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ARTICLE

Morphology, morpho-taxometric and molecular characterization of the invasive alien species Caribbean leatherleaf slug *Sarasinula plebeia* (Gastropoda: Veronicellidae): a first record in southern Philippines

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Abstract. Herein, we first report the comprehensive description of the terrestrial slug, *Sarasinula plebeia* (Gastropoda: Veronicellidae) by employing morphology, morpho-taxometrics and molecular analysis. A rapid survey on terrestrial slug invasive alien species (IAS) was conducted in La Dicha, Malangas, Zamboanga Sibugay, the Philippines. Obtained COI gene sequences shared 100% similarities to *S. plebeia* from Brazil (JX532107, KM489378), Dominica (KM489500) and Vietnam (KM489367) and further supported using Bayesian analysis thus designated as *S. plebeia* isolate LDZS. Notably, the first reported *S. plebeia* in 2013 from Batan island, Batanes, northern Philippines, characterized through COI gene markers (JQ582277, JQ582278, JQ582279) showed 100% sequence similarities to a closely related veronicellid slug, *Laevecaulis alte* isolates (LC636101, LC636102, LC636103, and LC636104) from Japan. Taken this into account, our *S. plebeia* LDZS isolated from an agricultural field is the first report in the Philippines with combined diagnostic tools for the taxon.

Keywords. COI gene; IAS; Morphology; Pneumostome; *Sarasinula* species; Terrestrial slug.

INTRODUCTION

Terrestrial gastropods have a geographic distribution from temperate to tropical regions comprising almost 35,000 species (Barker, 2001), occupying a diverse niche, and developing different life history strategies of survival (Klussmann-Kolb & Dinapoli, 2006). Most of these terrestrial gastropod species are free-living and take part in the nutrient cycle by feeding on living and organic plant matter and releasing nutrients back to the soil in the form of feces (Theenhaus & Scheau, 1996). However, several notable species of agriculturally important pests cause economic loss on agricultural crops.

As globalization steadily progresses allowing free movement of people and goods across the world, the possible infiltration and unintentional introduction of species to new habitats are irrefutable. Such species are usually non-native to the area and were introduced through various ways. These were labeled as invasive alien species (IAS) which pose a much greater threat to agriculture since they have no natural predator as population regulator. These IAS, of which gastropods are no exceptions, are threats to sustainable agriculture worldwide that account to agricultural loss resulting in economic loss in severe cases of infestation (Willis *et al.,* 2006), disruption in commerce, changing the natural environment and posing risk to human health (Staples & Cowie, 2001).

One of the IAS terrestrial gastropods is *Sarasinula plebeia* (Fischer, 1868) (Gastropoda: Veronicellidae), commonly known as Caribbean leatherleaf slug with neotropical range (Solem, 1964; Cowie, 1998; Robinson, 1999). This species is native to Central America and has now a worldwide distribution from the Americas, Pacific regions including the Philippine islands, and is considered a serious IAS (Barker *et al.,* 2005). In 2003, the United States Department of Agriculture

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(USDA) first intercepted *S. plebeia* (KM489380; Gomes *et al.,* unpublished) from the Philippines. The first record of *S. plebeia* in the country was in Batan island, Batanes, northern Philippines, identified using cytochrome oxidase subunit I (COI) gene markers (Batomalaque *et al.,* 2013) and another report was also in northern Philippines, from Masungi Georeserve in Tanay, Rizal with identification using morphological data (Valdez *et al.,* 2021).

Studies on gastropods are abundant, especially on invasive terrestrial gastropods. However, supplemental work is needed given the diversity of this class. For an increased resolution and reliability of species identification, integration of different diagnostic tools is highly suggested (Robinson & Fields, 2010). Thus, this study is conducted to identify IAS terrestrial slugs in the southern part of the Philippines by means of morphological, morpho-taxometrical, and molecular analyses. We have collected a total of six slug samples from selected agricultural areas in La Dicha, Malangas, Zamboanga Sibugay, however, assessment of severity of damage to the crops was not included in the present study. Our *S. plebeia* LDZS isolate (MZ598573) had 100% COI gene sequence similarities to *S. plebeia* from Brazil (JX532107, KM489378), Dominica (KM489500) and Vietnam (KM489367) and further supported using Bayesian analysis.

