



Study on Phylogenetic Status of Javan Plover Bird (*Charadrius*, Charadriidae, Charadriiformes) through DNA Barcoding Analysis

✉ Hidayat Ashari, Dwi Astuti

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Zoology Division, Research Centre for Biology, Indonesian Institute of Sciences Cibinong Science Centre, Indonesia

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Abstract

Javan Plover named *Charadrius javanicus* is taxonomically under controversy and phylogenetically unresolved yet. Through an analysis of DNA barcode, this study aims (1) to confirm whether Javan Plover is separated species named *Charadrius javanicus* or a subspecies of *C. alexandrinus* which named *C. a. javanicus* and (2) to determine a relationship within this genus. Totally 666 bp DNA sequences of COI barcode gene were analyzed. The results showed that a sequence divergence between Javan Plover and *C. alexandrinus alexandrinus* was only 1.2%, while sequence divergences between *C.a.alexandrinus* and others species, or between Javan Plover and others species were ranged from 9-12%. Neighbour-joining (NJ) and maximum-parsimony (MP) analyses showed that all individuals of both Javan Plover and Kenith Plover were clustered together, and supported by 99 % and 100 % of bootstrap value in NJ and MP, respectively. This study tends to support the previous findings that Javan Plover was not a separated species named *C. javanicus*, but it was as a subspecies of *C. alexandrinus*; named *C. a. javanicus*. There were two groups of Plover in this study; (*C. leschenaultii* and *C. javanicus* + *C.a.alexandrinus*), and (*C.dubius* and *C. melodus* + *C. semipalmatus*). DNA barcoding analysis can give certainty taxonomic status of the bird. Then, this study has implication as a basic data that can be used to provide and support the planning of Javan plover conservation programs.

How to Cite

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✉ Correspondence Author:
Jl. Jakarta-Bogor KM 46, Cibinong, West Jawa, Indonesia
E-mail: numenius.phaeopus@gmail.com

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INTRODUCTION

Shorebirds are classified in the Charadriiformes order. One of the families included in this order is Charadriidae, which is divided into 3 subfamilies and 10 genera (del Hoyo et al., 1996). The birds are known as the plover and spread globally, with the relatively short bill. *Charadrius* is one of the existing genera. *Charadrius* consists of several species, including *C. alexandrinus*, *C. javanicus*, *C. semipalmatus*, *C. melodus*, *C. dubius*, and *C. leschemaulti* (del Hoyo, 2004). *C. alexandrinus* was described has several subspecies, such as *C. a. alexandrinus*, *C. a. dealbatus*, *C. a. seebohmi*, *C. a. nivosus*, and *C. a. occidentalis* (Dickinson, 2003; del Hoyo, 2004). Although some avian taxonomist said that many species of plover belong to this genus (MacKinnon, 1998), but the grouping and the phylogenetic relationships among them are still controversies. For example, Javan Plover by Dickinson (2003) and del Hoyo (2004) was described as separated species under the name *Charadrius javanicus*.

Javan plover is one of the shorebirds (water birds) that in Indonesia spread only on the island of Java, Kangean Islands, and possibly also in Bali (del Hoyo, 2004). Last surveys informed that its presence on Bali, Sumatra, Sulawesi, Lombok, Meno Island (off Lombok), Sumbawa, Flores, Semaun Island (Kupang, Timor) and Timor-Leste (Iqbal et al. 2013). This bird has the morphological characteristic with small body size (15 cm). It is similar to the size and structure of the Malaysian Plover. The feather color of Javan plover is from dark brown to brown sand which is analogous to the Kentish Plover. The Similarity of the morphological characteristics led it into a taxonomic problem. It implicates to the taxonomic status to be unclear and debated. The possibility of *C. javanicus* as a subspecies of *Charadrius alexandrinus* (Kentish Plover) and is called *C. a. javanicus* (Chasen, 1938) or as a separate species under the name *Charadrius javanicus* as described by some others taxonomist (e.g. Monroe and Sibley, 1993; Dickinson 2003). So that, there is still much research that needs to be done to assess the status of the taxonomy of the Javan plover (del Hoyo et al., 1996). One of them is with molecular approaches to knowing the level of genetic variation in each species or subspecies, groupings, and relationships among species of *Charadrius*.

