



Research article

Mitogenomes provide insight into complex evolutionary history of freshwater and coastal Irrawaddy dolphin (*Orcaella brevirostris* Gray, 1866) in Thailand and Indonesia

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Article Info

Article history:

Received 8 February 2022

Revised 28 March 2022

Accepted 6 April 2022

Available online 30 June 2022

Keywords:

Divergence time,
Genetic connectivity,
Genetic diversity,
Irrawaddy dolphin,
Pleistocene

Abstract

Importance of the work: Irrawaddy dolphins have experienced demographic decline. The lack of genetic information might hamper management decisions necessary for restoring population viability.

Objectives: To apply population genetics and mitogenome phylogenetic analyses to assess genetic diversity and connectivity among Irrawaddy dolphin populations in Indonesia and Thailand.

Materials & Methods: Samples from stranded dolphins were collected from Mahakam River and East Kalimantan, Indonesia and from Songkhla Lake and the Lower and Upper Gulf of Thailand. Based on Bayesian time-scaled phylogeny and pairwise population differentiation, the divergence times were estimated between Irrawaddy dolphin lineages and potential historical factors influencing the observed genetic structure were inferred.

Results: The Indonesian populations had lower mtDNA diversity compared to Thailand populations. Pairwise population differentiation and analysis of molecular variance based on the control region supported high differentiation among fragmented Thailand and Indonesian populations ($F_{ST} = 0.6622$, $p < 0.0001$) with 66.21% variation among groups, except for the Songkhla Lake and coastal Lower Gulf populations. Phylogenetic analyses revealed close relationships between some of the Thai and Indonesian haplotypes, suggesting historical freshwater connectivity. There was a lack of monophyly despite the strong support for the Mahakam River clade in Indonesia. The Mahakam River population started to diversify during the late Pleistocene (~304.4 Ka; 95% highest posterior density (HPD) = 157.8–505.3 Ka), possibly due to sea-level fluctuation and changes in palaeodrainage systems.

Main finding: The results of the genetic diversity and complex evolutionary history of the Irrawaddy dolphin raise conservation concerns and support management strategies aiming to restore population connectivity and reduce the impacts of genetic and demographic stochasticity.

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<https://doi.org/10.34044/j.anres.2022.56.3.14>

Introduction

Southeast Asia is one of the global hotspots for freshwater and marine diversity; a complex evolutionary history in this region has resulted from long-term climatic fluctuation and geological events influencing changes in demography, genetic diversity and connectivity (Woodruff, 2010; de Bruyn et al., 2014). However, large-scale infrastructure development and agricultural conversion are rapidly disrupting evolutionary processes and threatening the ecosystem function across the region (Duckworth et al., 2012; Sheldon et al., 2015; Hughes, 2017). Within riverine and coastal habitat in particular, populations of Irrawaddy dolphin (*Orcaella brevirostris* GRAY, 1866) have substantially declined in range and numbers (Minton et al., 2018).

Their long-term survival has become a conservation concern due to habitat loss, chemical pollution, a slow reproductive rate, high mortality associated with plastic and debris ingestion, gillnet entanglement and vessel collisions (Minton et al., 2018). The small (< 100 individuals) and fragmented freshwater populations in Mahakam and Songkhla Lake in Thailand are classified as Critically Endangered on the IUCN Red List, while other coastal populations are Endangered (Smith and Beasley, 2005; Ponnampalam et al., 2013; Hughes, 2017; Minton et al., 2018). With restricted dispersal and population bottlenecks, these populations are subject to greater impacts of demographic stochasticity, genetic drift and inbreeding, leading to the potential loss of genetic diversity, population fitness and elevated risk of local extinction (Frankham, 2005; Banks et al., 2013; Ruiz-García et al., 2018; Vachon et al., 2018).

