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Research Article

The Oriental Tiny Frog of the Genus *Microhyla* Tschudi, 1839 (Amphibia: Anura: Microhylidae) Revealed across Geographical Barriers of the Wallace Line

Rury Eprilurahman[1]*, Vestidhia Y. Atmaja[2], Misbahul Munir[3], Amir Hamidy[4], Tuty Arisuryanti[1], Rosichon Ubaidillah[4]

1) Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Sleman, Daerah Istimewa Yogyakarta 55281, Indonesia
2) Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Bengkulu, Jl. W. R. Supratman, Kandang Limun, Bengkulu 38371, Indonesia
3) Graduate School of Global Environmental Studies, Kyoto University, Yoshida-Honmachi, Sakyo-ku, Kyoto, 606-8501, Japan
4) Museum Zoologicum Bogoriense, Research Center for Biology, Indonesian Institute of Science, Gd. Widyasatwaloka, Jl. Raya Jakarta Bogor Km 46, Cibinong, West Java 16911, Indonesia
* Corresponding author, email: rurybiougm@ugm.ac.id

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ABSTRACT

The frog genus *Microhyla* was considered as the South, East, and Southeast Asian frog species. *Microhyla orientalis* was described in 2013, distributed in Java and Bali, Indonesia. Thenceforth, it was known as the easternmost distribution of this genus within the oriental region, but recently this species was recorded from the Timor Island and Sulawesi on the Wallace regions. We applied molecular analysis to evaluate the taxonomic status and the origin of the Wallacean population. Phylogenetic analysis using the partial 16S mitochondrial gene demonstrated that the Java, Timor and Sulawesi populations were not significantly different from the Bali population. This Wallacean population of *M. orientalis* was originated from Java and possibly it is accidentally distributed by humans through the expansion of agricultural activity.

Keywords: introduced species, *microhyla*, molecular markers, species confirmation, identification

INTRODUCTION

Microhyla Tschudi, 1838, a genus of tiny narrow-mouthed frogs that consisted of 46 species which were widely spread from Japan (Ryukyu Islands), southern China across India to Sri Lanka, and through Southeast Asia region into the Indonesian archipelago (Matsui et al. 2005; Kurabayashi et al. 2011; Frost 2021; Garg et al. 2019; Gorin et al. 2020; Poyarkov et al. 2020). *Microhyla* were distributed along to Indonesia through the Sunda shelf region. It was spread from Sumatera, Kalimantan, Java, Bali, and its adjacent islands, Timor and Sulawesi (van Kampen 1923; Iskandar 1998; Inger & Frogner 1979; Inger & Stuebing 2005; McKay 2006). Previously, *M. orientalis* from Bali was known as the easternmost distribution of the genus *Microhyla* within the oriental region (Matsui et al. 2013). Recently, this frog was recorded from Java (Yudha et al. 2019) and across the Wallace line in Timor Island (Reilly et

al. 2020) and Sulawesi (identified as *Microhyla* sp. in Wiantoro et al. 2019) based on morphological characters.

The disjunct distribution of *Microhyla orientalis* from the western and eastern parts of Wallace line raised a question about the origin of this species. By using a molecular technique, we evaluated the species identification of the Sulawesi and Timor populations then reconstructed its phylogenetic relationships. The estimation on the phylogenetic relationship using molecular technique has succeeded to prove the origin of certain vertebrates (e.g. Moritz et al. 1993; Suzuki et al. 2011; Suzuki et al. 2014; Johnson et al. 2011, Hadi et al. 2020) as well as in the frogs (Kuraishi et al. 2009; Wogan et al. 2016; Reilly et al. 2017; Vences et al. 2017).

Here, we provide the distribution history encompassing the entire populations of *M. orientalis* in Indonesia. The presumption of *M. orientalis* genetic variation in the Wallace region, which is possibly associated with natural or human-mediated distribution, was appraised.

MATERIALS AND METHODS

Materials

We examined specimens of *Microhyla orientalis* stored in Museum Zoologicum Bogoriense (MZB), Research Center for Biology, Indonesian Institute of Sciences. A number of 25 partial 16S mtDNA sequences of the *M. orientalis* were analysed (Table 1). We compared several populations *viz.* Java, Bali, Timor (specimen from Reilly et al. (2020)), and Sulawesi Island (specimens from Wiantoro et al. (2019) previously identified as *Microhyla* sp.) (Figure 1 and Table 1).