Genetic variations in the intraspecific or interspecific levels necessary to recognize the limits of a species or subspecies of animals have attracted attention in a few decades ago (Cracraft 1983; Ball and Avise 1992; Astuti and Sulandari

2010, Astuti 2011, Astuti and Prijono 2015). Decisions on species and subspecies boundaries can have an impact on the conservation and management of a species (Shaffer et al., 2000). In theory, all adjacent populations of a species show genetic differences at some level (Avise, 1994). Thus, specific criteria used to justify a recognition of a species, subspecies, or any other taxonomy levels based on categories and even dependent on biological attributes of the taxa in question (Starkey et al., 2003).

Many studies aimed to determine the variation of boundaries between inter-and intra-specific using molecular data, mainly to test the hypothesis of relationships at the species level. More recently known for a theory and established the method of DNA barcoding using gene sequences and is believed to be able to answer the taxonomic status of an animal to the level of species or subspecies (Hebert et al., 2003).

COI gene has been using as a DNA barcoding. The gene is a part of the mitochondrial DNA with a length of about 2000 base pairs (bp). Approximately 648-660 bp length of this gene, known as DNA barcodes and the position COI gene barcode is near to the term-5 ' end of the gene (Hebert et al., 2003; 2004; Rach et al., 2008). COI gene barcode can be used to identify an animal species (Hebert et al., 2003; 2004; Nagy et al., 2013; Kaur S., 2015; Luo et al., 2011; Purty RS and Chatterjee S. 2016). The short DNA sequences can ensure the individuals of a species due to genetic variation among individuals within a species apart from the genetic variation among individuals in different species (Hebert et al., 2004). Studies to test the accuracy of species identification using COI DNA barcodes have been successfully carried out previously on birds (Hebert et al., 2004a) and Lepidoptera (Hajibabaei et al., 2006a)

The study was carried out to use the DNA sequences of COI gene barcodes: to confirm and to assess whether the Javan Plover is a different species named *C. javanicus* or a subspecies of *C. alexandrinus* called *C. a. javanicus*, and to determine the relationships among Plover (*Charadrius*).

METHODS

Sampling of genetic materials

Genetic materials in the form of blood samples from plovers were collected in the coastal areas of Northern (Jawa sea of Central Java), Western (Bekasi and Indramayu) and Southern (Yogyakarta) of Java Island. A total of 26 blood samples consisted of 11 samples of Javan plover

(*Charadrius javanicus*), 7 samples of *C. alexandrinus* (kentish plover), 2 samples of *C. dubius*, 4 samples of *C. leschenaultii*, and 2 samples of *Glareola maldivarum* were used in this study. List of samples was presented in Tabel 1. The others plover consisted of 2 samples of *C. melodus*, 3 samples of *C. semipalmatus* were not collected in this study, but the sequence data of them were used and adopted from GeneBank.

Around 0.05 – 0.1 ml of blood was taken from each individual bird by using 1 ml syringe. Then, each blood sample was preserved in the absolute ethanol in a 2 ml tube and stored in the refrigerator at 4 °C or at room temperature.

DNA extraction

Total DNA was extracted from each 5-20 mg of blood sample using DNA extraction Kit (Dneasy Blood & Tissue Kit) was obtained from Qiagen, and then used as a template in the PCR process.

PCR reaction and DNA Sequencing

The barcode COI gene fragment was amplified by PCR (Gene AmpR PCR System 2700 Applied Biosystem machine), using specific primers (David et al., 2009) were used in the condition of 94 °C for 3 min, followed by 35 cycles of (94 °C - min, 58 °C - min, 72 °C - 1.5 min), and the last at 72 °C - 5 min. Each mix-PCR was made with a composition of 2.5 ml 10 X Takara ExTaq buffer, with 20 mm MgCl₂, 1.2 ml of 10 mm each primer, 1 ml of 100 mm dNTPs, 0.2 ml of Takara ExTaq polymerase, 9.16 ml H₂O, and 2 ml DNA template, for 25 ml total reaction volume. DNA fragments from the PCR product were sent to Macrogen Company Korea to then be sequenced. DNA sequencing was done to determine the sequence of bases.

DNA sequence analyses

Each sequence data then was aligned

using BioEdit software, and 666 bp data was used for further analysis. MEGA-5 software is used to calculate genetic distances and sequence divergences among individuals Plover.