MATERIAL AND METHODS

Sampling site establishment

A survey for the occurrence of terrestrial slugs was conducted from an agricultural area in La Dicha, Malangas, Zamboanga Sibugay (07°40′8.832″N, 123°00′15.048″E) (Fig. 1). A purposive sampling utilizing the transect walk method was undertaken during sampling for terrestrial slug collection. Undergrowth of bushes, grasses, below fallen trees and stones were inspected for the presence of slugs. Collected samples were washed and transferred to sterile containers for further processing. The voucher sample specimens in 95% ethanol solution are deposited in the Department of Biological Sciences, Mindanao State University – Iligan Institute of Technology, Iligan City and the University of the Philippines Los Baños, Museum of Natural History (https://mnh.uplb.edu.ph).

Morphology and morpho-taxometrics analyses

Morphology was assessed by describing key characters along their morpho-taxometric values, such as total body length in stretched condition, circumference obtained at the widest region of the body using a thread (scaled to millimeter) and live weight (g) using digital weighing scale (Das & Parida, 2015).

Molecular analysis

For molecular analysis, COI gene was used as a marker for species identification. Tissue samples obtained from the slug were cut and submerged into 95% ethanol solution. Genomic DNA (gDNA) of the slug tissue samples was extracted using PureLink® Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific). Gene amplification by PCR was performed with the following components: template gDNA, universal HCO (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO