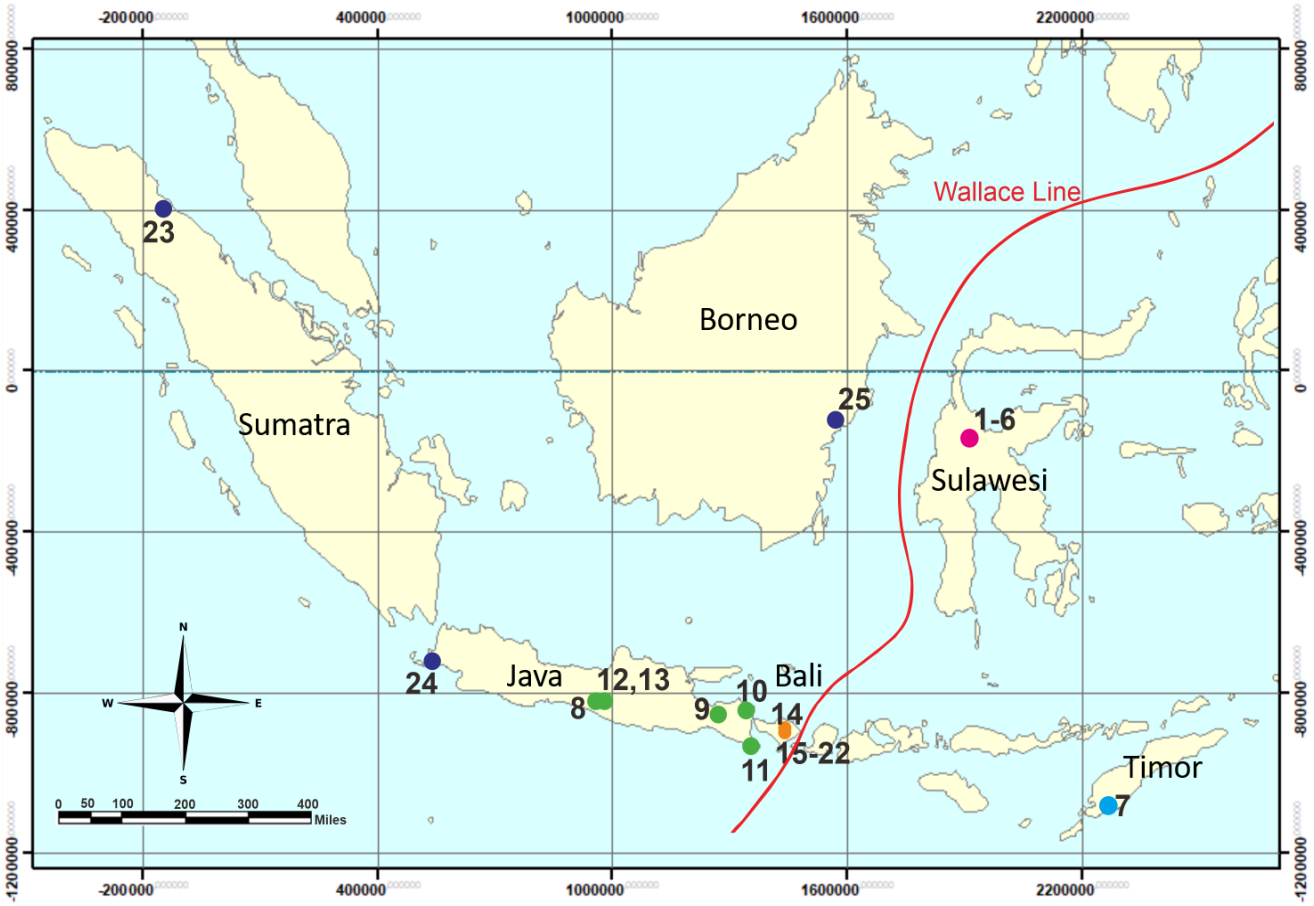

Figure 1. Indonesian Archipelago map of the localities of *Microhyla orientalis* specimens in Sulawesi (**purple**), Timor Islands (**cyan**), Java (**green**), and type locality, Bali (**orange**). Outgroups are shown by the **dark blue** circle. The specimen numbers represent those listed in Table 1. (Map modified from ArcMap 10.7.1, February 5th, 2021).

Table 1. Samples of Indonesian *Microhyla orientalis* and outgroups generated for 16S mtDNA analysis in this work with detailed information on Museum number, locality, GenBank accession numbers, and sources.

