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DNA Barcoding of Ornamental Crab *Geosesarma* in South-Slope Mount Slamet Central Java, Indonesia

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Abstract. *Geosesarma* shows intraspecific carapace color variation, which might lead to species misidentification. The problem can be solved using DNA barcoding. There is one research about *Geoserarma* from the southern slopes of Mount Slamet, but samples were only collected from the Banjaran River for morphological identification. Here, we collected samples from wider areas covering south slope and applied molecular identification. This research aims to assess *Geosesarma* diversity in south-slope Mount Slamet Central Java, Indonesia based on the cytochrome c oxidase 1 gene barcoding. Surveys were carried out at six sites. Taxonomic identification was done using the barcoding technique. Four morphotypes were obtained during the research. Three morphotypes with the square carapace were identified as *Geosesarma*, while the remaining one morphotype was included in *Parathelphusa*. The three *Geosesarma* morphotypes were barcoded as *Geosersarma dennerle* because their genetic identity was more than 97% of the *G. dennerle* sequence in Boldsystems. In contrast, the *Parathelphusa* morphotype was barcoded as *P. convexa* with a genetic identity of 97.50%. It can be concluded that the *Geosesarma* crab on the south-slope Mount Slamet only consists of one species but has carapace and claw color variations. The data are essential for *Geosesarma* market development and conservation in the region.

Keywords: crabs; diversity; freshwater; ornamental; similarity.

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INTRODUCTION

Indonesian freshwater crabs are classified into three families: Gecarcinucidae, Potamidae, and Sesarmidae. Sesarmidae comprises 41 genera (Shahdadi & Schubart, 2018; Schubart & Ng, 2020; Shahdadi et al., 2020). *Geosesarma* is a freshwater crab genus under Sesarmidae (Ng et al., 2008). It is a large genus *with* 73 species. This genus inhabits Southeast and East Asia, the Andaman Islands, New Guinea, and the Solomon Islands (Ng et al., 2008; Ng & Wowor, 2019; Shy & Ng, 2019; Naruse & Ng, 2020)

Geosesarma shows high intraspecific variation in body coloration (Ng et al., 2015). Morphological variation has caused misidentification in various animal groups (Nuryanto et al. 2020; Nuryanto et al., 2021). In addition, misidentification is also found in the ornamental trade because similar names are commonly used for different species. Simultaneously, various names are also commonly applied to single species (Dhar & Ghosh, 2015).

In the case of morphological variation, species status can be validated using the DNA barcoding technique (Bekker et al., 2016; Nuryanto et al., 2020; Palecanda et al., 2020). This technique is a reliable tool to unmask animal diversity into species categories, even without key morphological diagnostic characters (Purty & Chatterjee, 2016; Bhagawati et al., 2020). Previous studies have successfully revealed new species using molecular barcoding (Packer & Ruz, 2017), address taxonomic uncertainty (Kundu et al., 2018; Hu et al., 2018; Ceruso et al., 2018; Abdalwahhab et al., 2020), identify the pet trade of endangered taxa (Kundu et al., 2018), and biodiversity assessment (Nuryanto et al., 2017; Barman et al. 2018; Ali et al., 2020).

DNA Barcoding using the cytochrome c oxidase 1 (COI) gene is an effective and complementary tool in taxonomic research (Caputi et al., 2021; Ceruso et al., 2021; Mohammed-Geba et al., 2021). It has been proven that the COI gene could be used for the accurate identification of crustaceans up to the species level (Bilgin et al., 2015; Karanovic, 2015; Bekker et al., 2016; Palecanda et al., 2020), including crabs from the family Sesarmidae (Schubart & Ng, 2020). In DNA barcoding, species category can be determined based on intraspecies genetic distances with a value range of 1-3% (Ha et al., 2019; Abdalwahhab et al., 2020; Palecanda et al., 2020).