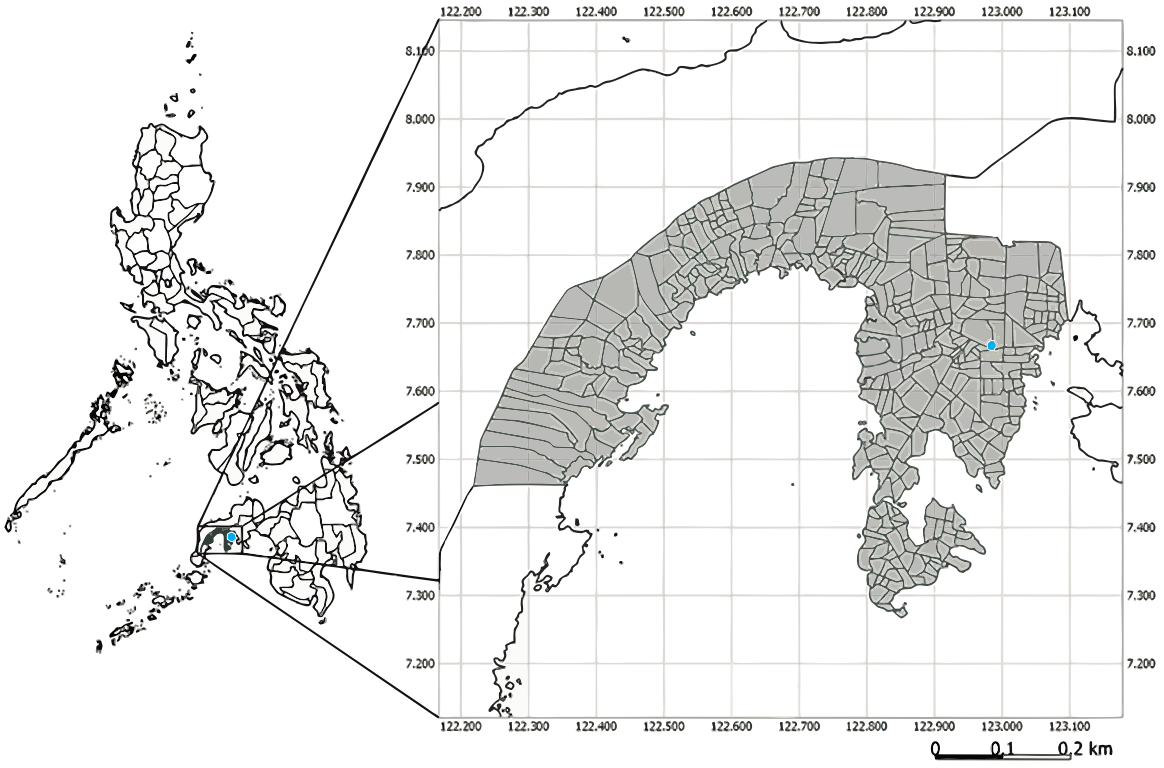

Figure 1. Map showing the sampling site (blue dot) in the selected area for terrestrial slug in La Dicha, Malangas, Zamboanga, Sibugay, southern Philippines.

(5′-GGTCAACAAATCATAAAGATATTGG-3′) primers, Taq Buffer, DNA Polymerase and dNTP Mix. Cycling parameters on the thermal cycler were set at 95°C for 45 sec, 70°C for 1 min and 5 min followed by 30 cycles of 95°C for 1 min, 58°C for a final extension at 72°C for 10 min. Amplicons generated had sizes ranging from 1,300-1,500 bp.

Capillary sequencing (bidirectional) was performed at the Philippine Genome Center-DNA Core Sequencing Facility (PGC-DCSF), University of the Philippines-Diliman, Quezon City. Fluorescent-labeled chain terminator dNTPs with the reaction components viz. amplicons, primers, and ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) were used. Cycling parameters on Bio-Rad T100 Thermal Cycler include: pre-hold at 4°C; 96°C for 1 min; 25 cycles of 96°C for 10 sec, 50°C for 5 sec, 62°C for 4 min and a hold at 4°C. Ethanol precipitation was carried out to remove unincorporated ddNTPs, excess primers and primer dimers. Capillary electrophoresis was done on the ABI 3730xl DNA Analyzer using a 50 cm 96-capillary array, POP7TM Polymer, and 3730xl Data Collection Software v3.1. Base calling was done on the Sequencing Analysis Software v5.4.

Sequence alignment and phylogenetic analysis

The generated COI gene sequences were assembled and trimmed using the BioEdit program (Hall, 1999). The assembled sequences were BLASTn-searched in National Centre for Biotechnology (NCBI) and compared with closely related sequences deposited. Phylogenetic analysis was done with other identified closely related species. Sequences were first aligned using MUSCLE in MEGA version 11 followed by manual trimming of poorly aligned ends, then pairwise distance analysis using Maximum Composite Likelihood model was used to determine the number of base substitutions between sequences (Tamura & Nei, 1993). Bayesian phylogenetic analysis (MrBayes 3.2.7) was employed for constructing the phylogenetic relationship inferred by obtained COI sequences and carried out using GTR+I+G model; analyses were run under 1×10^6 generations (4 runs) and Monte Carlo Markov chains were sampled every 100 generations and 20% of the converged runs were regarded as burnins (Huelsenbeck & Ronquist, 2001).

RESULTS

A total of six slug samples were collected from La Dicha, Malangas, Zamboanga, Sibugay. Combination of morpho-taxometric and molecular analysis showed that it is the Caribbean leatherleaf slug, *S. plebeia* and designated as *S. plebeia* isolate LDZS (MZ598573).

Morphological description

Sarasinula plebeia isolate LDZS has brownish dorsal notum without stripes (green), mantle is absent, body is flat and elongated when crawl, foot running from anterior to posterior end in a narrow line between the hyponotum. Colored arrows indicate the following characters: hyponotum (red), foot (orange), perinotum (yellow), entire notum (green) and ocular tentacle (blue) (Fig. 2).

Morpho-taxometric analysis

Sarasinula plebeia isolate LDZS has a mean body length of 48.18 mm, a circumference of 28.5 mm, and mean live weight of 1.28 g, respectively (Table 1).