No	Species	Museum Number	Locality	Accession Number	Sources
1	*Microhyla orientalis*	MZB Amph 26972	Sulawesi, Sigi, Saluki	MW683205	This study
2	*Microhyla orientalis*	MZB Amph 26973	Sulawesi, Sigi, Saluki	MW683206	This study
3	*Microhyla orientalis*	MZB Amph 26974	Sulawesi, Sigi, Saluki	MW683207	This study
4	*Microhyla orientalis*	MZB Amph 26975	Sulawesi, Sigi, Saluki	MW683208	This study
5	*Microhyla orientalis*	MZB Amph 26976	Sulawesi, Sigi, Saluki	MW683209	This study
6	*Microhyla orientalis*	MZB Amph 26977	Sulawesi, Sigi, Saluki	MW683210	This study
7	*Microhyla orientalis*	MZB Amph 21091	Timor, Kupang	MW683211	This study
8	*Microhyla orientalis*	MZB Amph 28435	Java, Kulon Progo	MW683212	This study
9	*Microhyla orientalis*	MZB Amph 26914	Java, East Java Mt. Argopuro	MW683213	This study
10	*Microhyla orientalis*	MZB Amph 12989	Java, East Java, Baluran NP	MW683214	This study
11	*Microhyla orientalis*	MZB Amph 12986	Java, East Java, Alas Purwo NP	MW683215	This study
12	*Microhyla orientalis*	ZMMUA 5067-1	Java, Yogyakarta	MN534556	Gorin et al. 2020
13	*Microhyla orientalis*	ZMMUA 5067-2	Java, Yogyakarta	MN534557	Gorin et al. 2020
14	*Microhyla orientalis*	MZB Amph 16259	Bali, Batu Karu	AB634679	Matsui et al. 2011
15	*Microhyla orientalis*	KUHE 55048	Bali, Wongaya Gede	AB781465	Matsui et al. 2013
16	*Microhyla orientalis*	KUHE 55049	Bali, Wongaya Gede	AB781466	Matsui et al. 2013
17	*Microhyla orientalis*	KUHE 55050	Bali, Wongaya Gede	AB781467	Matsui et al. 2013
18	*Microhyla orientalis*	KUHE 55072	Bali, Wongaya Gede	AB781468	Matsui et al. 2013
19	*Microhyla orientalis*	KUHE 55073	Bali, Wongaya Gede	AB781469	Matsui et al. 2013
20	*Microhyla orientalis*	KUHE 55074	Bali, Wongaya Gede	AB781470	Matsui et al. 2013
21	*Microhyla orientalis*	KUHE 55076	Bali, Wongaya Gede	AB781471	Matsui et al. 2013
22	*Microhyla orientalis*	KUHE UL-M11	Bali, Wongaya Gede	AB781472	Matsui et al. 2013
	Outgroup				
23	*Micryletta inornata*	MZB Amph 23947	Sumatra, Medan	LC208136	Alhadi et al. 2019
24	*Microhyla achatina*	ZMMUA 5054-2	Java, Banten, Ujung Kulon	MN534565	Gorin et al. 2020
25	*Microhyla malang*	MZB Amph 16364	Kalimantan, Balikpapan	AB634677	Matsui et al. 2011

Methods

Fragments of the 16S mtDNA (ca. 462) were employing a method as delineated by Matsui et al. (2011, 2013). DNA sequences obtained in this study were checked and edited using the ChromasPro software (Technelysium Pty Ltd., Tewantin, Queensland, Australia). The newly *M. orientalis* sequences were deposited in GenBank with accession numbers MW683205—MW683215 together with those from GenBank (Table 1) aligned applying Clustal W in MEGA X (Kumar et al. 2018). Phylogenetic trees were reconstructed using Neighbor-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI) analyses. The NJ tree was administered in MEGA X using p-distances with 1000 bootstrap replicates. The Akaike Information Criterion (AIC) performed using Kakusan 4 were used to identify the models of rate evolution for ML and BI analyses (Tanabe 2011). ML analysis was performed using Treefinder ver. March 2011 (Jobb et al. 2004) with general time-reversible (GTR) and a gamma shape parameter (G). The Bayesian analysis was performed in MrBayes 3.2.7 (Ronquist et al. 2012)

