Mitochondrial DNA suggests the existence of two distinct species in Moluccas and New Guinea within *Nyctimystes infrafrenatus* (Günther, 1867)

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Abstract. Sulaeman TN, Hamidy A, Farajallah A, Fouquet A, Riyanto A, Arida E, Mulyadi, Trilaksan W, Munir M. 2021. Mitochondrial DNA suggests the existence of two distinct species in Moluccas and New Guinea within *Nyctimystes infrafrenatus* (Günther, 1867). Biodiversitas 22: 3287-3297. *Nyctimystes infrafrenatus* is a widespread frog species distributed across northern Australia, New Guinea, Bismarck archipelago and in the eastern part of Indonesia archipelago. The species thus encompasses two biogeographic regions, Papua and Wallacea. We evaluated the phylogenetic relationships among taxa from Papua and Moluccas based on two mitochondrial loci (16S rRNA and Cytochrome Oxidase Subunit I). Two major subclades are recovered within *Nyctimystes infrafrenatus* with subclade A being represented by populations from New Guinea and northern Australia and subclade B by Moluccan populations (Halmahera and Tidore). Genetic distances (2.3-4.2% in 16S rRNA) between these subclades suggest they could belong to distinct species. Since New Guinea populations correspond to the nominal species and that *Nyctimystes tennuigranulatus* (Boettger 1895), currently considered as a junior synonym of *Nyctimystes infrafrenatus*, is available for the northern Moluccan populations, we proposed to remove this taxon from synonymy. However, samples used in this study come from northern Moluccas, further studies including samples from Ambon (where another synonym was described i.e. *Calamites dolichopsis*) is needed to determine the taxonomic status of the southern Moluccan population whether they are conspecific with northern Moluccan population or not.

Keywords: 16S rRNA, COI, Moluccas, *Litoria*, Papua

INTRODUCTION

*Nyctimystes infrafrenatus* (white-lipped tree frog) was originally described by Günther (1867) as *Hyla infrafrenata* (holotype BMNH 1947.2.24.11) based on a specimen collected from Cape York Peninsula, Australia. *Nyctimystes infrafrenata* was for long time placed in the genus *Litoria* Tchudi, 1838 (Tyler 1968; Tyler 1971; Tyler and Davies 1978). Based on molecular analysis (Wiens et al. 2010; Pyron and Wiens 2011; Duellman et al. 2016) the species has recently been transferred to *Nyctimystes* Stejneger, 1916 (Duellman et al. 2016). However, many other authors do not accept this revised generic arrangement for the Pelodyridae (e.g. Kraus 2018). Nevertheless, the name *Nyctimystes infrafrenatus* was later accepted by Dubois and Fretey (2016) and Frost (2020).

Many taxa have been synonymised with *N. infrafrenatus*. Two months after Günther’s description of *Hyla infrafrenata*, Cope (1867) described *Calamites dolichopsis* from Ambon, Indonesia. In 1878, two species that were similar to *H. infrafrenata* were described as *Pelodyras militarius* (Ramsay 1878) from New Ireland and *Litoria guttata* (Macleay 1878) from Katow, Papua New Guinea (PNG) which later considered as the synonym of *H. infrafrenata* (Fry 1913; Van Kampen 1923). Boulenger (1882) considered *P. militarius* (Ramsay 1878) as a junior name of *C. dolichopsis* (Cope 1867) and placed it in the genus *Hyla* as *Hyla dolichopsis*. Later, two variants of *H. dolichopsis* were described. *Hyla dolichopsis var. tennuigranulata* from Halmahera and Ternate, Moluccas (Maluku) (Boettger 1895) and *Hyla dolichopsis var. pollicaris* from Bismarck Archipelago (Werner 1898). *Hyla dolichopsis* and *H. dolichopsis var. tennuigranulata* were later considered as synonyms of *Hyla infrafrenata* (Boulenger 1912; Van Kampen 1919). In 1912, Boulenger considered *H. dolichopsis var. pollicaris* similar to which was firstly described as *Pelodyras militarius* (synonym of *H. dolichopsis*). He proposed the epithet name “militarius” which was proposed by Ramsay (1878) to be used in this New Ireland-type *Hyla* as *Hyla militaria* which was later considered as synonym of *H. infrafrenata*. In the same
publication, Boulenger also described a new species named *Hyla spengeli* from Dinawa, PNG which later synonymized to *N. infralateralis* by Loveridge (1948).