Table 1. Morpho-taxometric analysis of *Sarasinula plebeia* isolate LDZS (n = 5). Data are expressed in mean ± standard deviation (range). All measurements are in mm except live weight (g).

Maximum body length	Circumference	Live weight (g)
48.18 ± 0.30 (47.93-48.52)	28.5 ± 0.56 (28-29.1)	1.28 ± 0.19 (1.08-1.46)

Molecular and phylogenetic analysis

The COI gene sequence generated from *S. plebeia* isolate LDZS (MZ598573, 629 bp) shared 100% similarities with *S. plebeia* from Brazil (Gomes *et al.,* 2013; JX532107 and Gomes *et al.,* unpublished; KM489378), Vietnam (KM489367) which was intercepted by USDA, and Dominica (KM489500). Furthermore, *S. plebeia* isolate LDZS (MZ598573) shared 99% similarity with samples from India (MH819385 and MH819384), Venezuela (KM489393), Tahiti (KM489369) intercepted by USDA, USA (KM489374), and Japan (Hirano *et al.,* 2022; LC636113, LC636114, LC636115, LC636116, LC636117, LC636118, and LC636119). Phylogenetic relationship of *S. plebeia* isolate LDZS (MZ598573) with other closely related species as inferred by the COI gene sequences using Bayesian analysis showed a formation of a distinct clade with *S. plebeia* from Brazil, Vietnam, Dominica, India, Venezuela, Tahiti, and USA supported with a strong posterior probability of 1.

Likewise, all the samples from Japan formed a sister clade with the other *S. plebeia*. Interestingly, *S. plebeia* samples from Batan island, Batanes in northern Philippines (JQ582277, JQ582278, JQ582279) previously identified using DNA barcoding showed no relationship with other *S. plebeia,* instead they were closely related to another veronicellid slug *Laevicaulis alte* (Férussac, 1822) (Hirano *et al.,* 2022; LC636101, LC636102, LC636103, and LC636104), forming a separate clade with posterior probability value of 1 (Fig. 3).

Moreover, *S. plebeia* isolate LDZS (MZ598573), has shown that there was 0.00 number of base substitutions between closely related *S. plebeia* isolates as shown in Table 2. In contrast, a considerable amount of base substitution (0.76) between *S. plebeia* isolate LDZS (MZ598573) and closely related sequences to the sequences from Batan island, Batanes (JQ582277, JQ582278, JQ582279). Furthermore, the Batan island slug samples (JQ582277, JQ582278, JQ582279) showed no difference (0.00) on the

