is not as large as that produced by East Java Province, which is around 65.693 million tons/year in 2019, with a productivity of around 5.74% (BPS and Directorate General of Horticulture 2019). One of the important factors causing the low productivity of mangoes is disturbance from insect pests and plant diseases (Grechi et al. 2021; Karar et al. 2021). Some of the important pests reported to damage crops are thrips, mango seed beetle (Karar et al. 2022), and mango gall fly (*Procontarinia* spp.) (Augustyn et al. 2010; Singh 2018). Among these pests, the mango gall fly causes the most damage to crops in the field and is considered an important pest in various countries in the world, such as China (Jiao et al. 2018), Northern Australia, Papua New Guinea (Kolesik et al. 2009; Jiao et al. 2018), India (Vasanthakumar et al. 2020), and Pakistan (Karar et al. 2021). The most common symptom shown by this pest attack is gall-shaped mango leaves (Augustyn et al. 2013) which can interfere with plant metabolism and physiological processes during photosynthesis which adversely affects plant growth (Syawaluddin et al. 2019). These symptoms are a result of the destruction of plant leaf tissue when insects consume tissue and insert eggs into plant leaves (Hilker and Meiners 2011). Gall symptoms are histological symptoms of hypertrophy and hyperplasia (Carneiro et al. 2015; Richardson et al. 2017).

There are 22 known species of gall midge in mango, and 16 of these are members of the genus *Procontarinia* (Jiao et al. 2018). All Procontarinia species are reported to cause galls on mango leaves. Meanwhile, *P. mangiferae* can damage leaves and inflorescences (Vasanthakumar et al. 2020). The development of these pests has been successfully described to an adult stage by Cai et al. (2013). However, there are no good practices to control these pests. To obtain good practice for the management of these pests, it is necessary to have basic knowledge of these pests and methods that can be integrated into the farmer capacity-building process (Akotsen-Mensah et al. 2017; Deguine et al. 2021). One of the obstacles to implementing an integrated pest control program in the field is the limited insight into farmers' information and knowledge related to the bioecology of mango insect pests and their control techniques (Deguine et al. 2021; Supartha et al. 2022). The

basic knowledge possessed by farmers regarding the symptoms of damage caused by these pests can help develop integrated pest control techniques in the field (Grasswitz 2019; Supartha et al. 2021).

Information regarding gall-inducing species in mango leaves in Bali Province has not been reported. This study aims to map the distribution and abundance of the pest population and identify the pest species using the DNA barcode method using Cytochrome Oxidase Subunit I (COI) region on mango tree plantations in Bali. The results of this study are the initial data for the development of strategies and technology for controlling these pests on mango plants.

MATERIALS AND METHODS

Study area and sample collection

The study was conducted on a field and laboratory scale from May to September 2022. The field-scale investigation comprised locating the sampling site and collecting samples. The Integrated Pest Management Laboratory (IPMLab), Faculty of Agriculture, Udayana University, Bali, conducted laboratory-scale research, including specimen preservation and pest identification. The spelling took place at the Mango Plantation Center in Bali, Indonesia. Purposive sampling was used to gather mango leaf samples in Badung, Bangli, Buleleng, Gianyar, and Denpasar City (Table 1). Three to four location points were obtained at each district, and two mango plants with gall symptoms were identified at each location point. The total sample of mango plants observed was 68 trees. The number of plant materials gathered for each site varied between 50 and 100 leaves. The collected leaf samples were then placed in a polyethylene plastic bag, placed in a cooler box, and transported to the laboratory. Leaf samples containing larvae were then separated and housed in transparent plastic containers 10 cm and 12 cm in diameter. These rearing containers are then put on wooden racks until adult flies and/or parasitoids emerge (Yuliadhi et al. 2021, 2022). In addition, 20 adult midges reared from infected mango leaves were collected for genetic identification.

Procedures

Determination and distribution of Procontarinia robusta

Determination of the abundance of the *P. robusta* population was carried out after the insects emerged from the rearing area and adjusted to the morphological identification guidelines from Li et al. (2003).