Two subspecies were recognized as *Nyctimystes infralateralis infralateralis* and *Nyctimystes infralateralis militaris* (Tyler 1971). *N. infralateralis militaris* occurs in New Britain and New Ireland islands in the Bismarck Archipelago (Loveridge 1948; Tyler 1971; Iskandar and Colijn 2000; Menzies 2006). *N. infralateralis militaris* can be diagnosed by rudimentary pollux on the male (Tyler 1971; Ramsay 1878; Werner 1989). *Nyctimystes infralateralis infralateralis* is widely distributed from the Moluccas (including Kei, Ambon, Obi, Buru, Seram, Morotai and Halmahera), Talaud Island, Timor Island, the New Guinea (including Aru, Waigeo, Misol, and Tanimbar) and north-eastern Australia (Boulenger 1912; Brongersma 1948; Tyler 1978; Iskandar and Colijn 2000; Menzies 2006; Setiadi and Hamidy 2006; Hamidy and Mulyadi 2007; Koch et al. 2007; Iskandar et al. 2017; Karin et al. 2018; Nugroho et al. 2019; Frost 2020).

Interestingly, morphometric analysis showed slight variation in the size of the female between the Moluccas and the New Guinea populations of *N. infralateralis infralateralis* (Prafialdi et al. 2016). These two groups of populations are isolated by minimum 47 km of saltwater (Gebe Is. to Halmahera), a very efficient barrier for amphibians (Fonte et al. 2019). Moreover, past land bridges are ancient and these land masses (especially Halmahera) have not been in contact recently (Hall and Nichols 1990; Setiadi et al. 2010). Therefore, we assume that the previously observed morphological differences could be resulting from ancient events (Van der Kaars 1991; Hantoro et al. 1995; Voris 2000; Yokoyama et al. 2001) and possibly indicative of specific differentiation.

Here we examined phylogenetic relationships between the Moluccan and the Papuan populations of *N. infralateralis infralateralis* using 16S rRNA and Cytochrome Oxidase Subunit 1 (COI) genes which both are the most universally used genes for amphibians barcoding and phylogenetic analysis (Vences et al. 2005).

**MATERIALS AND METHODS**

**Study area**

Our study area was focused on Indonesian, including part of the Moluccas (Halmahera and Tidore) and Papua (including Raja Ampat). Additional data for both ingroups and outgroups were added from Papua New Guinea and Australia (Figure 1).

**Materials**

We generated partial sequence data of the mitochondrial DNA genes 16S rRNA (n = 25) and Cytochrome Oxidase Subunit 1 (COI) (n = 41) of *N. infralateralis*, representing populations from Moluccas, Papua and Australia. We added outgroups from the same genus e.g. *Litoria paludicola* [now *Nyctimystes paludicola* (Kraus 2018)], *Litoria purpureolata* [now *Nyctimystes purpureolatus* (Oliver et al. 2007)], from the different genera in the same family e.g. *Litoria rheocolus* [now *Ranoidea rheocola* (Liem 1974)], *Hyla nannotis* [now *Ranoidea nannotis* (Andersson 1916)], *Rana caerulea* [now *Ranoidea caerulea* (White 1790)], *Hyla gracilenta* [now *Ranoidea gracilenta* (Peters 1869)], *Litoria bellii* [now *Ranoidea bellii* (McDonald et al. 2016)], *Litoria auae* [now *Ranoidea auae* (Menzies and Tyler 2004)], *Hyla affakiana* [now *Litoria affakiana* (Peters and Doria 1878)], *Hyla angiana* [now *Litoria angiana* (Boulenger 1915)] and *Hyla amboinensis* [now *Litoria amboinensis* (Horst 1883)]. We also included outgroup from different family e.g. *Papura rana volkerjana* (Günter 2003), *Asterophrys turpica* (Schlegel 1837), *Callulops robustus* (Boulenger 1898), *Limnodynastes terraequorum* Fry, 1915 (Dubois and Fretey 2016; Duellman et al. 2016; Frost 2020) (Table 1). Specimens were collected from northern Moluccas, Waigeo, West Papua and Papua Province. Voucher specimens and/or tissues are stored in Museum Zoologicum Bogoriense (MZB), Research Center for Biology-Indonesian Institute of Sciences.

