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Research article

Genetic analysis evidence of population substructure within the endangered Irrawaddy dolphin (*Orcaella brevirostris* Gray, 1866) in Thailand and Indonesia

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Abstract

Importance of the work: The decline in numbers of the Irrawaddy dolphin threatens its viability. Genetic data scarcity imperils management decisions.

Objectives: To apply microsatellite-based analysis to assess the genetic diversity and population structure of the Irrawaddy dolphin in Thailand and Indonesia.

Materials & Methods: Skin and muscle samples were collected from stranded Irrawaddy dolphins in coastal and riverine areas of Thailand (n = 64) and Indonesia (n = 10). Individual DNA was analyzed using 12 microsatellite primers. However, DNA from only 15 individuals from Thailand and five from Indonesia could be successfully amplified. The data obtained were then used to assess population genetic parameters and population structure.

Results: A high level of genetic diversity was observed among Irrawaddy dolphin populations in Thailand and Indonesia. In total, 106 alleles were identified across four populations in Thailand and Indonesia, with mean observed heterozygosity (H_o) of 0.625 ± 0.048 and an allelic richness of 4.125 ± 0.182. Pairwise population differentiation and discriminant analysis of principal components supported significant population heterogeneity. These findings suggested a population structure within Irrawaddy dolphins across Thailand and Indonesia, contradicting the common assumption of panmixia in marine mammal populations.

Main finding: Genotyping data revealed a population substructure within Irrawaddy dolphin populations in Thailand and Indonesia, highlighting potential challenges for their long-term conservation.

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Introduction

The Irrawaddy dolphin (*Orcaella brevirostris* Gray, 1866) is a facultative cetacean that inhabits riverine, estuary and marine ecosystems, extending from the Bay of Bengal in India to the Philippines archipelago (Smith et al., 2007; Minton et al., 2018). Despite wide distribution and habitat adaptability, the Irrawaddy dolphin faces numerous threats, including habitat loss, pollution, low birth rates and high gillnet mortality (Kreb et al., 2004; Minton et al., 2018). Population estimates of the Irrawaddy dolphins vary substantially, from hundreds in the eastern Upper Gulf of Thailand to less than 30 in Songkhla Lake (Jutapruet, 2013; Hines et al., 2015).

From a conservation perspective, the Irrawaddy dolphin's distribution range has high anthropogenic disturbance due to massive infrastructure development and associated disruptions from agriculture and aquaculture (Duckworth et al., 2012; Hughes, 2017) leading to significant declines in the range and dolphin numbers (Minton et al., 2018). Population declines uncontestably lead to the potential loss of genetic diversity, population fitness and elevated risk of local extinction (Frankham, 2005; Vachon et al., 2018).

Other studies on Irrawaddy dolphin genetics focused on the mtDNA D-loop, revealing population-level diversity and structure within the Mekong River, the Gulf of Thailand and Indonesia (Krützen et al., 2018; Caballero et al., 2019; Dai et al., 2021; Budi et al., 2022). Microsatellite analysis in Thailand suggested a single gene pool with potential gene flow between neighboring populations (Dai et al., 2021). While control region-based studies indicate close genetic affinity between Thailand and Indonesia (Krützen et al., 2018; Caballero et al., 2019; Budi et al., 2022), a comparative assessment of genetic structure across both countries is lacking.

Bridging this knowledge gap is vital to assess population viability and set effective conservation priorities. Thus, the current study used microsatellite markers to investigate genetic diversity and structure among freshwater and coastal Irrawaddy dolphin populations in Thailand and Indonesia. This comprehensive approach should illuminate key population dynamics and inform critical conservation decisions needed to ensure the persistence of this endangered species.