Dispersal and gene flow among remnant populations could potentially reduce the effects of genetic stochasticity by increasing migration and colonization events, restoring evolutionary adaptation and the persistence of spatially structured metapopulations (Hanski and Gilpin 1991; Hanski, 1998). The magnitude and direction of gene flow depend greatly on habitat connectivity, intrinsic dispersal capability and an effective population size (Frankham et al., 2009; Frankham, 2015; Bell et al., 2019). During the 2008–2014 distance sampling surveys, population estimates of coastal Irrawaddy dolphin along the eastern Upper Gulf of Thailand were larger ($n = 423$; Hines et al., 2015) than elsewhere (Jefferson et al., 2008; Jutapruet, 2013; Noor et al., 2013; Teoh et al., 2013; Krebs et al., 2015, 2020; Rodríguez-Vargas et al., 2019; Tubbs et al., 2020) and might indicate potential genetic exchange with nearby populations along the Thailand-Cambodia border (Ponnampalam et al., 2013).

In contrast, the population estimates in Songkhla Lake, Thailand (< 30 individuals; Jutapruet, 2013) and Balikpapan Bay in East Kalimantan, Indonesia (< 100 individuals; Krebs et al., 2020) were smaller and these population potentially experienced disrupted movement due to human disturbance. There is no baseline information on whether gene flow could counteract the negative impacts of genetic drift and inbreeding, nor on which conservation actions would be appropriate to restore adaptive potential in response toward a changing environment. Rarely, have genetic data been integrated into ongoing demographic monitoring and this may underestimate extinction risk and hamper conservation efforts to delineate management unit (MUs; Moritz, 1994; Palsbøll et al., 2007; Funk et al., 2012), establish important marine mammal areas and promote survival of the species (Allendorf and Luikart, 2007; Harrisson et al., 2014).

The mitogenome data provide a higher resolution regarding evolutionary relationships with recent diversification during the Pleistocene or Holocene. Compared to a single-gene phylogeny, a larger number of single nucleotide polymorphisms (SNPs) in the mitogenome phylogeny provide a more reliable estimation of population-level genetic diversity, inference of divergence time, species origins and subsequent colonization events (Arnason et al., 2004; Galtier et al., 2009; Palmer et al., 2011; Bayas-Rea et al., 2018). The population-level genetic diversity and structure of Irrawaddy dolphins based on the control region of the mitogenome are available only for those in the Mekong River and the Gulf of Thailand (Krützen et al., 2018; Caballero et al., 2019; Dai et al., 2021). Control region-based phylogeographic studies support a close genetic affinity between Thailand and Indonesian populations; however, statistical support for such phylogenetic relationships is low (Krützen et al., 2018; Caballero et al., 2019). Therefore, a better understanding is needed of the factors driving diversification, historical connectivity and comparative genetic diversity between Thailand and Indonesia to assess their viability and set management priorities to prevent any further loss of adaptive potential in response to environmental changes.

The aims of the current study were to apply population genetics based on the control region and mitogenome: 1) to examine the historical impacts of climate fluctuation and the phylogeographic barriers to the evolutionary history of the Irrawaddy dolphin; and 2) to assess genetic diversity and historical population connectivity and to estimate the divergence time among freshwater and coastal populations of Irrawaddy dolphin in Thailand and Indonesia.

Materials and Methods

Sample collection

Genetic material samples were collected from stranded Irrawaddy dolphins in Thailand and Indonesia (Fig. 1). In Thailand, skin and muscle samples were collected by a veterinarian team of the Department of Marine and Coastal Resources during 2007–2019 from three localities: 1) Songkhla Lake ($n = 30$); 2) the Lower Gulf of Thailand, stretching along coastal Songkhla ($n = 22$); and 3) the Upper Gulf of Thailand ($n = 12$), along the coast of Samut Sakhon, Chachoengsao and Trat provinces. In Indonesia, samples were collected during 2010–2018 from three localities: 1) Mahakam River ($n = 8$); 2) Balikpapan Bay ($n = 1$); and 3) coastal Panajam Paser Utara ($n = 1$). Permission to collect the Indonesian samples was granted by the Ministry of Environment and Forestry of The Republic of Indonesia (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), and acquisition of the samples in Thailand was approved by the Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand (approved on 10 March 2021, Memo No. 0407/425).