**Procedures**

**Preparation of DNA, PCR and DNA sequencing**

Genomic DNA was extracted using a Phenol-Chloroform extraction procedure (Hillis et al. 1996). We homogenised tissues in 0.6 ml STE buffer containing 10 mM Tris/HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA, pH 8.0. We added Proteinase K (0.1 mg/ml) to the homogenate solutions and digested proteins between 4 (four) to 12 h at 55°C. The solution was treated with phenol and chloroform/ isomyl alcohol and DNA was precipitated with ethanol. DNA precipitates were dried and then resuspended in 0.6 ml TE (10 mMTris/HCl, 1 mM EDTA, pH 8.0) and 1 µl was used in polymerase chain reaction (PCR). The PCR cycle included an initial denaturation step of 5 min at 94°C and 33 cycles of denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 48-50°C, and extension for 1 min 30 seconds at 72°C. Primers used in PCR are shown in Table 2. The PCR products were purified using polyethylene glycol (PEG, 13%) precipitation procedures were used directly as templates for Cycle Sequencing Reactions with fluorescent-dye-labeled terminator (ABI Big Dye Terminators v.3.1 cycle sequencing kit). The sequencing reaction products were purified by ethanol precipitation following the manufacture's protocol and were then run on an ABI PRISM 3130 genetic analyzer. All samples were sequenced in both directions using the same primers as the PCR.

**Data analysis**

Both 16S rRNA and COI sequences were aligned and trimmed using MUSCLE in MEGA X (Kumar et al. 2018). The final alignment comprised of total of 419 nucleotide sites of 16S rRNA and 555 nucleotide sites of COI were examined. Phylogenetic relationships were estimated using maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood was performed in MEGA X with General Timer Reversible (GTR) and Gamma Shape Parameter (G) as best fit model for 16S rRNA and GTR.
with invariant site (I) for COI. This model was selected based on the results from the "Find Best Model" analysis on MEGA X with lower Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) (Nei and Kumar 2000; Kumar et al. 2018). The BI analysis was implemented using MrBayes 3.2.7a (Ronquist and Huelsenbeck 2003). The substitution model for Bayesian analyses was selected from the models with the lowest AIC scores (Akaike Information Criterion) in Kakusan 3 (Tanabe 2007), with GTR+Gamma for 16S rRNA and Hasegawa-Kishino-Yano (HKY85) + Gamma for COI. BI analyses were run 10000000 Markov Chain Monte Carlo (MCMC) generations with MCMC diagnosis frequency of 100000 and a burn-in of 25%. Tree topology was sampled every 10000 generations.

We used non-parametric bootstrapping (NPBS) to determine nodal support strength in ML analyses with 1000 replicates (MLBS). Nodes bootstrap values of 70% or higher were considered as strongly supported (Hillis and Bull 1993). While in the BI analysis, nodes with a Bayesian posterior probability (BPP) of 95% or greater were considered as well supported (Leaché and Reeder 2002). We also estimated the genetic distance (uncorrected p-distance) by both 16S rRNA and COI gene among populations of *N. infrafrenatus infrafrenatus* using MEGA X (Kumar et al. 2018).