Materials and Methods

Specimen collection

Skin and muscle specimens were collected from stranded dolphins in Thailand (Songkhla Lake (n = 30); the Lower Gulf of Thailand (n = 22); and the Upper Gulf of Thailand (n = 12)) and Indonesia (Mahakam River (n = 8); Balikpapan Bay (n = 1); and coastal Panajam Paser Utara (n = 1)) (Fig. 1; Table S1). DNA tissue collection were performed by local veterinary teams during necropsy and specimens were then subsequently buried. Specimens collection in Thailand were provided by the Department of Marine and Coastal Resources and approved by the Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand (approved on 10 March 2021, Memo No. 0407/425), while in Indonesia approval was provided by the Conservation Foundation for Rare Aquatic Species of Indonesia under the Ministry of Environment and Forestry permit (Animals/plants domestic transfer permit, Permit No: 04/SATS/BKSDA KALTIM/SKW.2/07/2018, 02/SATS/ BKSDA KALTIM/SKW.2/07/2018, 063/SATS/ BKSDA.18/ VI/2018).

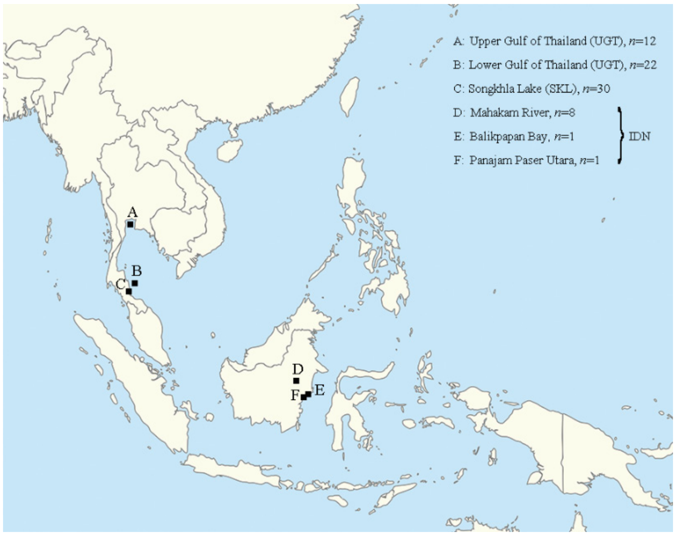

Fig. 1 Sampling locations for Irrawaddy dolphins in Thailand and Indonesia

DNA extraction and microsatellite loci amplification

Total genomic DNA extraction was performed using 10% Chelex 100 solution, Phenol-chloroform or Qiagen DNeasy® Blood and Tissue extraction kit (Qiagen; USA), as described in Budi et al. (2022). In total, 12 microsatellite primer pairs (Bp1357166, Bp1069071, Bp1057120, Bp1178841, Bp740180, GT211, EV37, GT122, LobsDi24, SCA9 and GATA417) were selected based on polymorphic information content (PIC) from Tardy et al. (2020). The 5′-end of the forward primer of each pair was labeled with fluorescent dye (FAM, HEX or TAMRA; IDT; Singapore). Polymerase chain reactions (PCRs) were performed in 10 µL reaction, consisting of 5 ng DNA template, 2x QIAGEN Multiplex PCR Master Mix (Qiagen; USA), 2 µM of each primer and 5X Q-Solution. The PCR reactions were run with the following parameters: a 15-min activation step at 95°C, followed by 45 cycles at 94°C for 30 s, 53–60°C for 90 s and 72°C for 90 s, with a final extension at 72°C for 10 min. The PCR reactions were visualized on 1.5% agarose gel stained with GelStar™ (Lonza; Switzerland). The PCR products were run on an ABI 3730XL automated DNA sequencer (Applied Biosystems; USA) using GeneScan LIZ 500 as the internal size standard at 1st Base Malaysia. Allele sizes were manually checked and scored using the PeakScanner version 1 software (Applied Biosystems; USA).