On Java Island, eleven *Geosesarma* species have been described, i.e., *G. noduliferum*, *G. bicolor*, *G. dennerle*, *G. hagen*, *G. lebak*, *G. sukabumi*, *G. robustum*, *G. confertum*, *G. shovel*, *G. cikaniki*, and G. *rouxi* (Ng & Wowor, 2019). Even though the genus *Geosesarma* is widely distributed, certain species have a minimal distribution capability, which sometimes leads to the finding of different species only 10 km apart between locations (Ng et al., 2015). This finding is proven by the discovery of 5 species of *Geosesarma* only from Mount Halimun, West Java (Ng & Wowor, 2019). This fact has become a challenge for taxonomists in uncovering the diversity of *Geosesarma* from various areas, including on the south slopes of Mount Slamet. There is only one study on the variety and relationships of *Geosesarma* crabs from the south slopes of Mount Slamet. However, they only took samples from the Banjaran River and identified them only to genus level, i.e., *Geosesarma* sp. (Hernawati, 2019) (*Geosesarma* sp.). Meanwhile, many rivers have springs on the south slopes of Mount Slamet. Therefore, Research by Hernawati (2019) has not comprehensively described the diversity of *Geosesarma* crabs on the south slopes of Mount Slamet and has yet to carry out DNA barcoding.

This study aimed to assess the species diversity of *Geosesarma* in the south slope of Mount Slamet, Banyumas, Central Java, Indonesia, based on molecular identification using the cytochrome c oxidase 1 gene.

METHODS

Sampling sites

Geosesarma samples were collected at six sites on the south slope of Mount Slamet, Central Java, Indonesia (Figure 1). Nevertheless, crab samples were only obtained from four sites. The geographical position and altitude of each area are presented in Table 1.

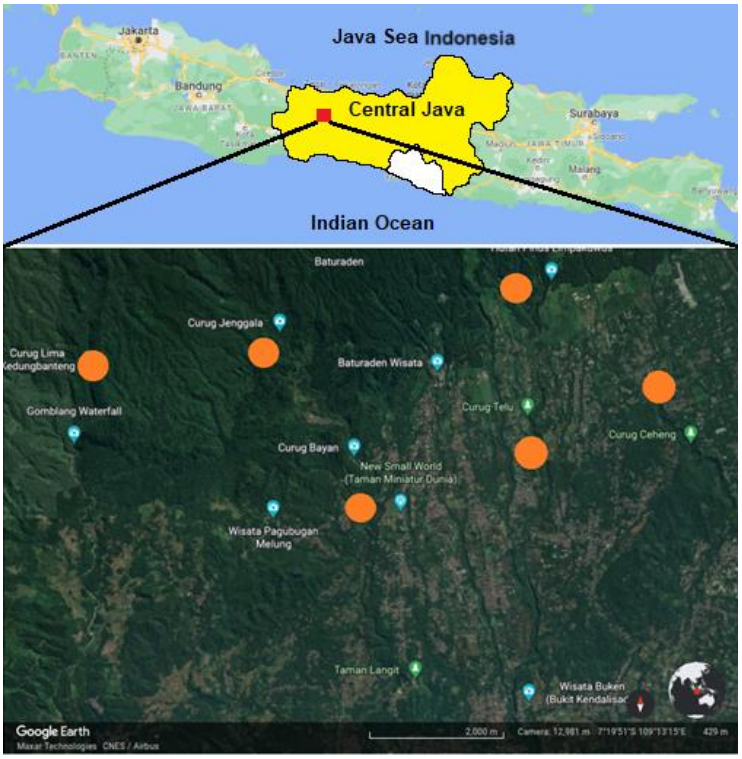

Figure 1. Sampling sites of *Geosesarma* on the south slope of Mount Slamet, Banyumas Regency, Central Java, Indonesia (modified from Google Earth).

Table 1. Geographic position and altitude of each sampling site

No	Name	Geographic Position	Altitude (m asl)
1	Limpakuwus pine forests	7°18'30.27838" S; 109°14'36.64144" E	687
2	Lima waterfall	7°20'2.08643" S; 109°11'34.28123" E	675
3	Telu waterfall	a. 7°19'17.54503" S; 109°14'25.12702" E	586
		b. 7°19'17.50097" S; 109°14'25.0552" E	581
4	Bayan waterfall	a. 7°19'31.31622" S; 109°13'15.12619" E	519
		b. 7°19'31.23309" S; 109°13'15.16904" E	518
		c. 7°19'31.34519" S; 109°13'14.43519" E	507
		d. 7°19'31.29405" S; 109°13'14.4898" E	507

Crab sample collection

Crab specimens were obtained during the field trips in June-July 2023. *Geosesarma* collection was carried out throughout the day at each sampling location. *Geosesarma* specimens were collected manually. The fresh samples were directly photographed before preservation in 70% ethanol. A portion of tissue samples was preserved in 96% ethanol before DNA analysis.