Relationships analyses

Furthermore, to determine the grouping and relationships among individuals or species observed, it created phylogenetic trees based on neighbor-joining (NJ) and maximum-parsimony (MP) analyses using MEGA-5 software. The NJ tree was constructed by considering all types of base substitutions both transition and transversion with Kimura 2-parameter model. Two individuals of *G. maldi* are used as outgroup species. DNA sequence data from 3 individual of *C. melodus* and 3 individuals of *C. semipalmatus* retrieved from GenBank with the accession number of each is DQ 433488, DQ 433490, DQ 433491, DQ 433493, DQ 433494, and DQ 433 495. To determine the level of confidence of the groupings, the bootstrap values were performed with 1000 replications for NJ and 100 times in MP were analyses.

RESULTS AND DISSCUSION

COI is a protein-coding gene and a part of the mitochondrial DNA. The sequence of DNA barcodes COI gene in all studied plovers has an average value of base composition of both total and at each codon position. In total, the base cytosine has the highest percentage, and guanine is the lowest (Table 2).

Analysis on all types of base substitutions (transitions and transversions) at all DNA sequence data from all individuals studied based on Kimura 2-parameter model, showed that the genetic distance between individuals in one species (intraspecific) ranged from 0.0000 (*C. semipalmatus*) to 0.0037 ± 0.0014 (*C. a. alexandrinus*) (Table 3). Whilst genetic distance between species one

Table 1. List of samples and locality of plover in Java Island which used in the study.

| Species name | Total sample | Sample code | Location |
|-------------------------|--------------|---|-----------------------------|
| <i>C. javanicus</i> | 11 | Dy1, Dy2, Dy3, Dy022, Dy023, MG0234, MG0235, MG0244 | Western, Northern |
| <i>C. alexandricus</i> | 7 | Dy4, Dy5, Dy6, Dy032, Dy033, Ind461, Ind466 | Western, Northern, Southern |
| <i>C. leschenaultii</i> | 4 | Dy10, Dy11, Dy12, Dy31 | Northern, Southern |
| <i>C. dubius</i> | 2 | Dy028, Dy029 | Southern |
| <i>G. maldivorus</i> | 2 | Dy8, Dy9 | Northern |
| <i>C. melodus</i> | 3 | DQ433488, DQ433490, DQ433991 | GenBank |
| <i>C. semipalmatus</i> | | DQ433494 | GenBank |

Table 2. Base compositions at the 666-bp of the COI barcode gene analyzed. T = thymine, C = cytosine, A = adenine, G = guanine.

| Codon position | T (%) | C (%) | A (%) | G (%) | Numbers of bases | AT (%) | GC (%) |
|----------------|-------|-------|-------|-------|------------------|--------|--------|
| Total | 26.3 | 31.7 | 25.4 | 16.6 | 666 | 51.7 | 48.3 |
| 1st position | 18.4 | 26.6 | 23.9 | 31.1 | 222 | 42.3 | 57.7 |
| 2nd position | 41.9 | 28.8 | 14.9 | 14.4 | 222 | 56.8 | 43.2 |
| 3rd position | 18.6 | 39.8 | 37.2 | 4.4 | 222 | 55.8 | 44.2 |

Table 3. Genetic distance (d) and standard error (SE) among individuals within species were based on analysis of all types of base substitution (transitions + transversions) on Kimura 2-parameter model.

| group | species/subspecies | Distance (d) | S.E |
|-------|-----------------------------|--------------|--------|
| 1 | <i>Charadrius javanicus</i> | 0.0013 | 0.0007 |
| 2 | <i>C. a. alexandrinus</i> | 0.0037 | 0.0014 |
| 3 | <i>C. leschenaultii</i> | 0.0020 | 0.0015 |
| 4 | <i>C. dubius</i> | 0.0030 | 0.0017 |
| 5 | <i>C. melodus</i> | 0.0020 | 0.0014 |
| 6 | <i>C. semipalmatus</i> | 0.0001 | 0.0010 |

Table 4. Genetic distance (d) and standard error (SE) between species, based on analysis of all types of base substitution (transitions + transversions) with Kimura 2-parameter model.