Microsatellite data analysis

Due to the low DNA quality, microsatellite genotyping was only successful on 20 samples representing four populations (Indonesia (IDN; $n = 5$), Songkhla Lake (SKL; $n = 5$), the Lower Gulf of Thailand (LGT; $n = 5$) and the Upper Gulf of Thailand (UGT; $n = 5$)) in all loci. Genetic diversity parameters and population structure analysis were performed following other studies based on microsatellite data (Budi et al., 2023; Wongloet at al., 2023). Genetic diversity was assessed based on allelic richness (AR), number of alleles per population (N_a), PIC, observed (H_o) and expected (H_e) heterozygosity, F statistics (F_{IS} and F_{ST}), individual relatedness (r) and pairwise Nei's genetic distance. Population structure analyses were used to investigate genetic differentiation. These analyses consisted of principal coordinate analysis (PCoA), discriminant analysis of principal components (DAPC), and STRUCTURE, following the methodology outlined in Budi et al. (2023) and Wongloet et al., 2023. Furthermore, the Wilcoxon signed-rank test was conducted to detect potential recent population bottlenecks. This test utilized both a two-phased mutation model (TPM) and a stepwise mutation model (SMM) to assess the likelihood of excess heterozygosity arising from small sample sizes. Additionally, tests for longterm bottlenecks based on the M ratio were performed, following the approach described in Budi et al. (2023).To investigate the possibility of selective sweep within the Irrawaddy dolphin populations, the individuals/population expected heterozygosity (H_e), observed heterozygosity (H_o) and inbreeding coefficient (F_{IS}) values based on genotype data were plotted for each microsatellite locus (12 loci in total).

Results and Discussion

Considerably high genetic diversity despite endangered status of Irrawaddy dolphin populations in Thailand and Indonesia

In total, 106 alleles with mean (± SD) number of alleles per locus of 4.583 ± 0.543, 4.167 ± 0.474, 4.083 ± 0.484 and 3.667 ± 0.432 were observed in the IDN, SKL, LGT and UGT populations, respectively. All allelic frequencies followed the Hardy-Weinberg equilibrium of the population, with no evidence of linkage disequilibrium. Null alleles were frequently found for the GATA417, GT211 and LobsDi24 loci; however, all the markers listed were similarly treated. All populations exhibited positive F values, except for the SKL population. The PIC of all populations was in the range 0.50–0.65, while Shannon's information index (I) was in the range 0.000–1.834. The mean H_o values were in the range 0.567–0.667 while the mean H_e values were in the range 0.548–0.663. All pairwise comparisons between H_o and H_e values within each population were not statistically significant (Table S2). The mean pairwise r values were –0.30±0.046 based on 190 pairwise combination from 20 Irrawaddy dolphin individuals (Table S3). The M-ratios for all populations were below 0.68 (Table S4), reflecting historical population decline. The Wilcoxon signed-rank tests for recent population bottlenecks generated using the stepwise mutation model (SMM) and a two-phased mutation model (TPM) showed sifted mode for all populations (Table S5). The genetic diversity parameters are presented in Table 1.

Table 1 Genetic diversity among 20 Irrawaddy dolphin individuals, based on 12 microsatellite loci

Population	n		N_a	AR	N_e	I	H_o	H_e	PIC	F
IDN	5	Mean	4.583	4.125	3.751	1.309	0.617	0.663	0.6204	0.064
		S.E.	0.543	0.182	0.448	0.151	0.111	0.065	0.2219	0.146
SKL	5	Mean	4.167	4.125	3.216	1.190	0.650	0.620	0.5737	-0.078
		S.E.	0.474	0.182	0.352	0.143	0.089	0.065	0.2471	0.120
LGT	5	Mean	4.083	4.125	3.238	1.163	0.567	0.600	0.5604	0.072
		S.E.	0.484	0.182	0.410	0.157	0.101	0.073	0.2221	0.133
UGT	5	Mean	3.667	4.125	2.590	1.017	0.667	0.548	0.4996	-0.227
		S.E.	0.432	0.182	0.304	0.128	0.090	0.059	0.1997	0.106
Overall	20	Mean	4.125	4.125	3.199	1.169	0.625	0.608	0.5635	-0.042
		S.E.	0.239	0.182	0.194	0.072	0.048	0.032	0.0498	0.064

n = number of individuals; [2]Number of alleles (N_a); [3]allelic richness (AR); [4]number of effective alleles (N_e); [5]Shannon's information index (I); [6]observed heterozygosity (H_o); [7]expected heterozygosity (H_e); [8]polymorphic information content (PIC); [9]fixation index (F); IDN = Indonesian population, SKL = Songkhla Lake population, LGT = Lower Gulf of Thailand population and UGT = Upper Gulf of Thailand population.