DNA extraction

DNA extraction was carried out according to Hamid et al. (2018) and Supartha et al. (2022). Adult specimens found within the study site were preserved in 70% alcohol and stored in a -20°C freezer until the material was required for isolation. The sample must be isolated, collected, and dried on a towel for 30 minutes. The larvae were then submerged in 85°C hot water for 30 minutes until they became a yellowish color. The locations in the two abdominal sections were then cut and put in a 1.5 µL tube. Proteinase K was added in the quantity of 5 µL and crushed until crushed. The crushed material (pH 7.5) was dissolved in 300 µL of TNES buffer containing 1 M Tris HCl, ddH_2O, and 20% SDS), homogenized, and incubated at 60°C for 3 hours. After the incubation time was fulfilled, 85 µL of 5 M NaCl was added and centrifuged for 10 minutes at 14000 rpm. The supernatant was collected in large amounts (up to 400 µL), deposited in a new tube with isopropanol up to 60% of the volume taken, placed in the freezer for 20 minutes, then 5 minutes of centrifugation at 14,000 rpm. After removing the supernatant, 500 µL of cold 70% alcohol was added before centrifuging for 15 minutes at 14,000 rpm. The supernatant was extracted once again and dried at room temperature for 24 hours. After drying, 20 µL of TE buffer was added. The DNA suspensions were stored at -20°C before being used.

Table 1. Sampling location of mango leaf samples used in the study

District	Sub-district	Coordinate point	Altitude (m asl.)
Badung	Kuta Selatan	$8^0$47'56" S 115^{0}10'22" E	85
	Mengwi	$8^0$37'46" S 115^{0}8'30" E	54
	Plaga/Petang	$8^0$17'48" S 115^{0}11'7" E	981
Bangli	Susut	$8^0$26'10" S 115^{0}20'37" E	519
	Kintamani	$8^0$18'32" S 115^{0}21'3" E	1138
	Bangli	$8^0$29'9" S 115^{0}21'0" E	328
Buleleng	Tejakula	$8^0$12'77" S 115^{0}28'97" E	250
	Kubutambahan	$8^0$8'8" S 115^{0}15'22" E	636
	Buleleng	$8^0$6'49" S 115^{0}6'3" E	34
Gianyar	Sukawati	$8^0$34'55' S 115^{0}16'41" E	87
	Ubud	$8^0$32'0" S 115^{0}15'57" E	164
	Blahbatuh	$8^0$31'53" S 115^{0}18'18" E	120
	Ginyar	$8^0$31'38" S 115^{0}19'4" E	220
Denpasar	Denpasar Utara	$8^0$37'57" S 115^{0}12'22" E	46
	Denpasar Timur	$8^0$38'15" S 115^{0}15'31" E	21
	Denpasar Selatan	$8^0$41'57" S 115^{0}13'43" E	16
	Denpasar Barat	$8^0$40'14" S 115^{0}13'7" E	24

DNA amplification and sequencing

We also sequenced the COI gene for *P. robusta* adults that emerged from the leaves of mango trees collected in the Bali orchard. The primers used for the amplification were as follows: forward, 5' - GGT CAA CAA ATC ATA AAG ATA TTG G -3' (LCO-1490) and reverse, 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA -3' (HCO-2198). Were used to amplify the Cytochrome Oxidase Subunit I (COI) region. The amplification technique was followed by Amouroux et al. (2013). The PCR was performed in a final volume of 12.5 µL, including 2.5 µL of five-time extract kappa buffer, 0.625 µL of primer forward, 0.625 µL of primer reverse, 0.125 µL of DNA polymerase, 0.875 µL of $MgCl_2$, 0.375 µL of DNTP, 7.375 µL of DdH_20.

We used the following procedure for amplification: predenaturation at 94°C for 3 min, 35 cycles consisting of denaturation at 94°C for 15 s, annealing at 54°C for 53 s, and elongation at 72°C for 60 s. The data was shown using a UV transilluminator (UVP, USA). The PCR findings were used to continue the sequencing procedure at 1st Base Malaysia. The sequencing data was examined at PT. Genetika Science Indonesia.

Data analysis

The population of *P. robusta* was determined and recorded based on the location of the sample. Furthermore, the spread of *P. robusta* in the Province of Bali was determined by documenting the sample size and the appearance of *Procontarinia* from that place (Table 1). The acquired data is then placed into a thematic map using the Q-GIS 3.16.16 program

Using the Bioedit Version 7.0.5.3 program, the base sequences of the two samples were evaluated in sequence to determine the difference in the base composition of the protein. The analytical findings were then sent to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to assess sample similarities and potential identities. To determine genetic distance, the MEGA program version 6.06 for Windows was utilized. The COI gene phylogenetic analysis was then performed using the Neighbor-Joining (NJ) technique with 1000 bootstrap replicates (Tamura-Nei model). The NCBI program (https://www.ncbi.nlm.nih.gov/) provided the reference strain utilized in this investigation (Wiradana et al. 2019; Supartha et al. 2022).