| group | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------------------------|--------|--------|--------|--------|--------|--------|
| 1. <i>Charadrius javanicus</i> | | 0.0040 | 0.0119 | 0.0153 | 0.0153 | 0.0154 |
| 2. <i>C.a.alexandrinus</i> | 0.0012 | | 0.0119 | 0.0154 | 0.0153 | 0.0155 |
| 3. <i>C. leschenaultii</i> | 0.0930 | 0.0881 | | 0.0135 | 0.0139 | 0.0161 |
| 4. <i>C. dubius</i> | 0.1441 | 0.1460 | 0.1105 | | 0.0141 | 0.0145 |
| 5. <i>C. melodus</i> | 0.1457 | 0.1403 | 0.1130 | 0.1148 | | 0.0125 |
| 6. <i>C.semipalmatus</i> | 0.1424 | 0.1410 | 0.1370 | 0.1241 | 0.0899 | |

with another species ranged from 0.012 ± 0.0040 (*C. a. alexandrinus* vs. *C. javanicus*) to 0.1469 ± 0.0154 (*C.a. alexandrinus* vs. *C. dubius*) (Table 4).

Genetic distance or nucleotide divergence between the javan plover (*C. javanicus*) and kentish plover (*C.a. alexandrinus*) is very small which is only 1.2%. Whilst, the genetic distances between species were more than 8% (Table 3).

The range of genetic distances and sequence divergences in the plover birds (genus: *Charadrius*) were very clear. All intraspecific genetic distances, intra, and interspecies were very clear difference, and between individuals of different species have a very large genetic distance of more than 0.80, while among the individuals of the same species or subspecies of (*C. semipalmatus*) is 0.0000 to 0.0037 ± 0.0014 . This is clearly seen that the genetic distances and sequence divergences among individuals of the same species is much smaller than the genetic distances between individuals of species. Previous study has shown that in 240 North American bird species DNA sequence variation of the COI gene among indi-

viduals is generally much more different types of variation within species, and no two bird species sharing its barcode sequence (Hebert *et al.*, 2004). In the 643 bird species included in 71 families and 284 genera, the average intraspecific and the genetic distance between different individuals and species were 4.3% higher than the average genetic distance between individuals on the same type or of the average maximum intraspecific genetic distances, and some types have a distance of more than 2.5% intraspecific (Kerr CR Kevin *et al.*, 2007).

Focus on *C. alexandrinus* and *C. javanicus*, the value of sequence divergence between them is only 1.2%. If both plovers are considered as two different species (as categorized by del Hoyo *et al.*; 1996; and Dickinson, 2003), they should have a sequence divergence more than 2%. In general, 98% of the sister species of vertebrate animals have mitochondrial DNA divergence are higher than 2% (Johns and Avise 1998).

Analysis based on gene sequence divergence of COI barcodes in this study showed that

