THE POTENCY OF ANGLE MEASUREMENT AND COMPARISON OF VEIN LENGTHS IN DISTINGUISHING BACTROCERA SPECIES COMPLEXES

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ABSTRACT

The potency of angle measurement and comparison of vein lengths in distinguishing Bactrocera species complexes*.* One of the most conspicuous features of Bactrocera fruit flies is their wing, which can be elaborated for identification. The distinctive wing patterns are used to separate species and classify species complexes. The wing shape can be used as a potential discriminator between closely related taxa. To develop wing applications in taxonomy, in this study, the degree measure of angles and the comparisons of vein lengths were investigated quantitatively to distinguish among important pest quarantine species of the *Bactrocera dorsalis* complex: *B. dorsalis* and *B. carambolae*; the *B. frauenfeldi* complex: *B. frauenfeldi* and *B. albistrigata*; and a species belonging to subgenus Zeugodacus, *B. cucurbitae*. The result showed that species complexes were clustered into significantly different groups based on the degree measure of cell br and the comparison between r-m and dm-cu cross-vein length. This conclusive evidence was supported by phylogenetic analysis using *COI* gene. This present result indicated that the cell br angle measure and the comparison between r-m and dm-cu vein length could be applied to distinguish species complexes in genus Bactrocera.

Key words: cryptic species, insect wing, morphometric, vein ratio, venation angle

INTRODUCTION

Within the family Tephritidae, the true fruit fly, the genus Bactrocera is the largest genera with over 500 species (Drew & Romig, 2013). Certain *Bactrocera* species are known to be most notorious agricultural pests that cause heavy losses in fruit and vegetable crops in tropical and subtropical Asia, Australia, and Pacific Islands. Fruit fly management usually has the problem in morphological identification of several taxa, especially within a species complex due to shared morphological characters. In addition, although distinctive traits have been described by Drew (1989), occurrence of colour polymorphism arose in distinguishing closely related species (Leblanc *et al*., 2013).

The reliable methods are necessary to distinguish cryptic taxa belonging to species complexes. There are various studies that has been applied independently to answer biological relationship among closely related species, such as molecular genetics (Nakahara & Muraji, 2008; Boykin *et al*., 2014), sexual compatibility (Schutze

et al., 2013), and chemoecology (Wee & Tan, 2005; Tan *et al*., 2011). Molecular phylogenetic analysis has better resolving power due to its rapid and precise result. The barcode region of the *COI* gene (mitochondrial cytochrome c oxidase I gene) is reliable molecular marker that has been intensively used in taxonomic classification and identification among closely related *Bactrocera* species (Clarke, 2019).

Morphometrics have been become alternative taxonomic method for quantitative descriptions in biology (Mitteroecker *et al*., 2013). Based on its development, morphometric divided into two methods. First method called traditional morphometric was done by measuring linear distances, such as length, width, and height. In Bactrocera fruit fly, morphometric approach based on male aedeagal length were studied as a discriminator of morphologically similar pest taxa within the *B. dorsalis* complex (Iwahashi, 1999a; Iwahashi, 1999b). However, these approaches showed substantial intraspecific variation and overlap data (Iwahashi, 2001). Krosch *et al.* (2013) reported that genitalia length of fruit flies

correlated with latitude which fruit flies in northern Asia had shorter aedeagi than fruit flies in southern Asia. Further, despite genitalia length, the measurements of wing vein characters of Bactrocera were investigated by Adsavakulchai *et al*. (1999) but it could not be categorized properly.

To overcome the weakness of traditional morphometric, the second method called geometric morphometrics was created. Geometric morphometrics have also been applied in detecting quantitatively morphological differences in order to assist identification. This technique is sensitively useful for visualizing dissimilar geometrical shapes from every single small distinction (Zaelor & Khittawee, 2018). According to Schutze *et al*. (2012), wing shape has a high degree of accuracy to discriminate fruit fly species. Wing shape has been applied to distinguish morphologically similar pest taxa within the *B. dorsalis* complex (Khamis *et al*., 2012; Schutze *et al*., 2012), *B. tau* complex (Kitthawee & Dujardin, 2010; Kitthawee & Rungsri, 2011; Zaelor & Khittawee, 2018). *Anastrepha fraterculus* complex (Perre *et al*., 2014), and *Blepharoneura* spp. (Marsteller *et al*., 2009).