Microsatellite genotyping based on the 12 loci revealed considerably high genetic diversity. The magnitude of the genetic diversity based on the microsatellites observed in this study was higher than in other published studies of the Irrawaddy dolphin in Thailand and the Mekong River (Krützen et al., 2018; Dai et al., 2021), probably due to the higher geographical coverage and higher loci number. The probabilities of identifying PID and PID-sibs values in the study were low (PID ~ 1×10^{-8} and PID-sib ~ 1×10^{-4}), as shown in Fig. S1 indicating the absence of bias due to close siblings that could lead to an underestimation of other genetic parameters, including heterozygosity, relatedness and population structure (Waits et al. 2001). The genetic diversity based on microsatellites showed higher diversity than the mitochondrial-based diversity reported elsewhere (Krützen et al., 2018; Caballero et al., 2019; Dai et al., 2021; Budi et al., 2022). These disparities in nuclear versus mitochondrial diversity could be attributed to the social structure of the Irrawaddy dolphin, whereby these mammals tend to exhibit maternal philopatry (Barragán-Barrera et al., 2017). Similarly, mtDNA tended to show lower diversity compared to nuclear DNA in the Australia snubfin dolphin (Parra et al., 2018).

Evidence of population differentiation despite single gene pool pattern observed in Irrawaddy dolphin populations in Thailand and Indonesia

Pairwise population differentiation based on the F_{ST} value between populations of the Irrawaddy dolphin in Thailand and Indonesia (Table 2) was low yet significant for all population pairs, except for LGT-SKL and IDN-SKL. The discriminant analysis of principal components (DAPC) revealed an independent cluster for all populations, except for LGT-SKL (Fig. 2). The PCoA (Fig. 3) revealed multiple clusters, with some evidence of sharing clusters among populations. The model-based Bayesian clustering algorithms implemented in STRUCTURE generated one population structure pattern based on a low to higher *K*-value ($K = 2–4$) with the highest posterior probability of ΔK and ln P(K) equal to 4 (Fig. 4).

Table 2 Genetic differentiation (F_{ST}) between four populations of Irrawaddy dolphins in Thailand and Indonesia, based on 12 microsatellite loci

F_{ST}	IDN	SKL	UGT	LGT
IDN	-			
SKL	0.062^{ns}	-		
UGT	0.105*	0.101*	-	
LGT	0.085*	-0.025^{ns}	0.108*	-

IDN = Indonesian population, SKL = Songkhla Lake population, LGT = Lower Gulf of Thailand population and UGT = Upper Gulf of Thailand population.

* = $p < 0.05$; ^{ns} = not significant.

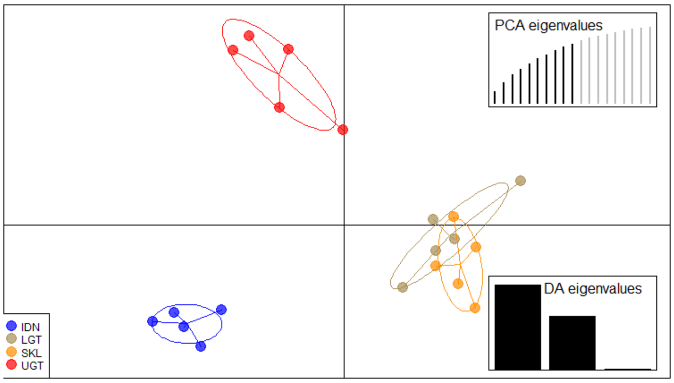

Fig. 2 Discriminant analysis (DA) of principal component analysis (PCA) results for Irrawaddy dolphin in Thailand and Indonesia, based on 12 microsatellite loci, where IDN = Indonesian population, SKL = Songkhla Lake population, LGT = Lower Gulf of Thailand population and UGT = Upper Gulf of Thailand population