### Table 2. Primers used in this study

| Target | Primer | Sequence (5'→3') | Reference |
|-------|--------|------------------|-----------|
| 16S rRNA | 16H-1 | CTCCGGTCTGAACTCAGATCACGTAGG | Hedges 1994 |
|        | 16L-1 | CTGACCCTGCAAAAGTACGGTAATCACT | Hedges 1994 |
| COI    | LCO1490 | GGTCACAAATCATAAAGATATTGG | Folmer et al. 1994 |
|        | HCO2198 | TAAACTTCAGGGTGACCAAAAAATCA | Folmer et al. 1994 |

**Figure 1.** Map of the Moluccas, New Guinea and Australia illustrating provenance of *N. infrafrenatus infrafrenatus* mentioned in this study. Sample numbers correspond to those in Table 1 (Map modified from ArcGIS 10.3 Desktop Map, September 27, 2020 (ESRI 2014))
Table 1. Samples of *Nyctimystes infrarenatus* and related species were used for mtDNA analysis in this study together with information on species identification, specimen voucher number, locality, GenBank accession numbers, and references

| Species       | Voucher     | Locality                                | Gene 16S rRNA | COI | Accession number       | Reference                  |
|---------------|-------------|-----------------------------------------|---------------|-----|------------------------|----------------------------|
| *N. infrarenatus* | MZB Amph 17641 | Indonesia, West Papua, Raja Ampat, Batanta Utara, Yensewai | +             | -   | MW410751               | This study                 |
| *N. infrarenatus* | MZB Amph 17640 | Indonesia, West Papua, Raja Ampat, Batanta Utara, Yensewai | +             | -   | MW410752               | This study                 |
| *N. infrarenatus* | MZB Amph 24414 | Indonesia, West Papua, Kaimana, Teluk Arguni Bawah, Urusa | +             | +   | MW410753, MW422136     | This study                 |
| *N. infrarenatus* | MZB Amph 24415 | Indonesia, West Papua, Kaimana, Teluk Arguni Bawah, Urusa | +             | +   | MW410754, MW422137     | This study                 |
| *N. infrarenatus* | MZB Amph 24399 | Indonesia, West Papua, Kaimana, Lobo     | +             | +   | MW410755, MW422132     | This study                 |
| *N. infrarenatus* | MZB Amph 24400 | Indonesia, West Papua, Kaimana, Lobo     | +             | +   | MW410756, MW422133     | This study                 |
| *N. infrarenatus* | MZB Amph 24944 | Indonesia, West Papua, Kaimana, Lobo     | +             | +   | MW410757, MW422135     | This study                 |
| *N. infrarenatus* | MZB Amph 24945 | Indonesia, West Papua, Kaimana, Lobo     | +             | +   | MW410758, MW422134     | This study                 |
| *N. infrarenatus* | MZB Amph UN-1  | Indonesia, West Papua, Tambrauw, Fef    | +             | -   | MW410759               | This study                 |
| *N. infrarenatus* | MZB Amph 26685 | Indonesia, West Papua, Tambrauw, Miyah   | +             | -   | MW410760               | This study                 |
| *N. infrarenatus* | MZB Amph 26694 | Indonesia, West Papua, Tambrauw, Miyah   | +             | -   | MW410761               | This study                 |
| *N. infrarenatus* | MZB Amph 26687 | Indonesia, West Papua, Tambrauw, Fef    | +             | -   | MW410762               | This study                 |
| *N. infrarenatus* | MZB Amph 30730 | Indonesia, Ternate, Ternate Selatan, Gambesi | +             | +   | MW410772, MW425689     | This study, BOLD 2020     |
| *N. infrarenatus* | MZB Amph 12696 | Indonesia, North Moluccas, Ternate, Ternate Selatan, Gambesi | +             | +   | MW410770               | This study                 |
| *N. infrarenatus* | MZB Amph 12697 | Indonesia, North Moluccas, Ternate Selatan, Gambesi | +             | +   | MW410772, MW425689     | This study, BOLD 2020     |
| *N. infrarenatus* | MZB Amph 12698 | Indonesia, North Moluccas, Ternate Selatan, Gambesi | +             | +   | MW410770               | This study                 |
| *N. infrarenatus* | MZB Amph UN-3  | Indonesia, North Moluccas, Ternate Selatan, Gambesi | +             | +   | MW410770               | This study                 |
| *N. infrarenatus* | MZB Amph 12699 | Indonesia, North Moluccas, Ternate Selatan, Gambesi | +             | +   | MW410770               | This study                 |
| *N. infrarenatus* | MZB Amph 12700 | Indonesia, North Moluccas, Ternate Selatan, Gambesi | +             | +   | MW410770               | This study                 |
| *N. infrarenatus* | MZB Amph 12706 | Indonesia, North Moluccas, Ternate Selatan, Gambesi | +             | +   | MW410770               | This study                 |
| *N. infrarenatus* | MZB Amph 12709 | Indonesia, North Moluccas, North Halmahera, Ruko | +             | +   | FJ952306               | Setiadi et al. 2010       |
| *N. infrarenatus* | MZB Amph 12710 | Indonesia, North Moluccas, North Halmahera, Seki | +             | +   | FJ952311               | This study, Setiadi et al. 2010 |
| *N. infrarenatus* | MZB Amph 12712 | Indonesia, North Moluccas, East Halmahera, Lolobata NP | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12713 | Indonesia, North Moluccas, East Halmahera, Lolobata NP | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12714 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12715 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12716 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12717 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12718 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12719 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12723 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12724 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12725 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| *N. infrarenatus* | MZB Amph 12726 | Indonesia, North Moluccas, East Halmahera, Soagimalaha | +             | -   | MW410763               | This study                 |
| Species                  | Location                           | Accession Numbers | Notes                  |
|-------------------------|------------------------------------|-------------------|------------------------|
| *N. infrafrenatus*       | MZB Amph 12735                     |                   |                        |
|                         | Indonesia, North Moluccas, South Halmahera, Kluting Jaya SP4 |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12736                     |                   |                        |