RESULTS AND DISCUSSION

Abundance of *Procontarinia robusta* in Bali

The study's findings revealed that the species responsible for mango midge galls in Bali was *Procontarinia robusta* (Figure 1). They produce galls (puru) on the top and lower surfaces of the leaves. Severe infections cause the leaves to curl, dry, and fall, decreasing agricultural output indirectly (Rehman et al. 2016). *Procontarinia matteiana* and *P. robusta* have been observed to attack mango plants in Indonesia, such as in Java, Sumatra, Sebesi Island, and Bali. The *P. matteiana* species varies from *P. robusta* primarily in the features of the two species' males (Kolesik and Gagne 2020).

Procontarinia robusta was discovered in all regencies/cities in Bali Province, with the greatest average being found in Denpasar City (314 adults), Badung District (109 adults), Gianyar District (67 adults), Buleleng District (20 adults), and Bangli District (18 adults) (Figure 2). Differences in population abundance seen in each district/city of Bali Province may be attributed to biotic and abiotic variables (Supartha et al. 2022). For example, the existence of plentiful host plants that meet the demands of insect pests to continue breeding may be an essential biotic element in the spread of this problem. Furthermore, the dynamics of host plant proliferation vary by region, resulting in various distributions of this pest in invasion and colonization (Greenlees et al. 2020). The high level of competition between species, the existence of natural enemies, and the variety of host plants all play an important role in the abundance of insect pests as biotic factors (Schulz et al. 2019). Temperature, humidity, and rainfall are all essential abiotic elements in raising the population of an insect pest (Supartha et al. 2021; Yuliadhi et al. 2021). Landscape management can help with pest control in a variety of demographics. Landscape composition may have a direct impact on pest abundance by affecting dispersion, material, or reproduction, or it can have an indirect impact by influencing natural enemies (Veres et al. 2013).

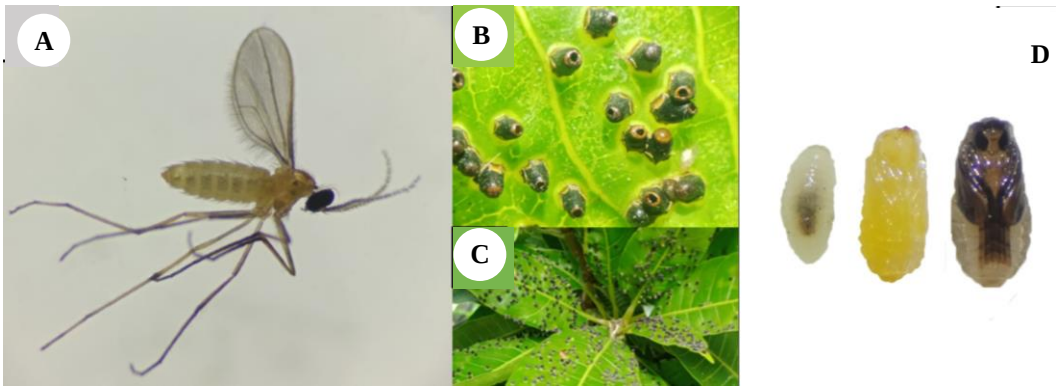

Figure 1. *Procontarinia robusta* and gall midges. A. *P. robusta*; B. Gall on mango leaf; C. Attack on mango leaf, D developmental stage of *Procontarinia* sp.

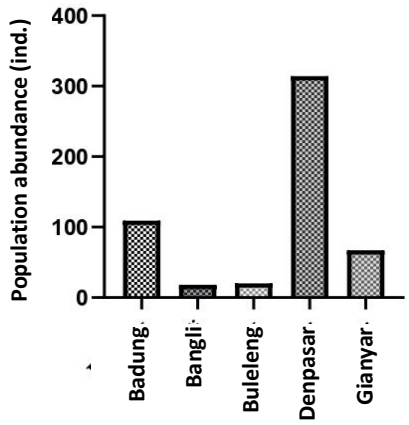

Figure 2. The abundance of adult *Procontarinia robusta* collected from Bali Province, Indonesia