Morphological identification

Morphological identification was carried out in the Animal Taxonomy Laboratory at the Faculty of Biology, Jenderal Soedirman University in Purwokerto, Central Java, Indonesia. The obtained crab specimens were identified based on carapace shape. This character was used to identify the sample into the genus category. The rectangular or square shape of the carapace is the diagnostic character for the Genus *Geosesarma*. Identification was carried out following the references by Ng & Wowor (2019) and Hernawati (2019).

DNA barcoding

All crab morphotypes were shipped to PT. Genetika Science Indonesia for molecular barcoding. The genomic DNA was extracted from each morphotype using a DNA Miniprep Kit (Zymo Research, D6016) following the manufacturer's protocol. The fragment of the COI gene was amplified using the MyTaq HS Red Mix (Bioline, BIO-25047) and universal primer pair LCO1490: 5' GGTCAACAAATCATA AAGATATTGG-3' and HC02198: 5'-TAAACTTCAGGGTGACCAAAA AATCA-3' (Folmer at al. 1994). The thermocycler was set up as follow. Double DNA bands were split into single bands at 96° Celsius (C). The amplification process was carried out for 35 cycles. Each step's thermal condition and duration were denaturation at 94° for 10 seconds, annealing at 52° for 30 seconds, and extension at 72° for 45. The final volumes of PCR reactions were 25 µl consisting of KOD FX Neo 1 µl, 2XPCR Buffer KOD FX Neo 12.5 µl, 2mM dNTPs 1 µl, 10pmol/µl of each primer was 1 µl, template DAN 1 µl, and ddH2O 6 µl. The amplification products were sequenced using standard big dye termination from the Sanger method using a bi-directional sequencing technique.

Data Analysis

The nucleotide base sequence of the COI gene fragment was aligned using the ClustalW program and edited manually with the help of the Bioedit program (Hall, 2011). Taxonomic status at the species level is based on morphological and molecular identification results. Morphospecies are determined by comparing the sample's carapace shape and the species' carapace shape contained in the reference (Ng & Wowor, 2019; Hernawati, 2019). The genetic species of the *Geosesarma* sample was determined based on the amount of sequence homology of the sample and sequences from reference species contained in international databases (GenBank and BOLDsystem). The genetic similarity used for species boundaries was 97% as commonly used in previous studies (Riani et al., 2021; Winarni et al., 2023) and also implemented as BIN used in the BOLD system (Ratnasingham & Hebert, 2013). The phylogenetic tree was reconstructed using the neighbor-joining algorithm and Kimura 2 parameter (K2P) model with the help of the MEGAX program (Kumar et al., 2018). The branching polarity of the tree was determined by comparison with *Parathelphusa convexa* HE794122, *P. batamensis* HE794121, and *P. maculata* KF201100 as the outgroup species. The confidence level in the branches formed will be obtained through repeated tree creation. The number of replications that were used was 1000 non-parametric bootstraps.

RESULTS AND DISCUSSION

Morphotype identification

Sixteen freshwater crab specimens were obtained during the field trips. Fifteen individuals have rectangular carapaces. Further examination revealed thirteen individuals with rectangular carapaces have orange claws (Figure 2A). One individual has brown claws (Figure 2B), and one specimen has black claws (Figure 2C).

The diagnosis character for *Geosesarma* is a rectangular carapace (Ng & Wowor, 2019; Hernawati, 2019) with variable carapace and claw color (Ng et al., 2015; Ng, 2017). The morphotype A, B, and C samples have rectangular carapace shapes with either orange, light brown, or black claws (Figure 2 The observed characters on the samples are similar to the *Geosesarma* characters, as described by Ng & Wowor (2019) and Hernawati (2019). Hence, morphotypes A, B, and C were morphologically identified as *Geosesarma*.

The remaining freshwater crab specimen had a flower pot carapace shape with light brown coloration (Figure 3), which was significantly different and was easily differentiated from the other specimens.

Figure 2. Freshwater crab specimens show rectangular carapaces with different claw colors.

Figure 3. Freshwater crab specimen showing flower pot-like carapace with light brown color (left: dorsal view, right: ventral view).