with general time-reversible (GTR) and a gamma shape parameter (G). Analysis of the Markov Chain Monte Carlo (MCMC) for the dataset was run for 5 million generations and every 100 cycles, trees were sampled. The convergence of the runs was determined by a split frequency of < 0.01 standard deviations and potential scale reduction factors of ~1.0. We discarded the first 20% of the sampled trees as burn-in and generate a majority-rule consensus tree using the remaining samples. Strong supports of tree nodes were considered when possessing bootstrap values of 70% or more for ML and NJ analyses (Hillis & Bull 1993). The genetic distances of the 16S mtDNA gene were computed using uncorrected p-distances with MEGA X. We regarded tree nodes with BI posterior probabilities values over 0.95 as strongly supported; values between 0.90 and 0.95 were considered as moderately supported; while the lower values were regarded to have no nodal support (Huelsenbeck & Hillis 1993).

Genetic Diversity Analysis

Twenty-two sequences of 462 bp 16S mtDNA gene from three sampling locations: Java, Timor (Reilly et al. 2020), and Sulawesi (Wiantoro et al. 2019) with additional sequences of the type specimen of *M. orientalis* from Bali were included in the generating process of 14 haplotype variations. Genetic diversity parameters including the haplotypes diversity (Hd) and nucleotide diversity (π) were computed using the DNASp v6.12.03 (Rozas et al. 2017) and Arlequin v.3.5.2.2 software (Excoffier & Lischer 2010).

Analysis on the structure of populations

Molecular variance (AMOVA) and fixation index (F_{ST}) (Wright 1951) analysis was performed for the group within intraspecific populations of *M. orientalis* using the Arlequin v.3.5 programs (set up, 1000 permutations; significance level threshold, $\alpha = 0.05$). The analyses allowed the approximation of the overall extent of the genetic variation and differentiation level within *M. orientalis* population. Furthermore, population differentiation and its significance between sampling locations were also calculated using pair-wise estimates (Weir & Cockerham 1984; Excoffier et al. 1992; Weir 1996).

Genetic haplotypes analysis

The median-joining method was generated to build the haplotype network using Network v10.2.0.0 program (Bandelt et al. 1999). Haplotypes distribution for each location was presented in the informative map (Figure 1) to show the recent genetic connectivity and distributions among populations.

RESULTS AND DISCUSSION

Morphologically, Timor and Sulawesi populations of *M. orientalis* showed high similarity in appearance with Java and Bali populations. The diagnostic character such as bright orange to pinkish brown on the upper forearm, a vertebral line at the dorsal of the body, and the web formulae of toes confirmed the species identification as *M. orientalis* (Figure 2).

We obtained 462 bp 16S mtDNA fragments of 25 samples including outgroups, 371 nucleotide sites were conserved, 87 site variables, and 28 sites parsimoniously informative. A topology with the highest log likelihood -1223.5300, gamma shape parameter 0.1407, and frequencies of the nucleotides: A=0.333, T=0.251, G=0.197, and C=0.217 resulted from the ML analysis. BI analysis with nucleotide frequencies: A=0.335, T=0.248, G=0.202, C=0.215, and a gamma shape parameter 0.3701. All analyses produced similar topologies, which differed only in several low-supported nodes. Thus, only the ML tree is shown (Figure 3).