Distribution mapping

The results showed that the gallmide *P. robusta* has expanded in practically all sample sites, particularly in mango-growing regions (Figure 3). In this study, the insect pest was discovered at several elevations in the Bali province, including the highlands (Bangli District), the middle elevation (Gianyar and Badung Regencies), and the lowlands (Denpasar City and Buleleng District). Mango tree farming has been impacted by a total of 181 pests, with around 80% of them being widespread in tropical places such as Indonesia (Siwi et al. 2006). As many as 48 distinct species of pests have been found worldwide, destroying mango tree leaves, including *Procontarinia* spp. (Kusrini et al. 2020). The finding of this insect infestation in numerous mangos growing sites in Bali Province has the potential to threaten economic success in trade partner nations. There are still limitations in pest control owing to a lack of resources to undertake large-scale studies to automate detection as an early warning system effort. Further research is urgently needed in the context of managing this pest, especially by utilizing biocontrol agents that are more environmentally friendly, including the use of natural enemies, vegetable pesticides, and the application of pheromones from natural materials that can reduce pest attacks at the field level.

DNA barcoding

The amplification of COI mitochondrial gene revealed that the primers employed produced DNA bands with a length of about 700 bp (Figure 4). The findings of this insect pest sequencing revealed a 99% similarity of species with *P. robusta* in the BLAST algorithm. The sequences and homology of insect pests from the Province of Bali were presented in Tables 1 and 2, respectively.

The *P. robusta* (JX110977.1) and *P. robusta* (JX110979.1) sequence data from GenBank exhibit 99% similarity with the sequence data of pest gall on mango leaf discovered in Bali. *Procontarinia robusta* (JX110978.1) scored 98%, while *P. robusta* (JX110976.1) scored 98%. Meanwhile, Cecidomyiidae sp. (KR667691.1) outperformed Cecidomyiidae sp. (KT102367.1). Cecidomyiidae sp. (KJ166174.1), Cecidomyiidae sp. (KJ445380.1), Cecidomyiidae sp. (KJ164850.1), Cecidomyiidae sp. (KJ166948.1), and Cecidomyiidae sp.

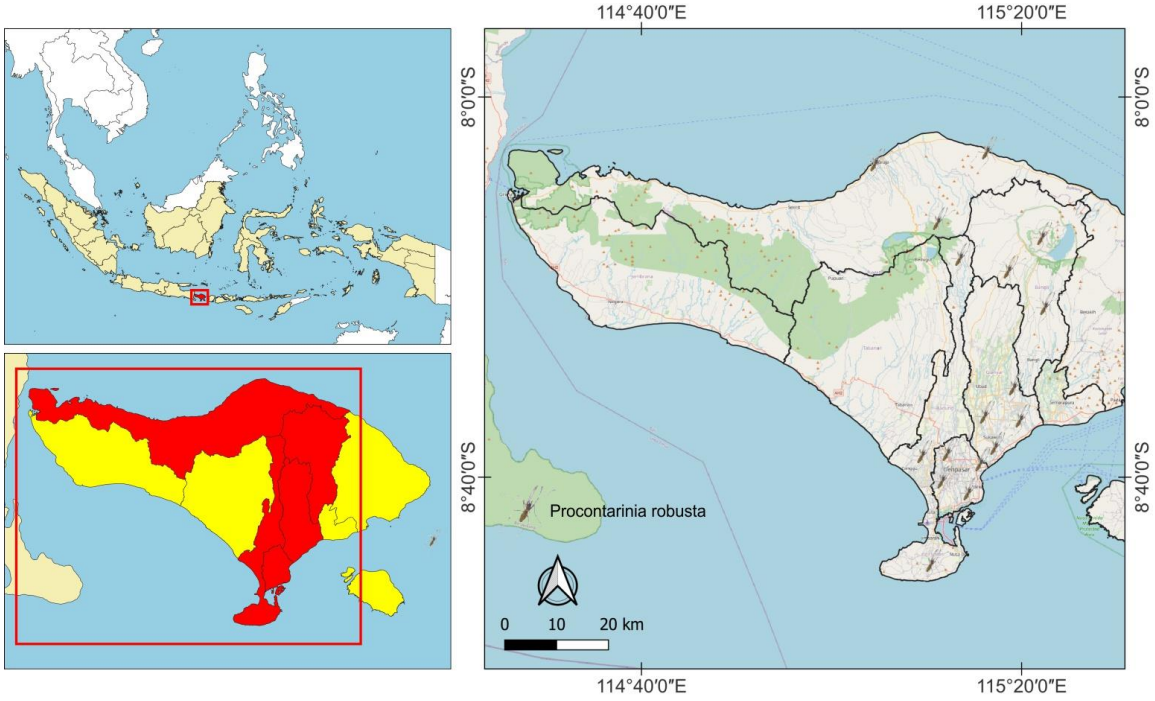

Figure 3. Distribution map of *Procontarinia robusta* causes of mango leaf gall in Bali Province, Indonesia