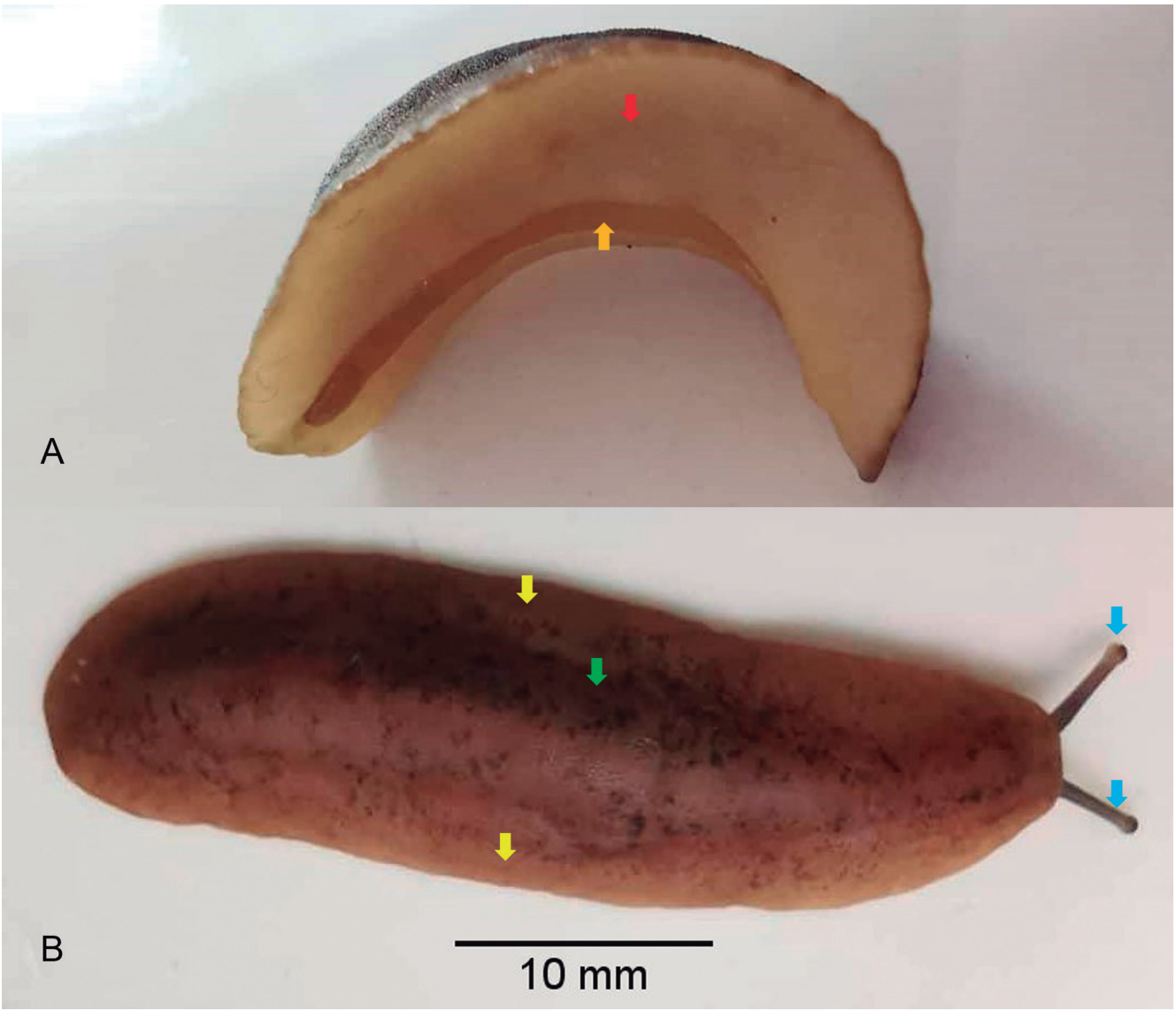

Figure 2. *Sarasinula plebeia* isolate LDZS morphological characters as indicated by arrows of the ventral region (A); hyponotum (red), narrow foot running from anterior to posterior end (orange); dorsal region (B) showing the notum (green), perinotum (yellow), and a pair of ocular tentacles (blue).