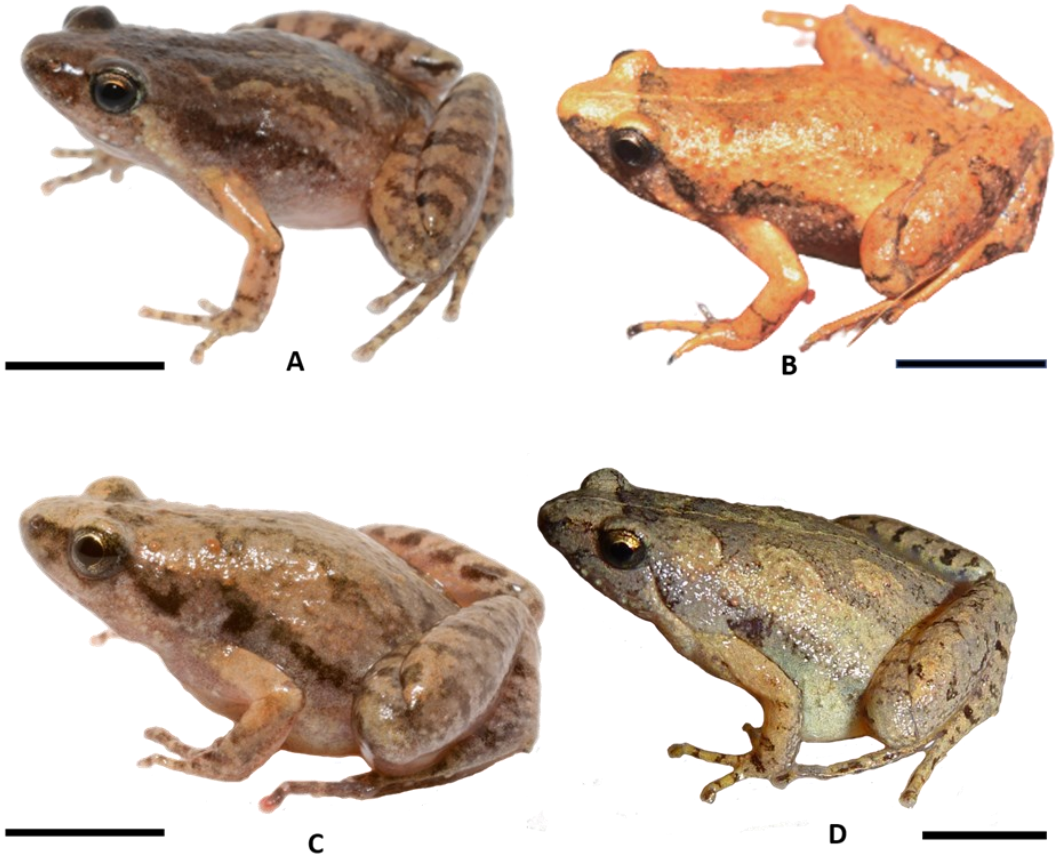

Figure 2. Color pattern variation on *Microhyla orientalis*. **A.** Bali (reproduced from Matsui et al. 2013), **B.** Java (Yogyakarta, Photographed by Misbahul Munir), **C.** Sulawesi (Central Sulawesi, Photographed by Farits Alhadi), **D.** Timor (reproduced from Reilly et al. 2020). Scale bar = 5 mm.