The results of genetic distance analysis between the Bali sequence sample and the nucleotide sequences of the COI gene *P. robusta* from other countries, namely the ingroup with *P. robusta* (China and Timor Leste) and the outer group *P. matteiana* (Australia and France), *P. fructiculi* (China), and *P. mangiferae* (Russia) obtained from GenBank (Table 3). Based on the homologous level of COI gene DNA sequences and the results of phylogenetic analysis, the genetic character of gall on mango trees found in Bali is similar to *P. robusta* found in GenBank. The results of the phylogenetic analysis showed that *P. robusta* Sequence from Bali was in the same group as *P. robusta* from Australia. Nubs JX110976, JX110977, JX110978, and JX110979 (Figure 5). The genetic similarity of pests that infect mango trees in Bali with *P. robusta* is confirmed by the results of research by Vasanthakumar et al. (2020) and Kolesik and Gagne (2020), who reported that *P. robusta* species is one of the causes of gall disease in mango plants. Mango plantations in Beijing, China were attacked by the pest *Syringa reticulata* subsp. *pekinensis*, which was identified morphologically and molecularly using the mitochondrial COI gene and the 12S ribosomal gene portion of the sequence. This gall pest causes severely diseased leaves to age and fall early (Jiao et al. 2020).

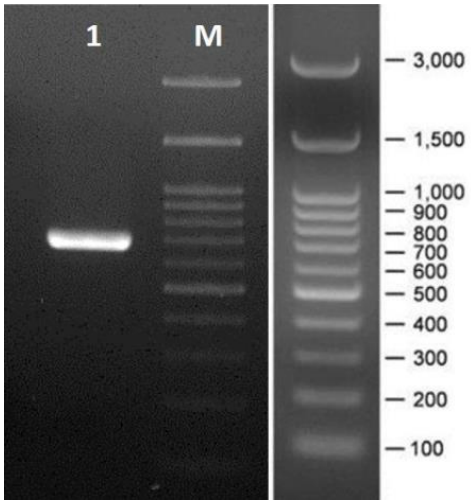

Figure 4. Results of DNA amplification using COI mitochondrial primer pair. M: Marker, (1) insect pests that cause mango leaf gall in this study

Table 1. Results of sequencing of gall pests on mango leaves in Bali Province, Indonesia

Sample	Sequences						
	Seq assembly 684 bp						
Insects cause gall on mango leaves in Bali	1	GACCAAAAAA	TCAAAATAAA	TGTTGATATA	GGACAGGGTC	TCCTCCTCCT	ATTGGGATCA
	61	TAAAATGATG	TATTTAAATT	TCGATCTGTT	AATAATATAG	TAATTGCTCC	TGCTAATACA
	1121	GGTAAAGATA	GTAATAATAA	TACTGTAGTT	ACTAAAATTG	ATCATACAAA	TAAAGAAATT
	1181	TGGTCAAATT	TTAAAAATCT	AATTTTTATA	TTTATTATAG	TTGAGATTAA	ATTAATAGCC
	2241	CCTATAATTG	ATGAAATTCC	TGCAATATGT	AAAGAAAAAA	TTGAAAAATC	TACTGATGAT
	3301	CCGGTATGAG	CAATAATTGA	AGATAAAGGT	GGATAAATTG	TTCATCCTGT	TCCTGCACCA
	3361	GTTTCTACAA	TTCTTCTTGT	TAATATTAAA	ATTATTGAAG	GCGGCAATAA	TCAAAATCTT
	4421	ATGTTATTTA	TTCGTGGGAA	AGCTATATCT	GGAGCGCCTA	ATATTATTGG	TACTAATCAA
	4481	TTTCCAAATC	CTCCTACTAT	AATAGGTATA	ACTATAAAAA	AAATTATTAT	TAATGCATGA
	5541	GATGTTACTA	ATACATTATA	AATTTGATCA	TTTCCAATTA	TATTAGAAAT	AGTACTTAAT
	6601	TCTATTCGAA	TTAAGATTCT	TAAAGAAGTT	CCTAATATTC	CTGATCAAAT	ACCAAATATA
	6661	AAATATAAAG	TTCCAATTAT	CTTT			