|                         | Indonesia, North Moluccas, South Halmahera, Kluting Jaya SP4 |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12737                     |                   |                        |
|                         | Indonesia, North Moluccas, South Halmahera, Kluting Jaya SP3 |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12738                     |                   |                        |
|                         | Indonesia, North Moluccas, South Halmahera, Kluting Jaya SP3 |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12739                     |                   |                        |
|                         | Indonesia, North Moluccas, South Halmahera, Kluting Jaya SP3 |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12740                     |                   |                        |
|                         | Indonesia, North Moluccas, West Halmahera, Sahu Timur, Goal |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12741                     |                   |                        |
|                         | Indonesia, North Moluccas, West Halmahera, Sahu Timur, Goal |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12744                     |                   |                        |
|                         | Indonesia, North Moluccas, Tidore Kepuluan, Oba Utara, Sofifi |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12751                     |                   |                        |
|                         | Indonesia, North Moluccas, Tidore Kepuluan, Oba Utara, Sofifi |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12752                     |                   |                        |
|                         | Indonesia, North Moluccas, Tidore Kepuluan, Oba Utara, Sofifi |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12754                     |                   |                        |
|                         | North Moluccas, East Halmahera, Wasile Selatan, Musambo |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12755                     |                   |                        |
|                         | North Moluccas, West Halmahera, Jailolo Selatan, Dodinga |                   |                        |
| *N. infrafrenatus*       | MZB Amph 12756                     |                   |                        |
|                         | North Moluccas, West Halmahera, Jailolo Selatan, Braha |                   |                        |
| *N. infrafrenatus*       | SAMA R34970                        |                   |                        |
|                         | Australia, Queensland, Cairns      |                   |                        |
| *N. purpureolatus*       | MZB Amph 30684                     |                   |                        |
|                         | Indonesia, Papua, Sarmi, Bonggo    |                   |                        |
| *L. amboinensis*         | MZB Amph 24313                     |                   |                        |
|                         | Indonesia, West Papua, Kaimana, Lobo |                   |                        |
| *L. arfajiana*           | TNHC 51936                         |                   |                        |
|                         | Papua New Guinea, Madang, Kaironk Village |                   |                        |
| *L. angiana*             | Clone 16La1                        |                   |                        |
|                         | Papua New Guinea, Western Province |                   |                        |
| *R. rheoidea*            | Clone 16Lr2                        |                   |                        |
|                         | Australia, Queensland, Mt. Lewis, 16.7 km |                   |                        |
| *R. nanotis*             | Clone 16Ln5                        |                   |                        |
|                         | Australia, Queensland, O'Keefe Ck |                   |                        |
| *R. caerulea*            | SAMA R33448                        |                   |                        |
|                         | Australia, Queensland, Townsville  |                   |                        |
| *R. caerulea*            | AY883980                           |                   |                        |
|                         | NA                                 |                   |                        |
| *R. gracilenta*          | SAMA R34600                        |                   |                        |
|                         | Australia, Queensland, Cairns      |                   |                        |
| *R. bellia*              | QM J74476                          |                   |                        |
|                         | Australia, Queensland, Coen, Lankelly Ck |                   |                        |
| *R. aure*                | SAMA R69326                        |                   |                        |
|                         | Papua New Guinea, Gulf Province, Kopi |                   |                        |
| *P. volkerjane*          | RG 7724                            |                   |                        |
|                         | Indonesia, Papua, Mts. Wondiwoi     |                   |                        |
| *P. volkerjane*          | MZB Amph 24409                     |                   |                        |
|                         | Indonesia, West Papua, Kaimana, Lobo |                   |                        |
| *A. turpina*             | ABTC 90180                         |                   |                        |
|                         | Indonesia, West Papua, Raja Ampat. |                   |                        |
| *C. robustus*            | ABTC 45377                         |                   |                        |
|                         | Papua New Guinea, Southern Highland Province, Magidobo |                   |                        |
| *L. terraereginae*       | J789                               |                   |                        |
|                         | Australia, Queensland, Rockhampton  |                   |                        |