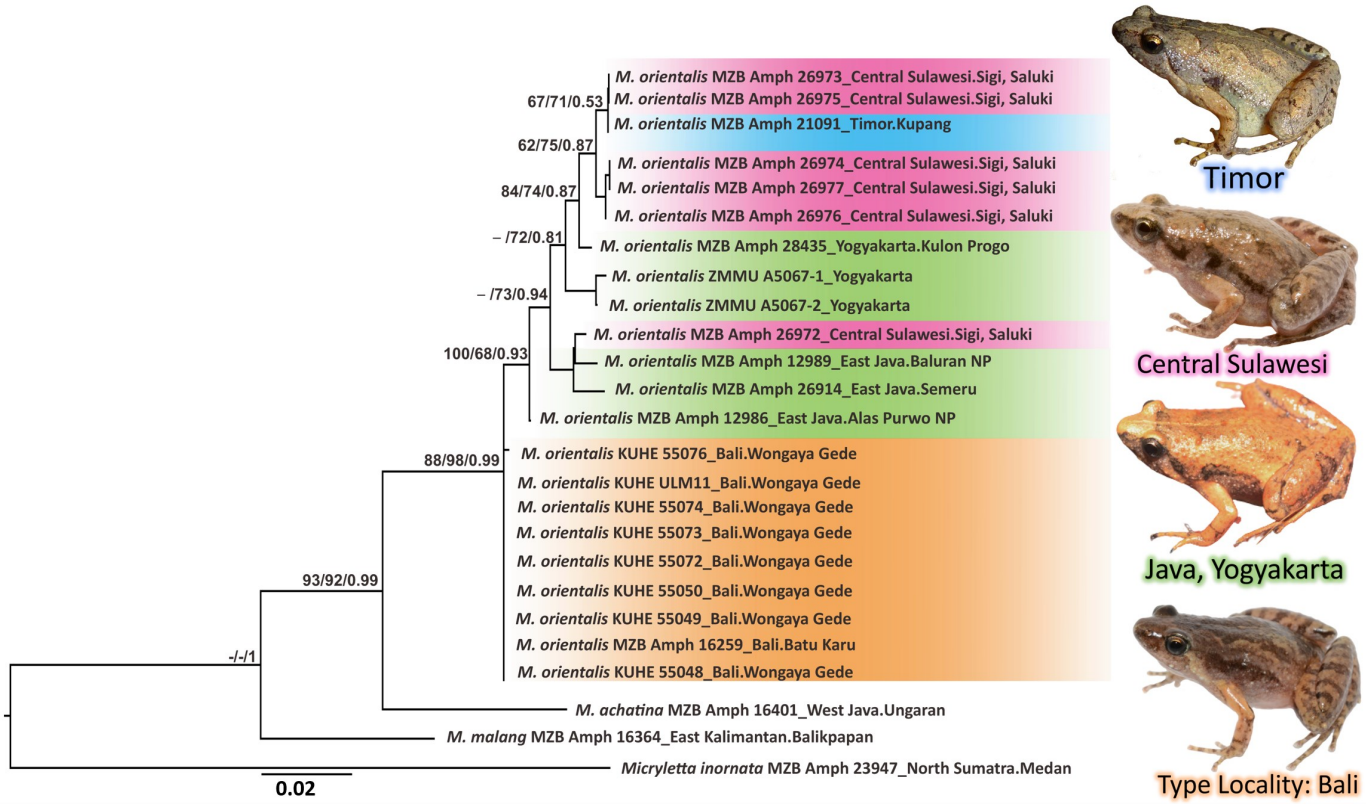

Figure 3. Maximum likelihood phylogeny tree of *Microhyla orientalis* samples and outgroups based on 462 bp of the 16S mtDNA gene. The Numbers shown above the branches represent bootstrap support for NJ/ML/and BI.

The population of *Microhyla* from Sulawesi and Timor examined here formed a well-supported monophyletic with *M. orientalis* from Java and Bali (NJ: 93; ML: 92; BPP: 99). Population from Timor and Sulawesi are closely similar to the population from Java with high significant supports (NJ: 88; ML: 98; BPP: 99). The tree showed the consistent clade of *M. orientalis* as the sister taxa of *M. malang* and *M. achatina*, although their relationships were not fully resolved.

Genetic Diversity

Sequences (n= 22) from four localities (Java, Timor, Bali, and Sulawesi) resulting in 462 bp 16S mtDNA was gained. All sequences, including samples from Bali as type locality of *M. orientalis* resulted in 14 haplotype variations with 17 polymorphic sites (Table 2).

The contrast of the genetic diversity among person tests of *M. orientalis* uncovered the nearness of variety (Table 3). Among the samples, it was obtained that the haplotype diversity (Hd) ranged from 0.222 to 1.0 and the nucleotide diversity (π) ranged from 0.000 to 0.015. Samples from Java were observed as the highest genetic diversity (Hd = 1.0; π = 0.015), the second is Timor, which shows a single haplotype and nucleotide diversity (Hd = 1.0; π = 0.000). The *M. orientalis* population from Sulawesi revealed a fairly high genetic diversity (Hd = 0.933; π = 0.008) and the lowest was from Bali (Hd = 0.222 and π = 0.011).

Table 2. Seventeen polymorphic sites of 14 haplotypes derived from 22 samples of the 16S mitochondrial DNA of *M. orientalis* from four localities.

Nucleotide position*	10	11	21	76	114	176	180	187	200	238	240	252	264	270	285	296	339
Haplotype 1–Bali	A	C	T	C	G	C	T	G	T	T	A	C	C	A	T	A	A
Haplotype 2–Bali	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
Haplotype 3–Sulawesi	G	T	.	.	A	.	.	A	.	.	.	T	.	.	.	.	G
Haplotype 4–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 5–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 6–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 7–Sulawesi	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 8–Java	G	T	.	T	A	T	.	A	.	.	G	T	.	.	.	.	.
Haplotype 9–Java	G	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
Haplotype 10–Java	G	T	G	.	A	.	.	A	.	.	.	T	.	.	.	G	.
Haplotype 11–Timor	G	T	.	.	A	.	C	A	.	C	.	T	.	G	.	.	.
Haplotype 12–Java	.	.	.	T	A	.	.	A	C	C	.	T	.	.	.	.	.
Haplotype 13–Java	.	.	.	.	A	.	.	A	C	C	.	T	.	.	.	.	.
Haplotype 14–Java	G	T	.	.	A	.	C	A	.	C	.	T	.	.	C	.	.

Notes:
*Nucleobase at each position is for Haplotype 1-Bali while those differences are written for all other haplotypes. Nucleobase identical to Haplotype 1-Bali are shown with dots (.).

Table 3. Genetic diversity samples of *Microhyla orientalis* derived from sample size (n), haplotype number (Hn), Haplotype diversity (Hd), and nucleotide diversity (π).