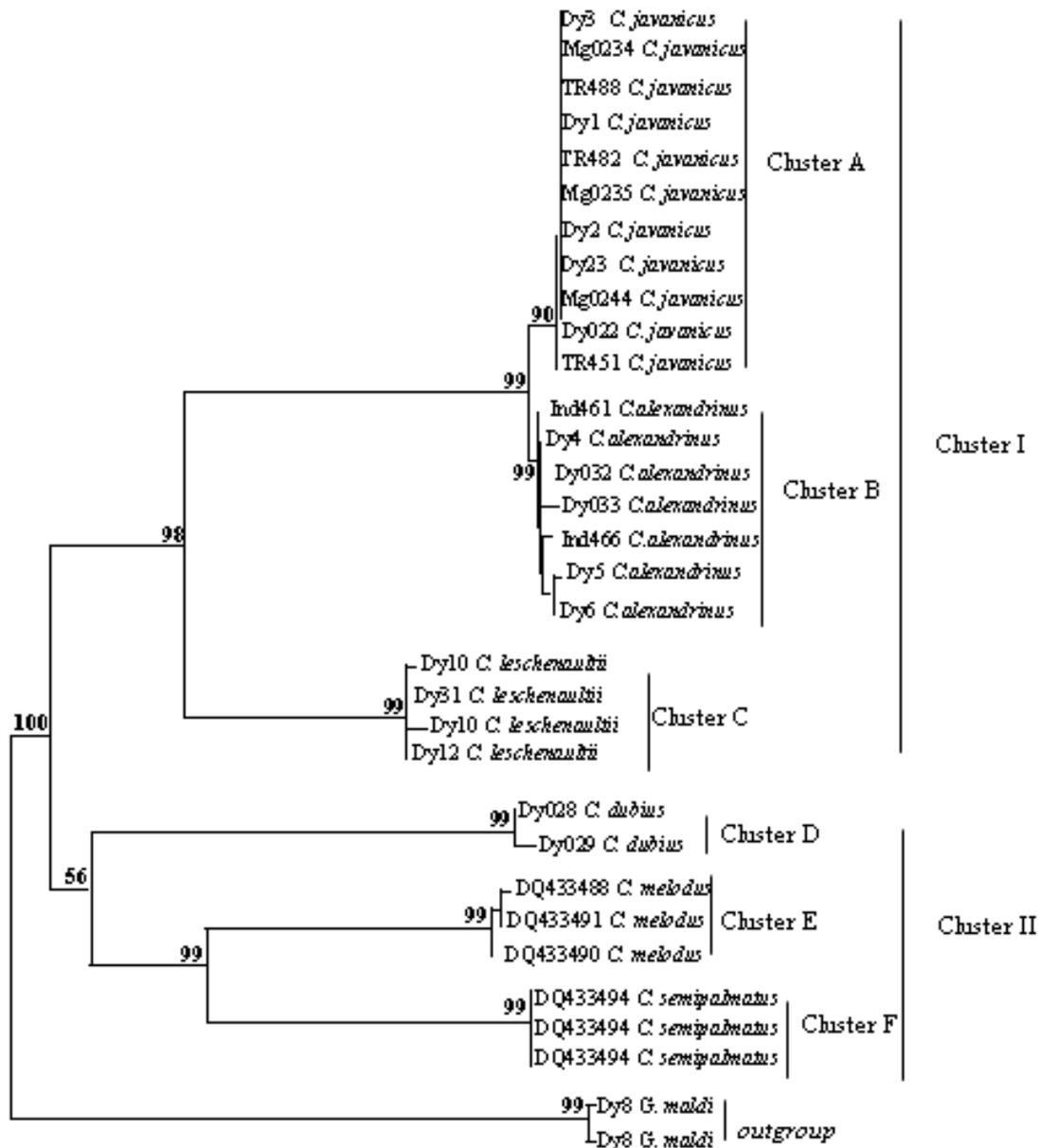


Figure 1. Neighbor-joining (NJ) tree of the plover species studied constructed on the basis of all transitions and transversions base substitutions in the 666-bp of DNA barcodes COI gene with Kimura 2-parameter model. The numbers above are bootstrap values > 50%.

between the two species (*C. a alexandrinus* and *C. javanicus*) there was a very small of divergence value. While, on the other plover species the average sequence divergence between the two species has a very high value of divergence (8-14%) is even much greater than 2%. Therefore, based on the genetic distance or sequence divergence from COI gene barcodes in this study, *C.a. alexandrinus* and *C. javanicus* were as two different species was not supported, although individuals of *C. alexandrinus* are clustered in different clusters with *C. javanicus*. The results of this study were more likely to assume that javan plover was not a separated

species with the name *C. javanicus*, but still one species with *C. a. alexandrinus* or in other words javan plover was still included in the species of *C. alexandrinus*, so it was a subspecies of *C. alexandrinus*, which if refers to Chasen (1938) named as *C. a. javanicus*.

According to the results, a high genetic divergence between individuals in different species, it is assumed that plover (genus: *Charadrius*) has a rapid substitution rate of nucleotides in COI barcode. In some other bird groups, genetic divergence between species within a genus has a value that varies widely. DNA Barcoding is based on the