The diagnosis character of *Parathelphusa* is a flower pot or ovoid carapace shape with a light brown color (Hernawati, 2019). The remaining obtained sample has a flower pot carapace shape and light brown color. These characteristics were more similar to th*ose* described by Hernawati (2019). Therefore, the current study identified morphotype D as *Parathelphusa.*

This study has confirmed the presence of freshwater ornamental crabs Genus *Geosesarma* on the south slope of Mount Slamet Central Java, Indonesia. The presence of *Geosesarma* in the south-slope Mount Slamet was previously reported by Hernawati (2019). Nonetheless, the current study reported more comprehensive information about the existence of *Geosesarma* in the south-slope of Mount Slamet than that was reported by Hernawati (2019) because this study has broader geographic sampling coverage than the sampling coverage of Hernawati (2019). Nevertheless, this study and a previous study (Hernawati, 2019) only morphologically identified the crab samples at the general level. Therefore, the findings need to be validated through molecular identification to ensure the species status of the fish samples.

Species barcoding

Genetic identity test

Since this study focuses on *Geosesarma*, further discussion is emphasized on the result of *Geosesarma* barcoding. Initially, the taxonomic status of all morphotypes was tested through a genetic identity test using the basic local alignment search tool (BLAST). The *Geosesarma* samples' query cover ranged between 99% and 100% with an e-value of 0.00. At the same time, genetic identity ranged from 92.63% to 93.17% for *G. faustum* MZ725940 available in GenBank. The complete genetic identity, query cover, expected value, and reference species for each sample are presented in Table 2.

The three parameters, as shown in Table 2, indicated that the obtained data are reliable as a basis for determining the taxonomic status of the samples because the query covers ranged from 99% to 100%, and the e-value was 0.00 for comparison. However, the ranges of genetic identity values between 92.63% and 93.17% could not be utilized as a genetic border to assign samples into species category; instead, it indicates that the samples are genetically barcoded into the genus category, i.e., *Geosesarma*. The assignment into the genus category was based on the fact that the homology, even below 95%, the highest genetic species border, has been used in crustacea (Karanovic et al., 2015; Lin et al., 2015). According to Kusbiyanto et al. (2020) and Bhagawati et al. (2021), the specimens could be assigned to the genus levels if the genetic identity is below 95%. Since this study used 97% homology value as a species border, the assignment of the crab's samples into the genus category (*Geosesarma*) is highly reliable because the obtained genetic identities were below 97%.

Table 2. Query cover, e-value, and genetic identity of *Geosesarma* samples compared to its reference species in GenBank

Sample Code	Accession Number	Query Cover (%)	e-value	Genetic identity (%)	Reference Species
CB-1	OR516562	100	0.00	92.91	*Geosesarma faustum* MZ725940
CB-2	OR516563	100	0.00	92.74	*Geosesarma faustum* MZ725940
CB-3	OR516564	99	0.00	92.63	*Geosesarma faustum* MZ725940
CB-4	OR516565	99	0.00	92.63	*Geosesarma faustum* MZ725940
CB-5	OR516566	100	0.00	92.91	*Geosesarma faustum* MZ725940
CB-6	OR516567	100	0.00	92.91	*Geosesarma faustum* MZ725940
CL-2	OR516569	99	0.00	92.91	*Geosesarma faustum* MZ725940
CT-1	OR516570	100	0.00	92.74	*Geosesarma faustum* MZ725940
CT-2	OR516571	100	0.00	92.74	*Geosesarma faustum* MZ725940
CT-3	OR516572	100	0.00	92.74	*Geosesarma faustum* MZ725940
CT-4	OR516573	100	0.00	92.74	*Geosesarma faustum* MZ725940
CT-5	OR516574	100	0.00	92.74	*Geosesarma faustum* MZ725940
CT-6	OR516575	100	0.00	92.74	*Geosesarma faustum* MZ725940
HPL-1	OR516576	100	0.00	93.17	*Geosesarma faustum* MZ725940
HPL-2	OR516577	100	0.00	92.74	*Geosesarma faustum* MZ725940

Taxonomic tree reconstruction

Support for the BLAST test was obtained from the monophyletic test between samples and reference species in the GenBank. The monophyly of the samples and their reference species is illustrated in the K2P-neighbor-joining (NJ) taxonomic tree (Figure 4).