Note: MZB (including TR, BJE): Museum Zoologicum Bogoriense, SAMA: South Australian Museum, QM: Queensland Museum, ABTC: Australian Biological Tissue Collection, TNHC: Texas Natural History Collection. +: present, -: absent.
RESULTS AND DISCUSSION

Sequence and statistics

The aligned 16S rRNA data set consisted of 419 characters, in which 234 sites were variable and 161 parsimony informative. Genetic distances for the 16S rRNA are provided in Table 3. The aligned COI data set consisted of 555 characters, in which 221 sites were variable and 186 parsimony informative. Genetic distances for the COI are provided in Table 4.

Phylogenetic relationships

All analyses resulted in the same topologies on ML and BI for 16S rRNA and slightly differed with ML and BI for COI. The Bayesian tree (Figure 2) inferred the following sets of relationships: (i) Monophyly of *N. infrafrenatus infrafrenatus* as sister group of the clade formed by *N. pallidofemora* and *N. purpureolatus* (MLBS= 99% and BPP= 100%). (ii) Monophyly of *N. infrafrenatus infrafrenatus* with respect to *N. pallidofemora* in COI was supported in all trees (MLBS= 100% and BPP= 99%). (iii) The clade of *N. infrafrenatus infrafrenatus* consisted of two subclades, subclade A (Papuan and Australian) (16S rRNA MLBS= 93% BPP=97%, COI MLBS=100% BPP=99%) and subclade B (Moluccan) (16S rRNA MLBS= 93% BPP= 99%, COI MLBS 100%, BPP= 99%). (iv) An extensive genetic distance exists between the Moluccan subclade and the Papuan subclade (without Australian) (16S rRNA p-distance= 2.3-2.8%, COI= 9.9-10.8%). (v) In 16S rRNA, the Australian N. infrafrenatus infrafrenatus (single sample) is genetically closer to the Papuan (p-distance 1.3-1.8 %) than to the Moluccan subclade (p-distance 3.9-4.2%).

Table 3. Genetic distance (p-distance) (%) among Papuan and Moluccan population of *N. infrafrenatus infrafrenatus* and outgroup taxa based on 16S rRNA

|          | 1  | 2  | 3  | 4  |
|----------|----|----|----|----|
| Australia|    |    |    |    |
| Papua    | 1.3-1.8 |    |    |    |
| Moluccas | 3.9-4.2 | 2.3-2.8 |    |    |
| *N. pallidofemora* | 11.3 | 10.4-10.9 | 10.9-11.4 |    |
| *N. purpureolatus* | 11.5 | 10.4-10.6 | 10.4-10.6 | 10.7 |