Population	bp	Number of samples (n)	Haplotype number (Hn)	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
Bali	457	9	2	0.222 ± 0.166	0.011 ± 0.001
Java	458	6	6	1.000 ± 0.096	0.015 ± 0.009
Timor	457	1	1	1.000 ± 0.000	0.000 ± 0.000
Sulawesi	458	6	5	0.933 ± 0.122	0.008 ± 0.005
Overall	**462**	**22**	**14**	**0.814 ± 0.064**	**0.011 ± 0.001**

Populations of *Microhyla orientalis*

The fixation index (F_{ST}) and *P*-values analysis between and within *M. orientalis* populations (Java, Bali, Timor, and Sulawesi) are presented in Table 4. The comprehensive F_{ST} value within four populations were highly significant (F_{ST} = 0.605; $P < 0.001$). This was predicted due to multiple subdivisions.

Table 4. The percentage of variation (%), F_{ST} value, and significance level (*P*-value) using the molecular variance (AMOVA) analysis for *M. orientalis* specimens.

Source of Variation	df (degrees of freedom)	Percentage of Variation (%)	F_{ST} value	*P*-value
Interpopulation	3	60.53	0.605	0.000
Intrapopulation	18	39.47		
Total	21			

Values of the pair-wise F_{ST} between the populations of *M. orientalis* are provided in Table 5. Significant differentiation between Bali and the other three populations (Java, Sulawesi, and Timor) was detected through the overall pair-wise analysis using the distance method.

Table 5. Pairwise F_{ST} values (**black**) and *P*-values (**blue**) between *M. orientalis* populations from Java, Bali, Timor, and Sulawesi.

Sample sites	Bali	Sulawesi	Java	Timor
Bali	-	0.000	0.009	0.990
Sulawesi	0.848	-	0.018	0.990
Java	0.573	0.231	-	0.990
Timor	0.972	-0.275	-0.211	-

Genetic Connectivity of *M. orientalis*

The haplotype network analysis recognized a complex ancestor or origin of Wallacean *M. orientalis* population (Timor and Sulawesi) (Fig 4). Two major groups of haplotypes are named clade Bali and Java-Timor-Sulawesi. Bali as the type locality of *M. orientalis* shows two different haplotypes H1 and H2. Timor (one haplotype) and Sulawesi (five haplotypes) have mixed connectivity with each other, several probabilities of genetic connection of various ancestor points evolved from the Java population (six haplotypes).

Two confined haplotype groups between Bali and Java-Timor-Sulawesi *M. orientalis* were differentiated by 6 discrete nucleotide bases due to the mutations. Concerning the significant value of F_{ST} among populations (F_{ST} = 0.605; $P < 0.001$) as well as haplotype network (Figure 4) and phylogenetic tree (Figure 3).

Discussion

Here, we confirmed the species identification of Yudha et al. (2019), Wiantoro et al. (2019) and Reilly et al. (2020) as *Microhyla orientalis* genetically . Genetic variation has not been significantly detected among the sundaic and wallacean population of *M. orientalis*. This assumed that the Wallacean *M. orientalis* were genetically mixed, feasibly limited in number, and accidentally introduced, as stated earlier by Reilly et al. (2020). It is predicted that oceanic dispersal to adjacent islands must also have occurred in association with human activities, such as the condition on some frogs species in Japan (Ota et al. 2004; Kuraishi et al. 2009).