The 15 crab samples formed a monophyletic group and are sister taxa to *Geosesarma faustum* MZ725940, as shown in Figure 4. This monophyly indicates that the fifteen samples were not *G. faustum* because monophyly between samples and *G. faustum* has a long branch topology. Monophyly could assign specimens as single taxa with their reference species if a short branch in the tree supports it. Nevertheless, the monophyly between samples and *G. faustum* MZ725940 can be utilized to assign that all the samples belong to the Genus *Geosesarma*. Sample CL-1 forms a monophyletic group with *Parathelphusa convexa* HE794122 but with long branches. This branch topology proves that the CL-1 sample belongs to the Genus *Parathelphusa*. According to Xu et al. (2015) and Kusbiyanto et al. (2020), monophyly can determine whether research specimens fall into the same group as the reference species.

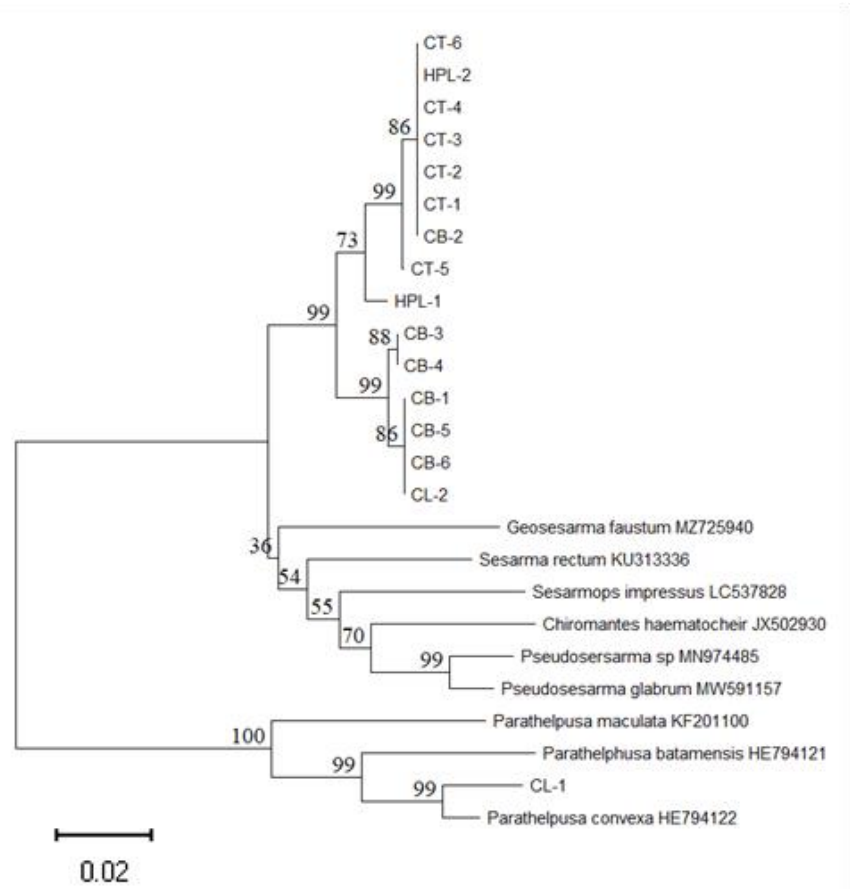

Figure 4. Taxonomic tree showing crab specimens from south-slope Mount Slamet belong to Genera *Geosesarma* and *Parathelphusa*

Genetic Similarity test

This study also performed a similarity test to sequences available in BOLDsystems (Ratnasingham and Hebert 2007) to ensure that Geosesarma samples can be identified into species categories. The sequence similarity test showed that the samples have genetic similarities ranging from 98.02% to 99.11% to conspecific reference in BOLDsystem, i.e., *Geosesarma dennerle* BOLD:AFF6306. Nevertheless, the sequences were inaccessible because they still needed to be published. Therefore, we could not reconstruct a taxonomic tree using *G. dennerle* sequences. The exact similarity values of the 15 *Geosesarma* specimens are presented in Table 3.

Table 3. Genetic similarity between samples and their conspecific references in BOLD systems.