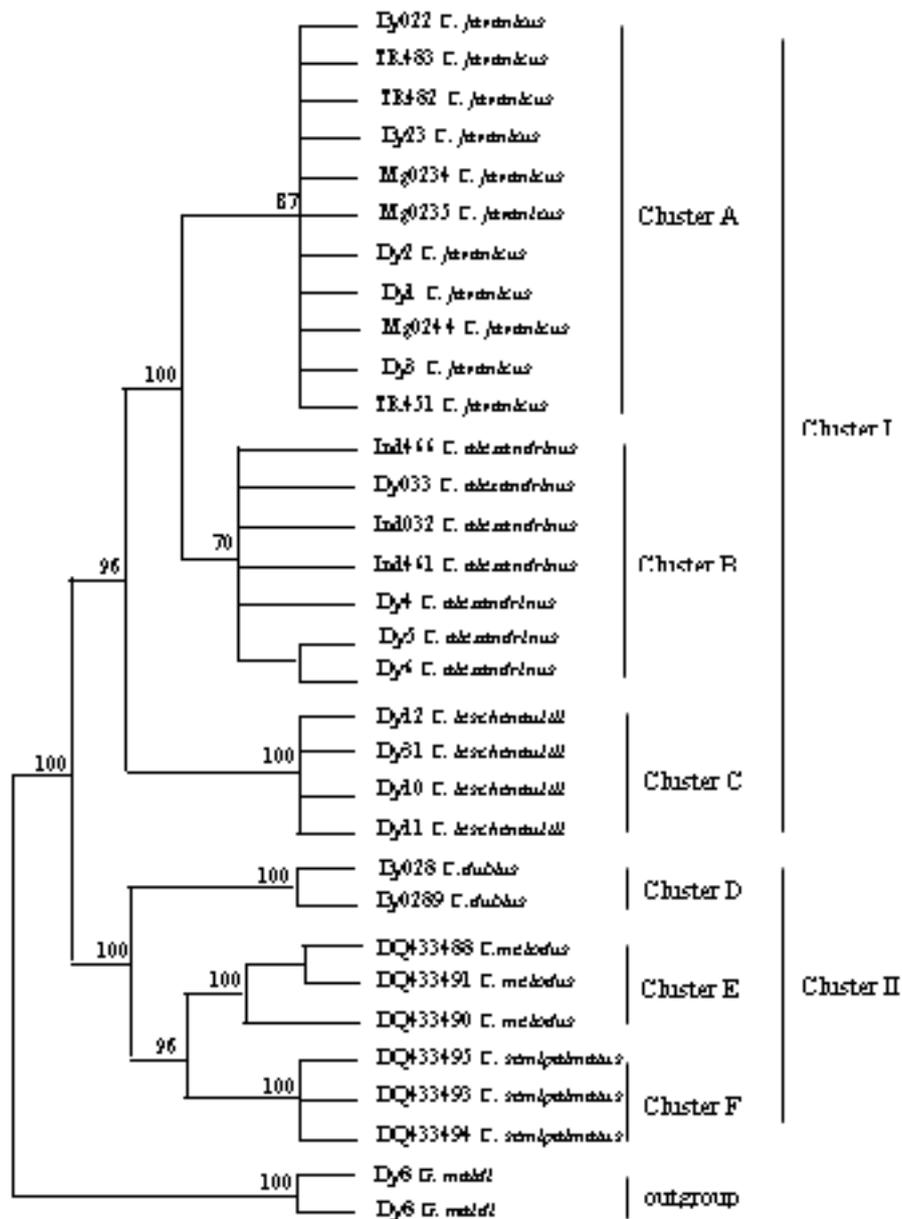


Figure 2. Maximum parsimony (MP) tree of the plover studied, constructed on the basis of all transitions and transversions base substitutions in the 666 bp of DNA barcodes COI gene. The numbers above are bootstrap values > 50%.

idea that the standard short DNA sequences can distinguish the individuals of a species because of variation between species exceeds the variation within species (Hebert et al., 2003).

COI sequence difference between closely-related species is much higher than differences within species; differences among species are 7.93%, and 0.43% within species. Most of the con-generic species pair showed divergences are well above the value of it (7.05%) (Hebert et al., 2004). Most animal species have intraspecific variation /divergence in COI was 0.24%, (Weibel and Moore; 2002), less than 2 % (Hebert et al.,

2003b; Mutanen et al., 2012), and the sister-bird species average found genetic distance of 5.1% (Klicka and Zink, 1997), 3.5% (Johns and Avise, 1998), and 4 % (Hebert et al., 2003a; Mutanen et al., 2012). In most mammal species average intra-specific divergence were less than 2-3% and inter-specific distances within most genera were more than 3% (Luo et al., 2011).

Plover (*Charadrius*) relationships

Relationships between plover species in this study, shown by NJ and MP analysis are described in the phylogeny trees (Figure 1 and 2).