As wing shape successes in discriminating among the morphologically cryptic species, it rises assumption that the angle measures and comparison between two vein lengths tend to be consistent. Furthermore, the ratios and angle measures are not correlated with size that makes quantitative descriptions difficult (Mitteroecker & Gunz, 2009). The analysis of degree angle and ratio resulting from comparison between two vein lengths was expected to explain dissimilar geometrical shapes presented by geometric morphometric analysis. Perhaps, these approaches sensitive enough in clustering at least species complexes. Therefore, the aim of this present

study was to use angle measures and comparison between two vein lengths to distinguish previously identified specimens of *B. dorsalis*, *B. carambolae* (the *B. dorsalis* complex), *B. frauenfeldi*, *B. albistrigata* (the *B. frauenfeldi* complex), and *B. cucurbitae*. Subsequently, these approaches will be compared with molecular genetic to see their consistency. If vein ratios and degree angles accurately reflected the species or species complex groupings as phylogenetic analysis using *COI* gene, these approaches could be a potential discriminator in certain taxonomy level.

MATERIALS AND METHODS

Research Site. The *B. frauenfeldi* samples were obtained by field trapping using cue lure trap from various locations in Sorong City, West Papua for a month, in August 2019. Individuals *B. carambolae, B. dorsalis*, *B. albistrigata*, and *B. cucurbitae* were obtained from Pest Forecasting Institute, Karawang, Indonesia. Wild and mass reared samples were preserved and transported to the Entomology Laboratory at Universitas Gadjah Mada, Yogyakarta, Indonesia for further analysis. This study was conducted from August to October 2019.

Morphometric. The 100 male wings from each species were dissected for the measurement of their veins. The right wing dissected was mounted on a microscope slide and covered with a glass coverslip. The slides were photographed with stereomicroscope Olympus SZ61 and saved in JPEG format. For analysis, six measurements of the veins and angles were taken using TpsDig2 V2.32 software (Figure 1). These measurements were used to calculate comparison of vein lengths for each individual. The comparisons were
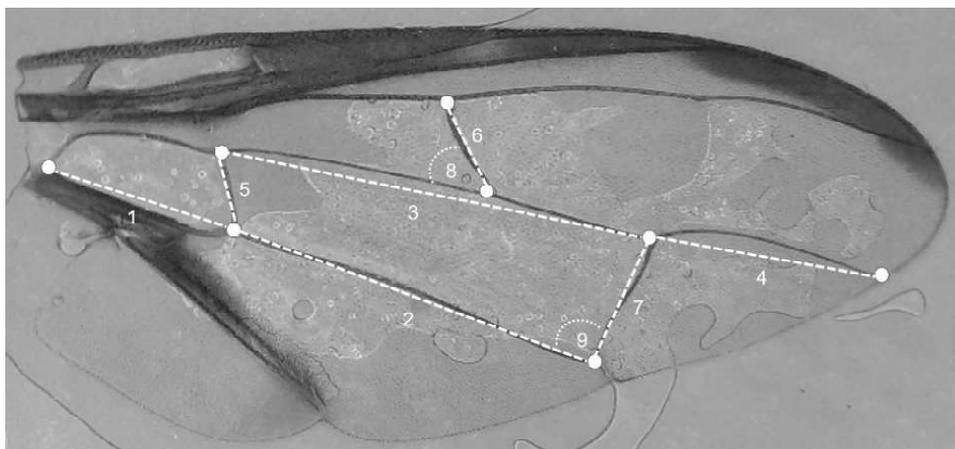
Figure 1. The measurements in wing venation. (1) Bm vein; (2) CuA_1 vein; (3) Dm vein; (4) M vein; (5) bm-cu cross-vein; (6) dm-cu cross-vein; (7) r-m cross-vein; (8) angle of cell br; and (9) angle of cell dm.