Table 2. The level of similarity of the mango leaf gall pest in Bali Province, Indonesia with identical data sourced from GenBank

Description	Identity	Accession
Cecidomyiidae BOLD-2016 voucher BIOUG01512-F05 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	89%	KT102367.1
Cecidomyiidae BOLD-2016 voucher BIOUG01616-B05 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	89%	KR667691.1
Cecidomyiidae BOLD: ABV1316 voucher BIOUG03511-G08 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	88%	KJ166174.1
Procontarinia robusta voucher PK-PR-02 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	99%	JX110977.1
Cecidomyiidae BOLD: ACE9303 voucher BIOUG03823-E06 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	88%	KJ445380.1
Cecidomyiidae BOLD: ACE9303 voucher BIOUG03513-A11 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	88%	KJ164850.1
Procontarinia robusta voucher PK-PR-03 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	98%	JX110978.1
Cecidomyiidae BOLD: ACE9303 voucher BIOUG03510-D07 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	88%	KJ166948.1
Procontarinia robusta voucher PK-PR-04 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	99%	JX110979.1
Procontarinia robusta voucher PK-PR-01 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	98%	JX110976.1

Table 3. The genetic distance among *Procontarinia robusta* and other species downloaded fron GenBank

Number	Sequence	Genetic Distance															
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1	Sequent_Bali	0.000															
2	JX110976	0.423	0.000														
3	JX110977	0.427	0.993	0.000													
4	JX110978	0.423	0.989	0.995	0.000												
5	JX110979	0.405	0.918	0.924	0.922	0.000											
6	JQ823236	0.323	0.672	0.674	0.670	0.691	0.000										
7	FJ820171	0.394	0.825	0.831	0.829	0.760	0.635	0.000									
8	FJ820170	0.390	0.827	0.834	0.831	0.762	0.635	0.995	0.000								
9	FJ820172	0.392	0.827	0.834	0.831	0.762	0.635	0.997	0.997	0.000							
10	MG637426	0.388	0.776	0.782	0.780	0.832	0.714	0.770	0.770	0.772	0.000						
11	MG637425	0.388	0.776	0.782	0.780	0.832	0.714	0.770	0.770	0.772	1.000	0.000					
12	JQ823233	0.319	0.659	0.661	0.657	0.681	0.899	0.631	0.631	0.631	0.730	0.730	0.000				
13	JQ823234	0.319	0.661	0.663	0.659	0.683	0.902	0.633	0.633	0.633	0.732	0.732	0.997	0.000			
14	JQ823232	0.319	0.661	0.663	0.659	0.683	0.902	0.633	0.633	0.633	0.732	0.732	0.997	1.000	0.000		
15	MN894529	0.346	0.645	0.651	0.651	0.701	0.546	0.635	0.635	0.637	0.683	0.683	0.520	0.522	0.522	0.000	
16	MN894528	0.400	0.766	0.772	0.772	0.801	0.668	0.754	0.754	0.756	0.797	0.797	0.640	0.642	0.642	0.831	0.000

Note: Ingroup: *Procontarinia robusta*: Sequence_Bali; *P. robusta* Australia: JX110976; *P. robusta* Australia: JX110977; *P. robusta* Australia: JX110978; *P. robusta* Australia: JX110979; *P. matteiana* Prancis: JQ823236; *P. matteiana* Australia: FJ820171; *P. matteiana* Australia: FJ820170; *P. matteiana* Australia: FJ820172; *P. fructiculi* China: MG637426; *P. fructiculi* China: MG637425; *P. mangiferae* Russia: JQ823233; *P. mangiferae* Rusia: JQ823234; *P. mangiferae* Rusia: JQ823232. Outgroup: *Dasineura jujubifolia* Korea: MN894529; *D. jujubifolia* Korea: MN894528