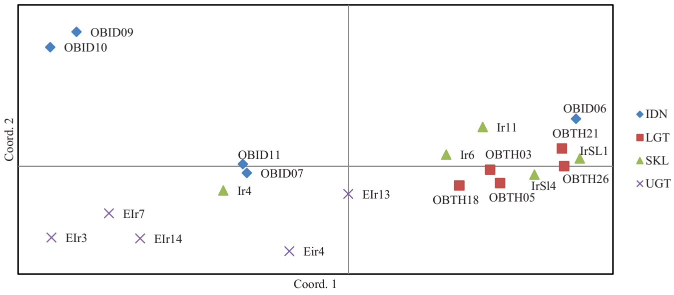

Fig. 3 Principal component analysis results for Irrawaddy dolphins in Thailand and Indonesia, based on 12 microsatellite loci, where IDN = Indonesian population, SKL = Songkhla Lake population, LGT = Lower Gulf of Thailand population and UGT = Upper Gulf of Thailand population

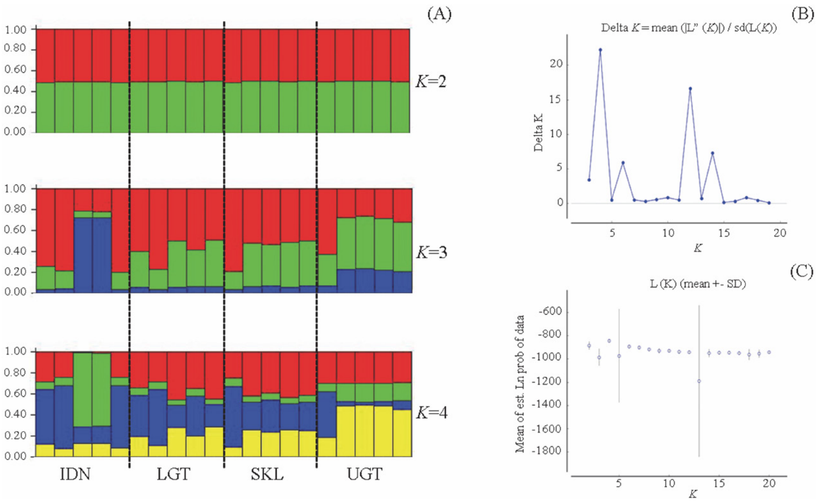

Fig. 4 (A) Population structure; (B) plot of ln P(K); (C) plot of Evanno's Δ*K* for Irrawaddy dolphins in Thailand and Indonesia, based on 12 microsatellite loci, where each vertical bar on x-axis represents one dolphin, y-axis represents proportion of membership (posterior probability) in each genetic cluster and each population of dolphins is superimposed on plot, with black vertical lines indicating boundaries

While panmixia has been a common assumption for marine mammal populations, this study on Irrawaddy dolphins in Thailand and Indonesia reveals a more nuanced picture. Pairwise F_{ST} values, though low, were statistically significant, indicating a tendency towards population differentiation. Notably, the non-significant F_{ST} value between SKL-LGT and SKL-IDN populations suggests a higher degree of historical or ongoing gene flow between these populations. However, a dispersal barrier, likely established within the last 3–4 generations, appears to currently disrupt connectivity between SKL and the LGT population. For long-lived species like the Irrawaddy dolphin, a time lag exists between barrier formation and detectable genetic structuring (Vachon et al., 2018; Louis et al., 2021; Budi et al., 2022). The non-significant difference between SKL and IDN population might also be attributed to limited sample size, given the substantial environmental separation. Therefore, a larger sample size is crucial for definitively assessing genetic structuring across all populations. Interestingly, DAPC analysis revealed clear clustering among these populations, further supporting population differentiation. The presence of population structure with possible historical connectivity between Thailand and Indonesian populations is also shown based on mtDNA studies (Budi et al., 2022).