DNA extraction and polymerase chain reaction amplification

Genomic DNA was extracted using a 10% Chelex 100 solution, Phenol-chloroform or a Qiagen DNeasy® Blood (Qiagen; USA) and a Tissue extraction kit (Walsh et al., 1991; Rivero et al., 2006). Initially, 770 bp of control region fragments were amplified using the published primers L15812 (Rosel et al., 1994) and Dlp8G (Garrigue et al., 2004) to assess genetic diversity and assign haplotypes to each sample. Subsequently, the representative haplotypes were extended to the mitogenome level using previously published (Rosel et al., 1994; Leduc et al., 1999; Kosuch et al., 2001; Dalebout et al., 2005; Morin et al., 2010) and newly designed (Table S1) primer sets. Each 10 μ L polymerase chain reaction (PCR) reaction consisted of 5 ng DNA template, 5 μ L of 2x Phire Tissue Direct PCR master mix (Thermo Scientific™; USA) and 0.5 μ M of each primer. The PCR reactions were run with the following parameters: a 3-min activation step at 98°C, followed by 40 cycles at 98°C for 5 s, 53–60°C for 5 s (Table S1) and 72°C for 30–60 s (depending on the amplicon sizes), with a final extension at 72°C for 5 min. The second PCR phase of the successfully amplified PCR products was performed using the same primer set and different *Taq* polymerase. The 40 μ L PCR reactions involved: 30 ng of 1st PCR product as template, 0.4 μ M of each primer, 2 U of *Taq* DNA polymerase (Thermo Scientific™; USA), 4 μ L of

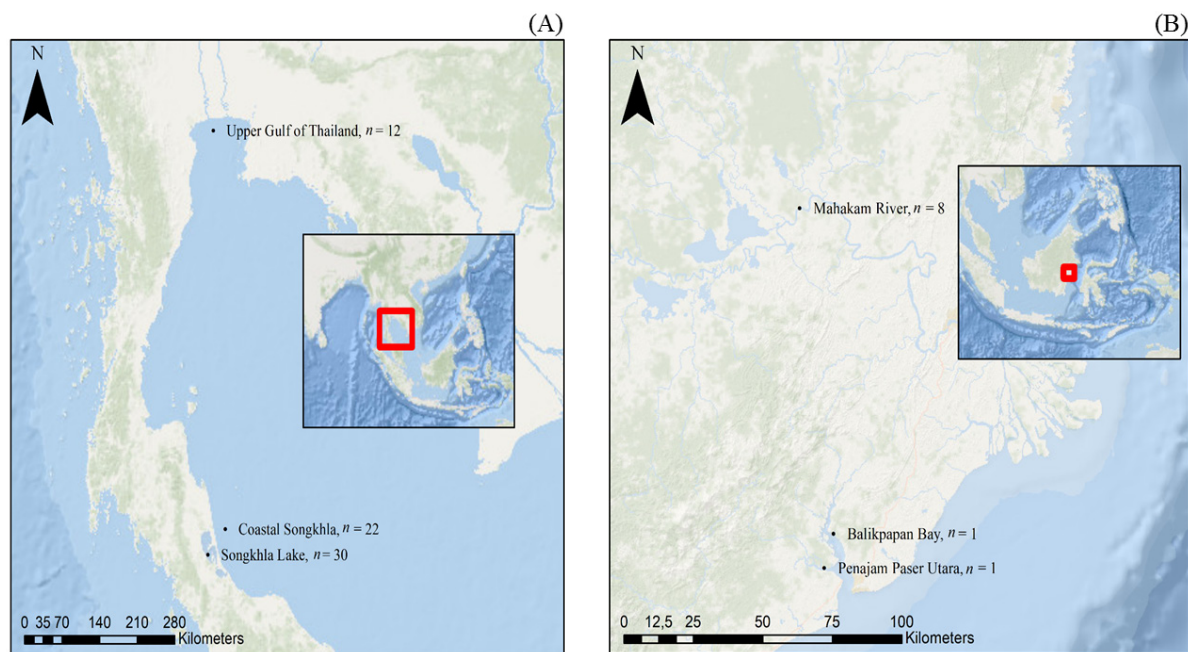


Fig. 1 (A) Locations of samples from Irrawaddy dolphins: (A) in Thailand from Songkhla Lake, Lower Gulf of Thailand (stretching along coastal Songkhla) and Upper Gulf of Thailand (along the coast of Samut Sakhon, Chachoengsao and Trat provinces); (B) in Indonesia from Mahakam River, Balikpapan Bay and coastal Panajam Paser Utara