Table 2. Pairwise distance analysis using Maximum Composite Likelihood model, showing number of base substitutions (below diagonal) per site from between sequences using COI gene sequences of *Sarasinula plebeia* isolate LDZS (bold) and related taxa. Standard error estimates (above diagonal) are shown.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. ***Sarasinula plebeia* (MZ598573)**		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**19.31**	**19.31**	**19.31**	**18.56**	**18.56**	**18.56**	**18.56**
2. *Sarasinula plebeia* (JX532107)	0.00		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**19.40**	**19.40**	**19.40**	**18.56**	**18.56**	**18.56**	**18.56**
3. *Sarasinula plebeia* (KM489367)	0.00	0.00		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**19.06**	**19.06**	**19.06**	**18.56**	**18.56**	**18.56**	**18.56**
4. *Sarasinula plebeia* (KM489378)	0.00	0.00	0.00		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**19.16**	**19.16**	**19.16**	**18.56**	**18.56**	**18.56**	**18.56**
5. *Sarasinula* sp. (KM489500)	0.00	0.00	0.00	0.00		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**19.57**	**19.57**	**19.57**	**18.75**	**18.75**	**18.75**	**18.75**
6. *Sarasinula plebeia* (MH819385)	0.00	0.00	0.00	0.00	0.00		**0.00**	**0.00**	**0.00**	**0.00**	**19.18**	**19.18**	**19.18**	**18.60**	**18.60**	**18.60**	**18.60**
7. *Sarasinula plebeia* (MH819384)	0.00	0.00	0.00	0.00	0.00	0.00		**0.00**	**0.00**	**0.00**	**19.18**	**19.18**	**19.18**	**18.60**	**18.60**	**18.60**	**18.60**
8. *Sarasinula plebeia* (KM489393)	0.00	0.00	0.00	0.00	0.00	0.00	0.00		**0.00**	**0.00**	**19.07**	**19.07**	**19.07**	**18.57**	**18.57**	**18.57**	**18.57**
9. *Sarasinula plebeia* (KM489369)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01		**0.00**	**19.06**	**19.06**	**19.06**	**18.56**	**18.56**	**18.56**	**18.56**
10. *Sarasinula plebeia* (KM489374)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		**19.20**	**19.20**	**19.20**	**18.33**	**18.33**	**18.33**	**18.33**
11. *Sarasinula plebeia* (JQ582279)	0.76	0.76	0.75	0.75	0.79	0.75	0.75	0.75	0.75	0.76		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
12. *Sarasinula plebeia* (JQ582278)	0.76	0.76	0.75	0.75	0.79	0.75	0.75	0.75	0.75	0.76	0.00		**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
13. *Sarasinula plebeia* (JQ582277)	0.76	0.76	0.75	0.75	0.79	0.75	0.75	0.75	0.75	0.76	0.00	0.00		**0.00**	**0.00**	**0.00**	**0.00**
14. *Laevicaulis alte* (LC636104)	0.68	0.68	0.68	0.68	0.70	0.68	0.68	0.69	0.68	0.68	0.00	0.00	0.00		**0.00**	**0.00**	**0.00**
15. *Laevicaulis alte* (LC636103)	0.68	0.68	0.68	0.68	0.70	0.68	0.68	0.69	0.68	0.68	0.00	0.00	0.00	0.00		**0.00**	**0.00**
16. *Laevicaulis alte* (LC636102)	0.68	0.68	0.68	0.68	0.70	0.68	0.68	0.69	0.68	0.68	0.00	0.00	0.00	0.00	0.00		**0.00**
17. *Laevicaulis alte* (LC636101)	0.68	0.68	0.68	0.68	0.70	0.68	0.68	0.69	0.68	0.68	0.00	0.00	0.00	0.00	0.00	0.00	