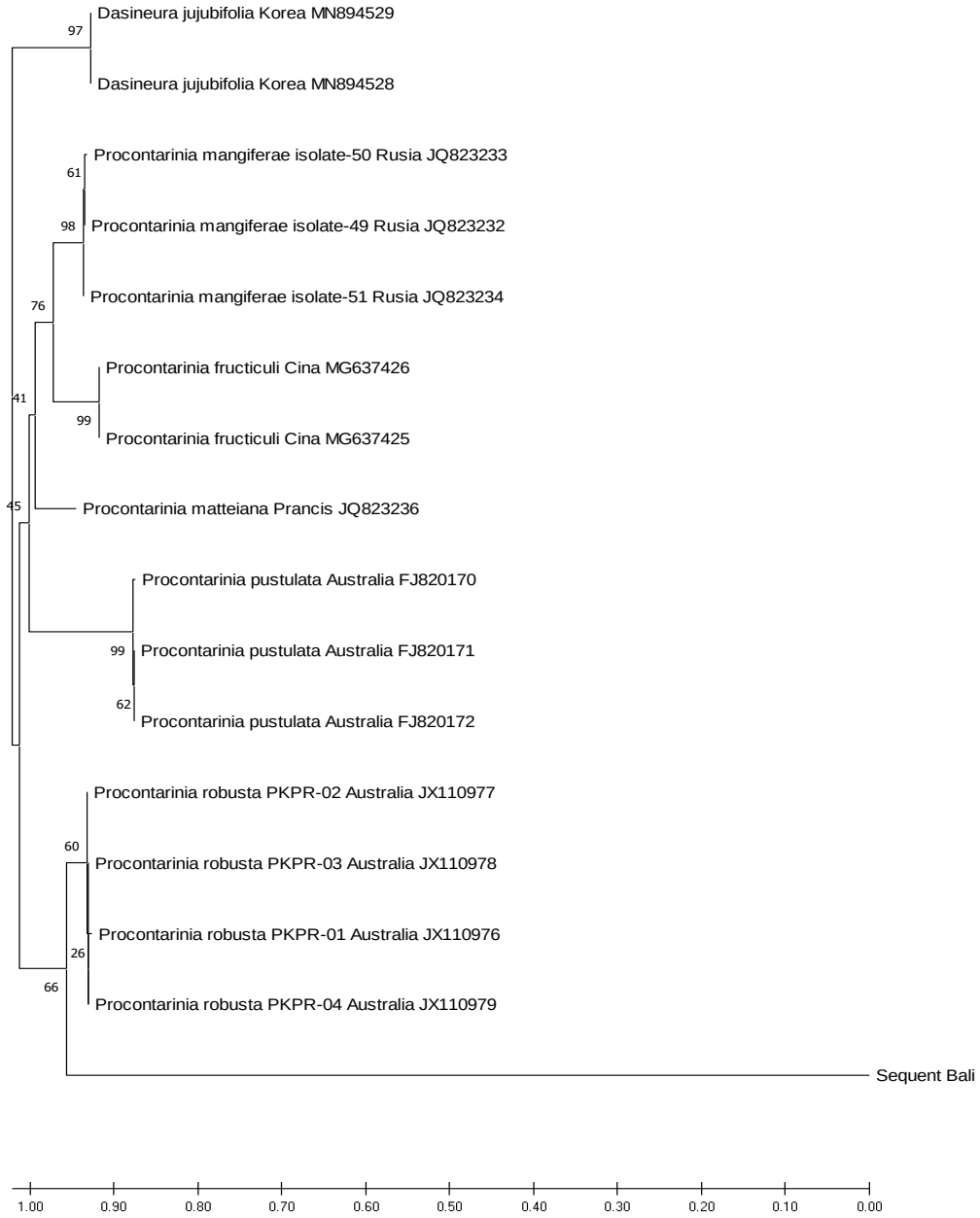

Figure 5. The phylogenetic tree was developed based on the COI gene using the Maximum Likelihood method (1000×; Tamura-Nei model)

In conclusion, the population of *P. robusta* has the highest abundance in Denpasar City, which is indicated by the results of mapping the distribution of the pest in all regencies/cities in Bali Province. The insect pest responsible for mango leaf gall disease in Bali was *P. robusta*, which was identified as similar to the same insect pest found in China and East Timor. The results of our study are the first information that reveals the presence and role of *P. robusta* as the cause of mango leaf gall disease in Bali Province. The potential loss caused by these pests is very significant, therefore, integrated pest management efforts, such as the use of sex pheromones, natural enemies, and other biocontrol agents, need to be carried out for future monitoring and control on a field scale.

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