as follows: (1) vein bm : CuA$_1$ vein; (2) vein Dm : CuA$_1$ vein; (3) Dm vein : M vein; (4) bm-cu cross-vein : dm-cu cross-vein; and (5) r-m cross-vein: dm-cu cross-vein. The size of venation cell angle of cell br and dm were also calculated (Figure 1). The all data were checked for normality by using the Kolmogorov-Smirnov test. Subsequently, data were analyzed using one-way analysis of variance to compare data from each species. The Tukey post hoc test was used to compare the means (P < 0.05). All statistical analyses were performed in SPSS. 23.

Molecular Analysis. DNA isolation of imago was based on the CTAB (cetyl-trimethyl-ammonium-bromide) protocol described by Doyle & Doyle (1990) with several modification. Each fruit fly thorax was ground in 500 µL of 60 °C CTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, and 0.2% β-mercaptoethanol) using sterile micro pestle in a 1.5 mL centrifuge tube. The suspension was vortexed and incubated for 45 min at 65 °C with intermittent mixing. The sample was centrifuged for 10 min at 10,000 rpm at 4 °C. The supernatant was transferred into 1.5 mL centrifuge tube and equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and repeatedly inverted. The suspension was centrifuged for 10 min at 10,000 rpm at 4 °C and the supernatant was transferred into 1.5 mL centrifuge tube. The DNA was precipitated by adding an equal volume of absolute ethanol (-20 °C) and mixing it. The suspension was incubated for overnight at -20 °C and then DNA was pelleted by centrifugation for 20 min at 12,000 rpm at 4 °C. The supernatant was removed and 70% alcohol was added to wash pellet. Subsequently, the suspension was centrifuged for 5 min at 4,000 rpm then alcohol was discarded. Finally, the pellet was air-dried in silica gel and re-suspended in 40 µL nuclease-free water.

A mitochondrial DNA, Cytochrome Oxidase subunit I (*COI*), was analysed. PCR was conducted using universal primers reported by Folmer *et al*. (1994) (Table 1). DNA amplification in PCR method was carried out in a 30 mL volume containing 15 µL taq DNA polymerase (Bioline), 3 µL DNA template, 3 µL forward primer, 3 µL reverse primer, and 12 µL ddH$_2$O. PCR cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation

at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min. There was a final run-out extension step at 72 °C for 7 min. The 3 µL of samples from PCR products and 1000 bp marker were run in 1.5% of agarose gel to determine the existence and size of amplified DNA.

The Qualified PCR product was then sequenced to determine the nucleotide sequence in cytochrome oxidase I region. The products were sent to the Integrated Research and Testing Laboratory, Gadjah Mada University. The forward and reverse sequences obtained were assembled and edited manually using BioEdit v. 7.0.9 (Hall, 1999), and then analyzed using BLAST (Basic Local Alignment Search Tool) NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to clarify the homology from closest species. The Maximum-likelihood tree were constructed using MEGA (Molecular Evolutionary Genetics Analysis) software version X (Kumar *et al.*, 2018).

RESULTS AND DISCUSSION

Vein Lengths. The male wings from all species (100 right wing/species) were dissected for the vein measurement. The vein lengths resulting among the five species studied were significantly varied in seven vein measurements (P =0.001) (Table 2). Overall, the shortest vein length was recorded on *B. albistrigata* and was followed by *B. frauenfeldi*. On the other hand*, B. cucurbitae* had longest vein lengths, except on M vein. The measurements clearly demonstrated that the vein lengths highly correlated with wing size. However, the result of vein lengths could not separate the five species studied according to species complexes because it showed ranges of overlap. Adsavakulchai *et al*. (1999) reported that vein lengths were correlated with the wing size which made identification difficult. In addition, Schutze *et al.* (2012) also discovered that vein length and wing size could not effectively discriminate one species from the other due to there was no consistent significant difference in wing size of Bactrocera fruit flies from various countries.