Table 4. Genetic distance (% p-uncorrected) among Papuan and Moluccan population of *N. infrafrenatus infrafrenatus* and outgroup members based on COI

|          | 1  | 2  |
|----------|----|----|
| Papua    | 9.9-10.8 |    |
| Moluccas | 18.0-18.4 | 17.8-18.2 |

Discussion

Our results showed that *Nyctimystes infrafrenatus infrafrenatus* forms a monophyletic group divided into two subclades: Moluccas vs. Papua and Australia (Figure 2) among which we recovered large genetic distance (16S rRNA 2.3-4.2%, COI 9.9-10.8%). Using 16S rDNA, Fouquet (2007) suggested a threshold value of 3% p-distance could be used to flag candidate species. Moreover in Pelodryadidae, McDonald et al. (2016) described a new species of *Ranoidea bella* from Australia with 2% genetic distance to its closest relative *Ranoidea auae* Menzies and Tyler, from New Guinea. Our finding (2.3-4.2% p distance in 16S rRNA) between the Moluccan and the Papuan populations matches these values. The type locality of *N. infrafrenatus* is Cape York, Australia. Our subclade A thus corresponds to the nominal species *Nyctimystes infrafrenatus infrafrenatus*. In order to examine the taxonomic status of the Moluccan population, the long and complex taxonomic history of *Nyctimystes infrafrenatus* need to be carefully explored. There are two synonyms of *N. infrafrenatus infrafrenatus* which have type localities from Moluccas. There is *Calamita dolichopsis* (Cope 1867) from Ambon and *Hyla dolichopsis var. tenuigranulata* (Boettger 1895) from Halmahera. According to the article 23 of the International Code of Zoological Nomenclature (ICZN 1999) the valid name of a taxon is the oldest available name applied to it, unless that name has been invalidated or another name is given precedence by any provision of the Code or by any ruling of the Commission. Although *Calamita dolichopsis* had been published earlier (1867) but all Moluccan samples involved in this phylogenetic reconstruction come from the Halmahera and Ternate, so we suggest the *H. dolichopsis var. tenuigranulata* (1895) to be applied for the northern Moluccan population as *Nyctimystes tenuigranulatus* (Boettger 1895).

Both populations (Papua and Moluccas) shared similar morphological characteristics. Nevertheless, previous study on morphometrics showed that the females from Moluccas have shorter hind limb (HLL) than the ones from Papua. Moreover, in the description of *Hyla dolichopsis var. tenuigranulata* Boettger stated that the tympanum diameter of this species is smaller than the fingers disc while for the Papuan population (refer to *H. dolichopsis* from Papua) it is larger. From the live specimen (Figure 3), the width and length of white infralabial line appear to differ among the two populations. The Papuan population sample appears to have a thicker white lip than the Moluccan, but is not consistent across all specimens (Figure 4). Further examination of morphological characteristics of both populations is needed to determine the distinctive characteristics of the Moluccan population.
Figure 2. Bayesian phylogram of 419 bp of 16S rRNA and 555bp COI mitochondrial genes for samples of *N. infrafrenatus* and outgroup species. Numbers above branches represent bootstrap supports for ML and Bayesian Inferences (BI). Closed circles indicate nodes with significant bootstrap supports for ML (>70%) inferences and Bayesian Inference (BI>95%)
The genetically separated populations of *N. infrafrenatus infrafrenatus* occurred in two biogeographic regions. Subclade A from Papua occurs in Australo-Papuan region. Subclade B is from Halmahera, the largest island of Moluccas (Setiadi et al. 2010) and Tidore, represented Wallacean, one of biodiversity hotspots in Indo-Australia archipelago (Myers et al. 2000; Lohman et al. 2011). Wallacea is broadly defined by Wallace’s line in the west and Lydakker’s Line in the east (Hall 1998; 2002; 2013; Michaux 2010; Lohman et al. 2011). It is a transition zone between Sahul and Sundaland. Halmahera and Papua (New Guinea) were the part of Australian zoogeographic region (Wallace 1876; Rueda et al. 2013) which were divided by Holt et al. (2013) into Australian and Oceanian including Moluccas, New Guinea, and Pacific Islands.