Both NJ and MP trees showed that all individuals in a same species, grouped together in the one cluster and supported with a bootstrap value of 90% -99% in NJ and 70% -100% in MP. Eleven individuals of javan plover (*C. javanicus*) grouped in cluster A is supported by 90% bootstrap values (NJ) and 90% (MP). All individuals of kentish plover (*C.a. alexandrinus*) grouped in cluster B, with a bootstrap value of 90 % (NJ) and 70 % (MP).

Javan plover and Kentish plover appeared to be a sister group, supported by 99% bootstrap values in NJ and 100% in MP. Morphological characters of Javan Plover is similar to Kentish Plover (Bamford et al., 2008).

Four individuals of *C. leschenaultii* grouped in one cluster C with 99% bootstrap values (NJ) and 100 % bootstrap value (MP). Two individuals of *C. dubius* grouped in one cluster D supported by bootstrap value 99% (NJ) and 100% (MP), three individual of *C. melodus* grouped in one cluster E with 99% bootstrap values (NJ) and 100% (MP), and four individuals of *C. semipalmatus* grouped in one cluster F supported bootstrap value 99% (NJ) and 100% (MP). Two individuals from other species that are used as outgroups grouped together and are far apart from the species analyzed.

NJ and MP trees described the relationships among plover species studied that both *C. javanicus* and *C.a. alexandrinus* closer to *C. leschenaultii* than with other species and is supported by bootstrap values of 99% in NJ and 96% in MP. *C. melodus* closer to *C. semipalmatus* supported by 99% bootstrap values in NJ and 96% in MP, and similar with Barth et al. (2013), Dos Remedios et al. (2015), Rheindt et al. (2011). Both species are closer to *C. dubius* but only supported by 58% bootstrap values in NJ and 56% in MP. So both NJ and MP demonstrate 2 main clusters, namely cluster I consists of *C. leschenaultii* and *C. javanicus* + *C.a. alexandrinus*, and cluster II consists of *C. dubius* and *C. melodus* + *C. semipalmatus*.

Grouping based on NJ and MP analyses and the genetic distance and divergence values of DNA sequences from all species studied showed that genetically javan plover (*C. javanicus*) really are far apart from the four other species (*C. dubius*, *C. melodus*, *c. semipalmatus*, and *C. leschenaultii*), but not far apart with kentish plover (*C.a. alexandrinus*). These results show a tendency that the javan plover and the kentish plover are not a separate species, in the other words that both species are still in one species, or as two different subspecies of *C. alexandrinus*. So the results of this study assumed that Javan plover is

not a separate species but a subspecies of *C. alexandrinus* called by Chasen (1938) as *C.a javanicus*. Therefore, this study supports the classification of Chasen (1938).

NJ and MP trees suggests that cluster I contains of cluster A, cluster B, and cluster C. Cluster C consisted of *C. leschenaultia* and separated from cluster B (*C. alexandrinus* and cluster C (*C. javanicus*)). That grouping may indicate the possibility of influence of the presence of distribution factors of these three species. Reported by Dickinson (2003) that *C. alexandrinus* can be found in Indonesia's western region, while *C. javanicus* in Java, and *C. leschenaultii* especially *C.l. leschenaultii* also spread to Indonesia.

C. dubius (cluster D) is separated from *C.melodus* (cluster E) and *C. semipalmatus* (cluster F) form a cluster II in NJ analysis. This suggests that *C. dubius* closer to *C. melodus* and *C. semipalmatus*, rather than with the others three species (*C. alexandrinus*, *C. javanicus*, and *C. leschenaultii*), although its proximity is only supported by 58% bootstrap value. *C. dubius* especially *C.d. curonicus* can also be found in Indonesia.

CONCLUSIONS

Analyses of 666 bp of COI barcode gene in this study tend to support the opinion of Chasen (1938) that, javan plover is not a separated species called *C. javanicus*, but still is a subspecies. Because of the sequence divergence between javan plover and *C. alexandrinus* is only 1.2 % and phylogenetically they group together and as closely related therefore the present study conclude that the javan plover is a subspecies of *C. alexandrinus*, and called *C. alexandrinus javanicus*. There are two main groups in this study were (*C. leschenaultii* and *C.a. javanicus* + *C.a. alexandrinus*), and (*C.dubius* and *C. melodus* + *C. semipalmatus*).

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