The adult size variation can clearly be determined by variation in the growth rate and the duration of developmental time during the last larval instar (Chown & Gaston, 2010). The quality and amount of larval food

Table 1. Primer mtDNA Cytochrome Oxidase subunit I (Folmer *et al*., 1994)

Primer name	Sequence
LCO1490 (Forward Primer)	5'-GGTCAACAAATCATAAAGATATTGG-3'
HCO2198 (Reverse Primer)	3'-TAAACTTCAGGGTGACCAAAAAATCA-5'

source affect larval growth rate, which in turn determines larval and adult size (Davidowitz & Nijhout, 2004). The different host plants influenced wing size of *B. dorsalis*, *Ceratitis capitata* (Pieterse *et al*., 2017), *Drosophila gouveai* and *D. antonietae* (Soto *et al*., 2010). The nutritional approach studied by Sentinella *et al*. (2013) revealed that the increasing body size of *Telostylinus angusticollis* (Diptera: Neriidae) was affected by carbohydrate in larval diet. In addition, the high temperature has an impact on biochemical reactions which triggers moulting, therefore the developmental time become shorter. Total larval duration of *B. dorsalis* and *B. carambolae* declined exponentially at 30–35 °C (Danjuma *et al*., 2014). Moreover, condition experienced during the immature stage may affect adult size as Benitez *et al*. (2013) found that the hind wing size of *Diabrotica virgifera* (Coleoptera: Chrysomelidae) changed according to the major types of soil.

The Angle Measurements and Comparison of Vein Lengths. Two cell angles (cell br and dm) were measured for each species. The degree angles of five fruit flies studied were statistically different for cell br (P=0.0001) and cell dm (P=0.0001). However, the potential discriminator among *Bactrocera* species complexes was showed in the angle measurement of cell br (Table 3). The Tukey post hoc test revealed that degree angle of cell br among species complexes was significantly different. The *B. frauenfeldi* complex samples had the significantly smallest degree angle of cell ber and was followed by *B. dorsalis* complex and *B. cucurbitae*, with the angle measure respectively, 33.27º–44.65º; 50.82º–65.10º; and 65.05º–74.35º. The obtained result explained quantitively differences found directly on the wing venation pattern among five species studied, especially in cell br shape (Figure 2). Further, degree angle of cell br had a role in distinguishing among species complexes based on comparison between vein r-m and dm-cu because it is related to length of dm-cu cross-vein. The ratios resulting from comparison between vein r-m and dm-cu significantly varied among five species studied (P=0.0001) (Table 3). Subsequently, the Tukey post hoc test showed that ratios were divided into three groups, namely *B. frauenfeldi* complex (0.79–0.96), *B. dorsalis* complex (0.65–0.81), and *B. cucurbitae* (0.56–0.68). On the other hand, comparison of other vein lengths did not separate species complexes.

Our results showed that degree angle of cell br and comparison between vein r-m and dm-cu explained

Table 2. The length of wing vein (mm) in different species *Bactrocera* spp.