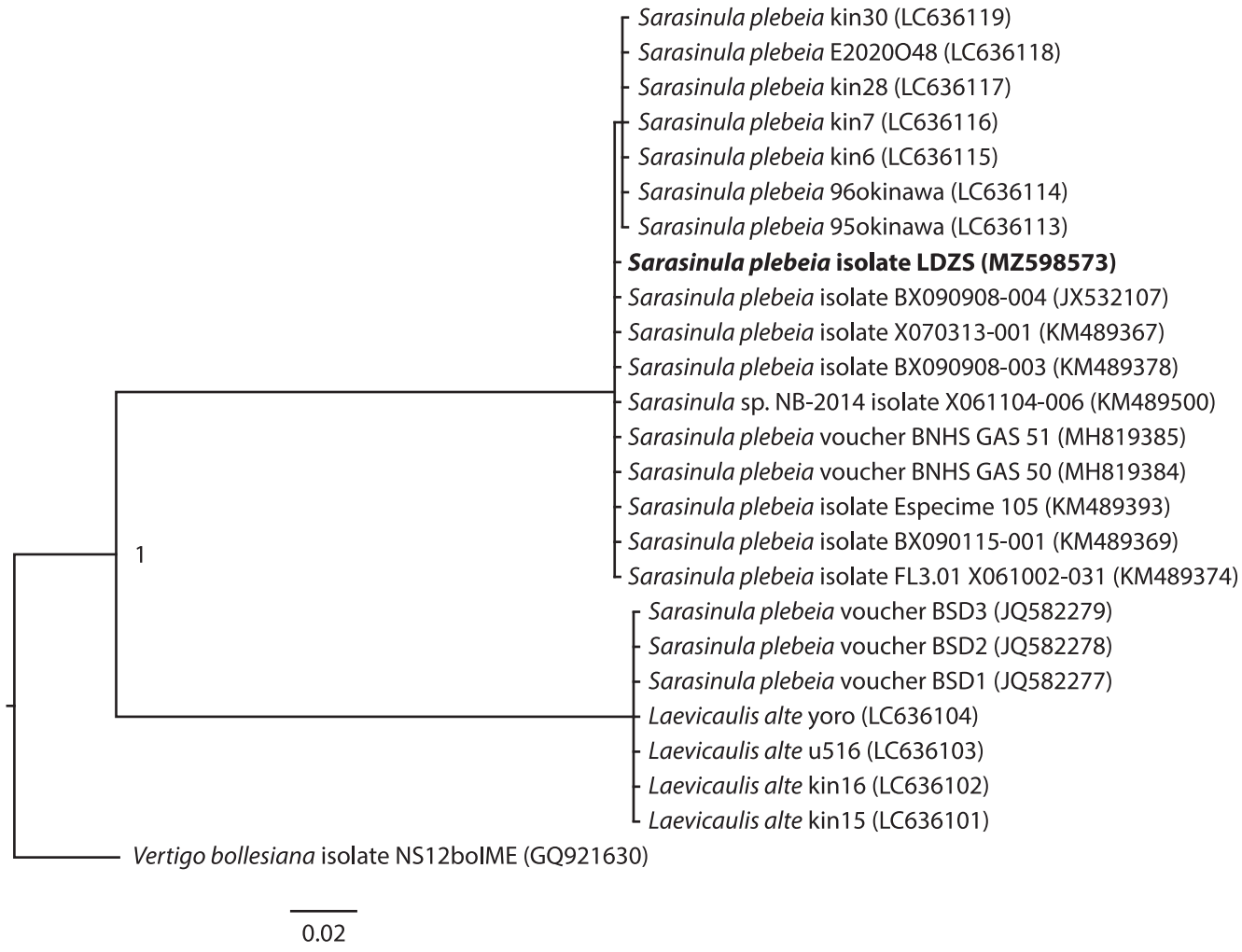

Figure 3. Phylogenetic relationship of *Sarasinula plebeia* isolate LDZS (bold) and related sequences inferred by the COI sequences through Bayesian analysis using GTR+I+G model showed a strong relation with posterior probability value of 1. Position of *S. plebeia* (JQ582279, JQ582278, JQ582277) also showed strong relation with *L. alte* (PP value of 1). Scale bar represents the estimated substitution per site.

number of base substitutions with *L. alte* (Hirano *et al.,* 2022; LC636101, LC636102, LC636103, and LC636104) from Japan. This supported that *S. plebeia* isolate LDZS and those from Batan island are not of the same species with the latter being closely related to *L. alte* having 0.00 number of base substitutions (Table 2).