In contrast, PCoA analysis showed evidence of sharing clusters among populations, indicating weak differences between Irrawaddy dolphin populations. In agreement with PCoA, a single gene pool pattern was observed within Irrawaddy dolphin populations in Thailand and Indonesia over various *K* values, as previously observed in Thai dolphin populations (Dai et al., 2021). These results indicate the absence of a population structure with a shared gene pool pattern among populations of the Irrawaddy dolphin in Thailand and Indonesia, indicating retained gene flow between populations (Quérouil et al., 2007). Alternatively, low sample numbers might obscure existing genetic differentiation among these populations. Interestingly, some individuals from IDN and UGT populations exhibited distinct gene pool patterns, hinting at potential substructures within population. The overall results of this study therefore rejecting panmixia population hypothesis for the Irrawaddy dolphin populations in Thailand and Indonesia. Similarly, based on the current population assessment of the Irrawaddy dolphin, it is likely that the populations in Thailand and Indonesia are experiencing population structuring. Moreover, long-term studies confirmed high site fidelity (Kreb, 2004) and relatively short daily movement in the Irrawaddy dolphin (Noor et al., 2013), further disfavoring the panmixia population.

On the other hand, selective sweep analysis indicates neutral or balanced selection for all populations (Fig. S2). Neutral or balanced selection possibly indicates a relatively recent radiation event, as previously estimated during the late Pleistocene (~500 KYA; Budi et al., 2022). The founder effect might also provide an appropriate explanation for the observed result because the founder effect might generate similar patterns. Alternatively, limited sample numbers also tend to mask selective sweeps (Dehasque et al., 2020).

This microsatellite analysis in this study is prone to bias due to the low sample size, which can lead to deviations from the actual value and individual probability assignment (Reiner et al., 2019). However, the strategy to utilize more loci (>10 loci) can be possible to counteract low sample number effects (Koskinen et al., 2004) with careful consideration of the polymorphic information content of the individual loci (Reiner et al., 2019). Therefore, additional samples from each population are required to confirm the observed result.

Implication for conservation

The Irrawaddy dolphin in Thailand and Indonesia is classified as both critically endangered (CR) for the low and isolated population in Songkhla Lake in Thailand and the Mahakam River in Indonesia and vulnerable (VU) for the coastal population in Thailand and Indonesia. This assessment reflected the current condition of those populations that are facing multiple threats to their survival (Minton et al., 2018). This study provides evidence suggesting population structure among Irrawaddy dolphins in Thailand and Indonesia.

Given the evidence for population structuring, managing Irrawaddy dolphins in Thailand and Indonesia as separate Management Units (MU) is warranted. Management of a metapopulation necessitates a focus on the entire network of populations, as local impacts can disrupt population dynamics and potentially lead to isolation (Durante et al., 2022). Functional movement among populations could improve genetic diversity and evolutionary potential within isolated populations. Increasing genetic diversity will improve species evolutionary potential to counteract the probability of genetic drift, inbreeding depression, and to cope with environmental change (Lacy, 1997; Frankham, 2005). However, promoting genetic exchange among populations through genetic rescue is not an option (Krützen et al., 2018).

The appropriate management strategies for the Irrawaddy dolphin are to manage and improve its habitat. This strategy has been adopted by the Indonesian government with assistance from the Rare Aquatic Species of Indonesia (RASI) to establish the Aquatic Conservation Area of Pesut Mahakam Habitat, which resulted in a stable population number during the last two years (RASI, personal communication). Spatial genetic diversity mapping throughout the Irrawaddy dolphin range is crucial to determining management units. It is also recommended to integrate a demographic and genetic approach to assess the human disturbance effects on the population's genetic diversity and connectivity. It is also needed to take into consideration the socio-economic dynamics of the locals to design appropriate and better action plans for the Irrawaddy dolphin conservation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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