Vein	*B. frauenfeldi*	*B. albistrigata*	*B. dorsalis*	*B. carambolae*	*B. cucurbitae*
Bm	0.92 ± 0.08 b	0.78 ± 0.05 a	0.94 ± 0.04 c	0.94 ± 0.06 c	1.14 ± 0.05 d
CuA1	1.52 ± 0.07 b	1.36 ± 0.07 a	1.86 ± 0.09 c	1.84 ± 0.10 c	2.05 ± 0.08 d
Dm	1.44 ± 0.10 b	1.30 ± 0.08 a	2.13 ± 0.08 c	2.09 ± 0.12 c	2.36 ± 0.10 d
M	1.43 ± 0.06 c	1.22 ± 0.07 b	1.13 ± 0.05 a	1.15 ± 0.07 a	1.42 ± 0.16 c
bm-cu	0.38 ± 0.03 b	0.35 ± 0.02 a	0.40 ± 0.02 c	0.40 ± 0.04 c	0.50 ± 0.03 d
r-m	0.56 ± 0.05 c	0.50 ± 0.04 a	0.52 ± 0.03 b	0.49 ± 0.04 a	0.53 ± 0.03 b
dm-cu	0.62 ± 0.05 b	0.58 ± 0.04 a	0.70 ± 0.03 c	0.70 ± 0.05 c	0.86 ± 0.03 d

Different letters in each row show significant different based on Tukey post hoc test (mean ± SD).

Table 3. The angle degree and comparisons of vein measurements in different species *Bactrocera* spp.

Measurement	*B. frauenfeldi*	*B. albistrigata*	*B. dorsalis*	*B. carambolae*	*B. cucurbitae*
Cell br	38.81 ± 2.18 a	40.81 ± 2.12 a	58.14 ± 3.16 b	59.66 ± 3.11 b	71.02 ± 2.10 c
Cell dm	58.62 ± 2.12 a	61.56 ± 3.04 a	93.66 ± 2.64 c	89.64 ± 2.90 c	87.51 ± 2.40 c
Bm : CuA1	0.60 ± 0.02 b	0.56 ± 0.02 b	0.51 ± 0.03 a	0.51 ± 0.02 a	0.56 ± 0.02 b
Dm : M	0.98 ± 0.05 a	0.98 ± 0.05 a	1.90 ± 0.07 b	1.81 ± 0.11 b	1.71 ± 0.08 b
Dm : CuA1	0.96 ± 0.02 a	0.97 ± 0.03 a	1.15 ± 0.04 b	1.15 ± 0.03 b	1.15 ± 0.01 b
bm-cu : dm-cu	0.60 ± 0.03 a	0.61 ± 0.03 a	0.58 ± 0.04 a	0.56 ± 0.03 a	0.58 ± 0.02 a
rm : dm-cu	0.89 ± 0.04 c	0.87 ± 0.04 c	0.75 ± 0.02 b	0.70 ± 0.03 b	0.62 ± 0.03 a

Different letters in each row show significant different based on Tukey post hoc test (mean ± SD).

its discriminating power among *Bactrocera* species complexes studied. According to Adsavakulchai *et al*. (1999) that comparison of vein lengths provided an effective means for recognizing the different shapes. In addition, angle and ratio of the two different vein lengths can describe a shape of an object which is unaffected by changes in the position, the orientation, and the size of the object. (Mitteroecker *et al*., 2013). The previous geometric morphometric studies showed that wing shapes differ between taxonomic groups and can be used for insect identification (Henry *et al*., 2010; Schutze *et al.,* 2012). In accordance with geometric morphometric, our finding is in line with the observations of Khamis *et al*. (2012) and Pieterse *et al.* (2017) who found that points in junction of vein R4+5 and r-m cross-vein and also junction of vein M and dm-cu influenced the landmark shifts in classifying *Bactrocera* and *Ceratitis* species. This present study provided conclusive evidence that clarified the differences of geometrical shapes in discriminating taxa for a quick diagnostic character of wing shape. Moreover, our result complemented previous diagnostic characters to categorize species complexes in the Genus Bactrocera. Initially, for classification purpose, the presence of a dark band in wing venation are classically used (Drew, 1989; Drew & Romig, 2013).