Sample Code	Accession Number	Similarity (%)	Reference Species
CB-1	OR516562	99.11	*Geosesarma dennerle* BOLD:AFF6306
CB-2	OR516563	98.02	*Geosesarma dennerle* BOLD:AFF6306
CB-3	OR516564	98.76	*Geosesarma dennerle* BOLD:AFF6306
CB-4	OR516565	98.76	*Geosesarma dennerle* BOLD:AFF6306
CB-5	OR516566	99.11	*Geosesarma dennerle* BOLD:AFF6306
CB-6	OR516567	99.11	*Geosesarma dennerle* BOLD:AFF6306
CL-2	OR516569	98.94	*Geosesarma dennerle* BOLD:AFF6306
CT-1	OR516570	98.02	*Geosesarma dennerle* BOLD:AFF6306
CT-2	OR516571	98.02	*Geosesarma dennerle* BOLD:AFF6306
CT-3	OR516572	98.02	*Geosesarma dennerle* BOLD:AFF6306
CT-4	OR516573	98.02	*Geosesarma dennerle* BOLD:AFF6306
CT-5	OR516574	98.38	*Geosesarma dennerle* BOLD:AFF6306
CT-6	OR516575	98.02	*Geosesarma dennerle* BOLD:AFF6306
HPL-1	OR516576	98.55	*Geosesarma dennerle* BOLD:AFF6306
HPL-2	OR516577	98.02	*Geosesarma dennerle* BOLD:AFF6306

Nevertheless, genetic identity and monophyly tests could not reveal the species status of the research samples. It was because no sequence of the same species is deposited in GenBank. Therefore, an additional genetic test was carried out on the database in BOLDsytems. The result of the genetic similarity test, as summarized in Table 3, proved that all samples had high genetic similarities (98.02% to 99.11%) to their reference species, *G. dennerle* in BOLDsystems. The obtained genetic similarity values were higher than a predetermined value of 97% as a threshold value for species border. The values indicated that the samples can be identified as the same species as their reference species in the BOLDsystem. Previous studies reported that genetic similarity and identity of 97% were commonly used in species determination during molecular barcoding of animal species (Nuryanto et al., 2018; Amatya, 2019; Kusbiyanto et al. 2020; Riani et al., 2021). Even as low as 95% of genetic similarity and identity were acceptable as a species boundary in species determination (Karanovic et al., 2015; Bhagawati et al., 2022; Setyaningrum et al., 2022). In such cases, additional consideration is needed (Candek & Kuntner, 2015; Lin et al., 2015). It is well known that variable genetic similarity and identity have been used in various animals (Aguilar et al., 2017; Ha et al., 2019; Abdalwahhab et al., 2020). It was because COI-high genetic distances are common among animal groups (Diaz et al., 2016; Ali et al., 2020; Sholihah et al., 2020). Therefore, 98.02 to 99.11% genetic similarities are reliable and acceptable values to be used as a genetic identity that the crab specimens delineated into *G. dennerle*.

Figure 2 shows that *Geosersarma* specimens collected during the field trips have different colors of their claws and carapace. However, genetic identification proved that all *Geosesarma* morphotypes belong to a single species, *Geosesarma dennerle*. This result was not surprising because a previous study demonstrated that *Geosesarma* shows the intraspecific variation of body coloration (Ng et al., 2015). Moreover, a single species of *Geosesarma* was observed in different sampling sites because the sampling sites were located at similar altitudes (Table 1) and had a close distance between one location and another, below 5 km. A previous study reported that different species of *Geosesarma* in Mount Halimun, West Java, were collected from various altitudes and more than five kilometers away (Ng et al., 2015). Previous studies reported that eleven *Geosesarma* species have been described, while this study only found *G. dennerle* from south-slope Mount Slamet. Therefore, further research is needed covering wide geographic altitudes and four side slopes of Mount Slamet to provide more comprehensive data about *Geosesarma* diversity in Mount Slamet Central Java, Indonesia. The data are essential for *Geosesarma* market development and conservation in the region.

CONCLUSION

Based on morphology, monophyly, genetic identity, and genetic similarity, this study has successfully identified ornamental freshwater crabs in the south-slope Mount Slamet Central Java, Indonesia, into single species *Geosesarma dennerle*. It can be concluded that ornamental freshwater crabs in the south-slope Mount Slamet

Central Java, Indonesia, only comprised a single species. The data are essential for *Geosesarma* market development and conservation in the region and provide additional data about crab diversity in the region. Further studies are needed covering wide geographic altitudes and four side slopes of Mount Slamet to provide more comprehensive data about *Geosesarma* diversity in Mount Slamet Central Java, Indonesia. The data are essential for *Geosesarma* market development and conservation in the region.

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