The genetic difference between two populations of *N. infrafrenatus infrafrenatus* might be a result of geological history of Papua and Moluccas. New Guinea and Moluccas, especially Halmahera, come from two different tectonic plates (Hall 1998; 2002; 2009; 2013; Michaux 2010; Lohman et al. 2011; Baldwin et al. 2012). New Guinea is a part of the Australian plate (Hall 2002; 2009; Baldwin et al. 2012) whilst Halmahera is a part of the Philippine Sea plate (Michaux 2010; Baldwin et al. 2012). During the Neogene, Halmahera was probably close to New Guinea in the north margin of Australian plate about 20-15 mya, small pieces of landmass only appeared in the northern part of current New Guinea. Most of New Guinea, region of the Bird Head and Halmahera were on carbonate platform. Halmahera’s landmass first appeared about 10 mya (Hall 1998; 2002; 2009; 2013; Lohman et al. 2011). Halmahera has been isolated from the mainland probably early during this process (Setiadi et al. 2010). Therefore, considering the level of genetic distance, the presence of *N. infrafrenatus* on Halmahera probably result from oversea dispersal during Late Pliocene or Pleistocene, followed by isolation.

Concerning the low divergence between Australian and Papuan samples of *N. infrafrenatus* (1.3-1.5% in 16S rRNA), a vicariance event is likely since. We know that New Guinea and Australia were connected recently. The lowest sea level during the last maximum glaciation (about 18000 BP) was up to 125 m below the present sea level (Hantoro et al. 1995). The exposed area, called Sahul shelf, was covered by humid lowland tropical forest (Hantoro et al. 1995). Woodland-open forest covered the southern part of New Guinea to the eastern part of Australia and was followed by grassland-shrubland on most of northern Australian side of Sahul shelf (Van der Kaars 1991). The Sahul shelf could become the land bridge of fauna distribution between Australia and New Guinea. There was also Pleistocene river system (Voris 2000) which was important to support faunal exchange through the Sahul shelf. At the end of glaciation, sea level rose separated New Guinea and Australia until present (Voris 2000; Yokoyama et al. 2001).

The wide distribution of *N. infrafrenatus* is often believed to have resulted from human-assisted transport because this species is commensal which can be found in the garden and urban area (Vences et al. 2003; Menzies 2006; Hamidy and Mulyadi 2007). Distinct genetic differences between Papuan and Moluccan populations could support the dispersal theory. Moreover, Halmahera is an oceanic island that has not been in contact with the mainland of New Guinea or Australia. Therefore, *N. infrafrenatus* most likely dispersed from New Guinea to Halmahera overseas. In Wallacea herpetofauna, Karin et al. (2020) found that northern water dragon (*Tropicagama temporalis*) colonized the Moluccan islands of Babar, Kur, Tam, and Taninbar from the New Guinea rather than from Australia via overwater dispersal.

**Figure 3.** *Nyctimystes infrafrenatus infrafrenatus* from the Moluccas of Ternate (A) and Halmahera (B) has the thinner white lip than their counterparts from the Papuan of Timika (C) and Lengguru (D). Live specimens photographed by M. Iqbal Setadi (A,B), Amir Hamidy (C) and Antoine Fouquet (D)
Finally, our finding showed that the populations of a single species *Nyctimystes infrafrenatus* which are geographically separated form distinct phylogenetic subclades with genetic distance in the range of species level and phenotypic differences (even though deserving more examination). We propose to restrict the nominal species *Nyctimystes infrafrenatus* (Günther, 1867) to the Papuan and Australian populations and to resurrect *Nyctimystes tenuigranulatus* (Boettger 1895) for the northern Moluccan populations. Further studies including samples from Ambon (southern Molucca), where the *Hyla dolichopsis* (Cope, 1867) was described is needed to determine the taxonomic status of the southern Moluccan population.

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Figure 4. Specimens of *Nyctimystes infrafrenatus infrafrenatus* in Museum Zoologicum Bogoriense (MZB). A-E from Papua, F-J from Moluccas.
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