DISCUSSION

Sarasinula plebeia is already recorded in the Philippines with introduction to a critical ecosystem such as in Masungi Georeserve in Tanay, Rizal (Valdez *et al.,* 2021), identified based on the morphological key characters for the species. Prior to this, *S. plebeia* was initially recorded from Batan island, Batanes, northern Philippines (Batomalaque *et al.,* 2013), identified using COI gene marker with sequences submitted to NCBI as *S. plebeia* voucher BSD1 (JQ582277), *S. plebeia* voucher BSD2 (JQ582278), and *S. plebeia* voucher BSD3 (JQ582279). However, we found a discrepancy in the designation of the said terrestrial slug from Batan island. The COI gene sequences of this first reported *S. plebeia* in 2013 (JQ582277, JQ582278, and JQ582279) were BLASTn-searched and found to be closely related to another veronicellid slug, *Laevicaulis alte* from Japan (Hirano *et al.,* 2022). Percent similarities between the misidentified *L. alte* as *S. plebeia* from Batan island with the *L. alte* isolates from Japan is 100%, showing 0.00 number of base substitutions using pairwise distance analysis and forming one clade in the phylogenetic tree. For this particular group, the use of only one tool to identify species is not sufficient and robust. Solely depending on either COI gene marker or morphological key characters may result in the misidentification of the species.

For instance, the group Systellommatophora: Veronicilloidea have degree 2 limacization (Simone, 2018), with almost indistinguishable morphological characters among the taxa. The high intraspecific morphological variation among veronicellid slugs (Kim *et al.,* 2016) renders identification of the species based on morphology alone difficult, requiring supplemental molecular analysis (Robinson & Fields, 2010). We therefore propose a re-description of the terrestrial slug species found in the Batan island (identification using COI gene marker only) and in the Masungi Georeserve (identification using morphology only), pending on the availability of voucher specimens. We also recommend dissecting for

the reproductive part of our slug, which is an important key character to increase resolution of species identification, and also including DNA barcoding of more *S. plebeia* samples in the area.

Aside from being a successful IAS, another risk that *S. plebeia* poses is on human health; *S. plebeia* is sometimes found in areas near human settlements (Garcia *et al.,* 2007). This terrestrial slug is known to be the vector for rat lungworm nematode species like *Angiostrongylus cantonensis* and *A. costaricensis* which are causative agents of meningoencephalitis and abdominal angiostrongyliasis (Laitano *et al.,* 2001). This association with a parasitic nematode coupled with its preference close to the human population, poses serious risk in health with successful infection sometimes leading to death if left untreated. Like other veronicellid slugs, *S. plebeia* is a hermaphroditic herbivore that feeds on a wide variety of plants. It devastates agricultural, horticultural fields as well in suburban areas worldwide due to its non-discriminate feeding habit as a polyphagous species (Hata *et al.,* 1997; Robinson & Hollingsworth, 2004). Generally, this species is found in moist areas such as below leaf litters and decaying matters to avoid desiccation, they are also known to burrow in soil with depths ranging from 25 cm to 1 m during dry season. They rapidly reproduce during the wet season with, at its peak, juvenile maturity reaching at ~ 2.5 months (Garcia *et al.,* 2007).

Here, we recovered *S. plebeia* partially buried underground, from an orchard of mango trees beside several plots of rice paddies; however, the severity of damage caused by this species to agricultural crops was not assessed and is an excellent research outlook. This terrestrial slug, along with other gastropods, are pests in agriculture causing reduced leaf surface area, stagnant growth, contaminating crops with their feces (Port & Port, 1986, South, 1992; Glen & Moens, 2002; Port & Ester, 2002) and consuming seeds, stems and meristems through voracious feeding habits, which results to reduction of growth and loss of plant vigor (South, 1992). Though they have relatively limited individual mobility over a large span of area and distances, trade in food, horticulture commodities, and people facilitated their spread (Howlet, 2012).

CONCLUSION

This study showed that one of the top IAS worldwide is established in the Philippines, wherein *S. plebeia* were identified using morphology, morpho-taxometric and molecular analysis with COI gene as marker. To our knowledge, this is the first recorded *S. plebeia* in southern Philippines.

AUTHORS' CONTRIBUTIONS: LBD: Software, Data curation, Formal analysis, Writing – original draft, NHNS: Supervision; LBD; NHNS: Conceptualization, Methodology; LBD; MABD: Visualization, Investigation, LBD; MABD; NHNS: Writing – review & editing. All authors have actively participated in the discussion of the results, they reviewed and approved the final version of the paper.

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