Phylogenetic. The phylogenetic tree was generated according to Maximum-likelihood analysis with Tamura 3-parameter method (Tamura, 1992) and 1000x bootstrap repetition. Based on the highly sensitive comparison of nucleotide sequences in the mitochondrial genes, all species were essentially distinguishable and classified into a monophyletic group (Figure 3). The analysis revealed that the five *Bactrocera* species studied were separated into three major lineages, the first lineage was *B. frauenfeldi* complex consisting of *B. frauenfeldi* and *B. albistrigata*. Then, the second lineage was *B. dorsalis* complex consisting of *B. dorsalis* and *B. carambolae*, and the last lineage was *B. cucurbitae*. Two *Bactrocera* species both in the first and the second major lineages were considered as sibling species, which was supported by bootstrap analysis > 90% confidence level. This result also showed that *B. cucurbitae* had large genetic distance with both species complexes as it belonged to different Subgenus. The generated phylogenetic tree was in accordance with previous phylogenetic analysis of *COI* reported by Nakahara & Muraji (2008), Boykin *et al.* (2014), and Jiang *et al*. (2014).

Our result was congruent with the evolutionary history shared by species studied. The morphometric approaches investigated in this research consistently
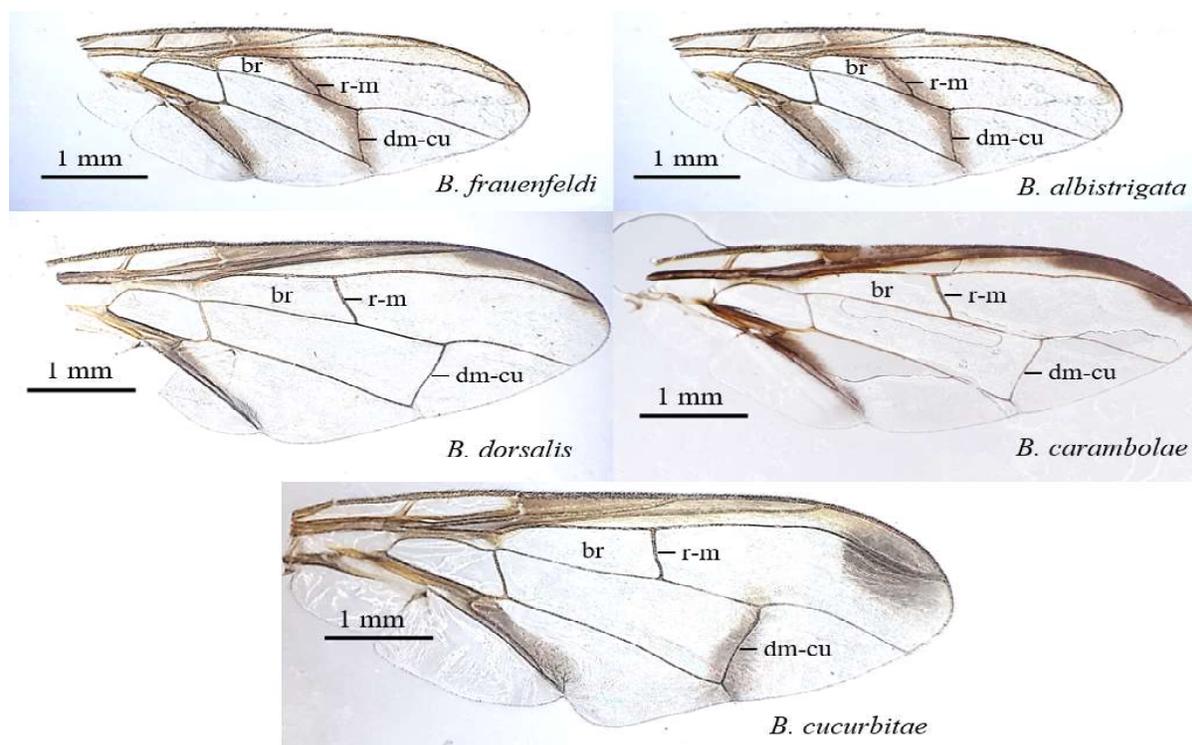
Figure 2. The wing venation of five species studied.
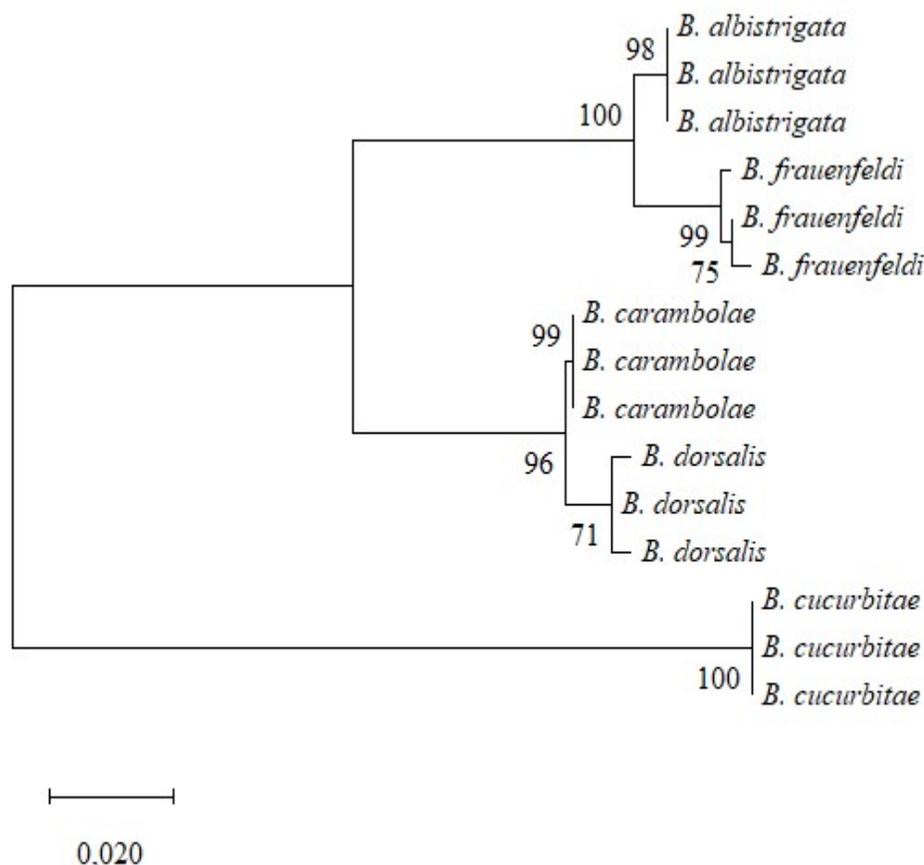