This introduction of *Microhyla orientalis* to Timor and Sulawesi was

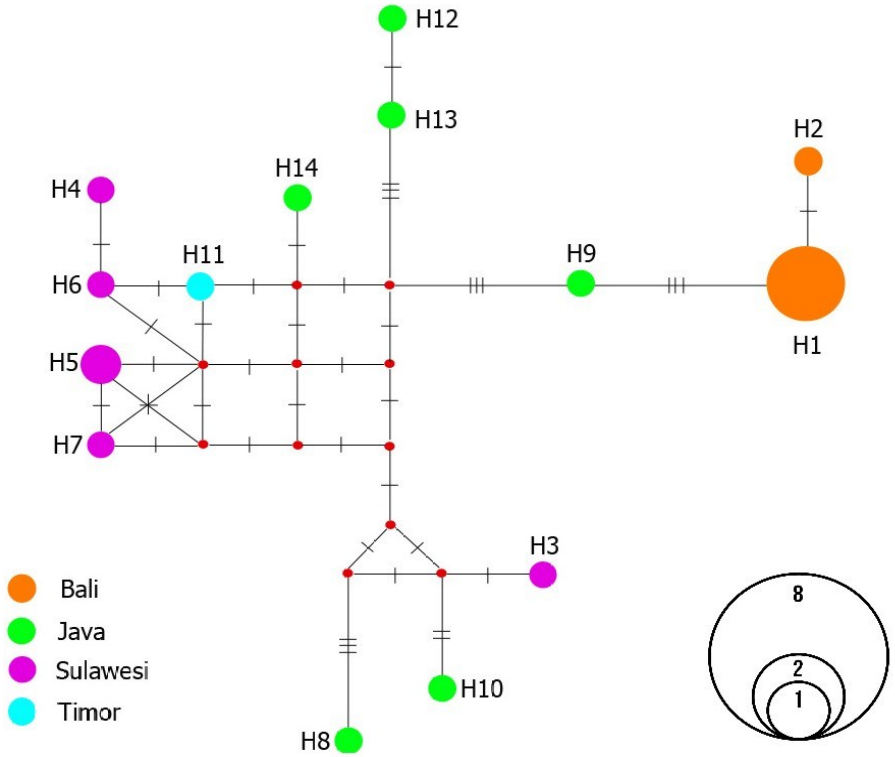

Figure 4. Results of median-joining methods for *M. orientalis* population (n = 22) haplotype network from Indonesia: Java, Sulawesi, Timor, and type locality, Bali.

similar to the tracing result of the origin of *Duttaphrynus melanostictus* in Madagascar (Wogan et al. 2016; Vences et al. 2017) and Wallacean region (Reilly et al. 2017). Madagascar *D. melanostictus* was found to be a single origin from the Southeast Asian lineage. The Southeast Asia mainland population shows high haplotypes diversity than the island's population, 51 haplotypes in the mainland, two and four in the island's population (Wogan et al. 2016). As well as, Wallacean *D. melanostictus* were shared single haplotype originated from Sundaic region of Sumatra and Java (Reilly et al. 2017).

Microhyla orientalis from Java showed higher haplotypes diversity than Bali, Timor and Sulawesi. The haplotype network of *M. orientalis* showed two major haplotype groups, Bali and Java-Timor-Sulawesi, with low genetic diversity. Since the Wallacean population of *M. orientalis* is closest to Java than Bali, we assumed that this Wallacean population is originated from Java similar to Reilly et al. (2017) finding.

M. orientalis were suspected as an accidentally introduced species by the colonization event back to The Dutch East Indies era in 1905 (Kementerian Desa, Pengembangan Daerah Tertinggal dan Transmigrasi RI 2015a). One of the major sources of people who move to other areas of Indonesia is Java. It was mentioned that Timor and Central Sulawesi were the destinations of the colonization called "*transmigrasi*" program of the Indonesian government (Kementerian Desa, Pengembangan Daerah Tertinggal dan Transmigrasi RI 2015a; Kementerian Desa, Pengembangan Daerah Tertinggal dan Transmigrasi RI 2015b). Colonization is usually also involved with agriculture and fisheries for living. Possibly , people from Java also brought their cultural agriculture and fisheries to fulfill their basic needs in the new land. It is likely that the species was recently introduced by human activity, and genetic analysis has proven the human interfere of *M. orientalis* distribution. Moreover, the paddy field is the suitable habitat of *M. orientalis* (Matsui et al. 2013), the expansion of agricultural activity in the Wallace region might play an important role in distributing this species out from Java.

CONCLUSIONS

Both molecular analysis and morphological identification on the population of *M. orientalis* from Java, Timor and Sulawesi show similarities to Bali population. Two major groups were detected molecularly within the *M. orientalis* population, Bali and Java-Sulawesi-Timor. The Javan population of *M. orientalis* assumed accidentally introduced to the Wallacean region.

AUTHORS CONTRIBUTION

R.E. and V.Y.A. contributed equally to this work, formulated and designed the assessment, conducted the research, analysed the data, generated figures and tables, authored and reviewed drafts and approved the final draft; M.M. contributed to data collection, scrutinized the data, authored and reviewed drafts of this paper and approved the final draft; A.H. supervised all the process of this work, designed the experiments, recorded and scrutinized the data, authored and reviewed drafts of this paper and approved the final draft; T.A. and R.U. contributed to examining the data, authored and reviewed drafts of this paper and approved the final draft.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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