Figure 3. Phylogenetic tree generated by Maximum-likelihood analysis based on *COI* gene. Numbers at the nodes indicate bootstrap values.

explained the discriminating among species complexes studied as a phylogenetic tree. Perrard *et al.* (2014) confirmed that the genetic difference between wasp species had a stronger influence on the wing shape than other biotic or abiotic factors. Genetically, species complexes in genus Bactrocera are paraphyletic each other due to descent from different ancestral lines (Boykin *et al.*, 2014; Krosch *et al.*, 2020). However, the application of angle measure of cell br and ratio vein r-m : dm-cu could demonstrate had a lack in showing the relationship between subgenus Bactrocera and Zeugodacus as phylogenetic tree showed. According to Drew (1989) subgenus Bactrocera can be distinguished from Zeugodacus based on the presence of lateral and medial vittae, where subgenus Bactrocera only has two lateral vittae but subgenus Zeugodacus has three vittae include medial vittae.

CONCLUSION

Based on this result, the degree angle of cell br and comparison between vein r-m and dm-cu is an appropriate tool for *Bactrocera* species complexes

determination. For further study of these morphometric approaches, angle of cell br has advantage in a high degree ranges for clustering species complexes. To see its accuracy, these approaches must be tested with other species complexes, such as *B. tryoni* complex, *B. tau* complex, and *B. xanthodes* complex.

ACKNOWLEDGMENTS

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