(NH₄)₂SO₄ buffer, 3.2 µL of 25 mM MgCl₂ and 0.8 mM dNTPs. Reactions were run with the following parameters: a 3 min activation step at 95°C for 30 s, followed by 30 cycles at 95°C for 30 s, 53–60°C for 30 s (Table S1) and 72°C for 30–60 s (depending on the amplicon sizes), with a final extension at 72°C for 5 min. The PCR reactions were visualized on 1.5% agarose gel, purified using a FavorPrep™ Gel/PCR Purification Kit (Favorgen; Taiwan) and sent for Sanger sequencing at 1st BASE Laboratory, Malaysia. Laboratory workflows were performed in the country where the samples originated.

Sequence quality control, assembly and annotation

The mitochondrial sequences were visually inspected using the BioEdit program (Hall, 1999). The overlapping fragments of all sequences were examined to ensure complete sequence coverage and consensus sequences were aligned and mapped to the Irrawaddy dolphin mitogenome (Accession number NC019590; Vilstrup et al., 2011) using the Unipro UGENE software (Okonechnikov et al., 2012). The complete mitochondrial genome was annotated using MITOS (Bernt et al., 2013) and MITOS2 (Donath et al., 2019) and subsequently verified using a BLAST search against the Delphinidae mitogenome on the NCBI webservice at <https://www.ncbi.nlm.nih.gov/nucore/>, followed by manual checking for the start and stop codons of the protein-coding genes.

Mitochondrial DNA diversity and differentiation among populations

Due to the limited sample sizes from Balikpapan Bay ($n = 1$, BBY) and coastal Panajam Paser Utara ($n = 1$, PPU), the Indonesian population ($n = 11$) was classified as a pooled dataset of Mahakam ($n = 8$, MHK), BBY and PPU. For the Thailand populations, independent datasets were used: Songkhla Lake ($n = 30$, SKL); the Lower Gulf of Thailand ($n = 22$, LGT); and the Upper Gulf of Thailand ($n = 12$, UGT). Calculations were made of haplotype diversity (h), nucleotide diversity (π) and the number of polymorphic sites (S) of the 770 bp control region using the DnaSP v.6 software (Rozas et al., 2017). Population genetic differentiation based on pairwise F_{ST} was calculated using the control region sequences with 10,000 permutations and analysis of molecular variance (AMOVA). A hierarchical AMOVA was performed to assess the proportions of genetic variation within and among populations using five packages implemented in the R software (R Core Team, 2021 : apex (Jombart et al., 2017), adegenet

(Jombart, 2008), pegas (Paradis, 2010), mmod (Winter, 2012), and poppr (Kamvar et al., 2015).

Phylogenetic and haplotype network reconstruction

The control region and mitogenome sequences from this study were combined with those derived from other populations (Beasley et al., 2005; Jayasankar et al., 2011; Caballero et al., 2019; Dai et al., 2021) and deposited in the NCBI database (<https://www.ncbi.nlm.nih.gov/>). The best fitted nucleotide substitution model was estimated using ModelFinder (Kalyaanamoorthy et al., 2017) and a phylogenetic tree was reconstructed based on maximum likelihood with ultrafast 10,000 bootstrap replicates (Hoang et al., 2018) on the IQ-TREE web server (<http://iqtree.cibiv.univie.ac.at>; Trifinopoulos et al., 2016) and visualized using the iTOL software (Letunic and Bork, 2019). Templeton-Crandall-Sing (TCS) haplotype networks were constructed using the PopART software (Leigh and Bryant, 2015)

Time-calibrated phylogeny and divergence dating

To estimate a calibration point and improve divergence time estimation in delphinids, Irrawaddy dolphin mitogenome sequences from this study and those from the Family Delphinidae and Balaenopteridae (Table S2) were downloaded from GenBank and included in the time-calibrated phylogenetic analyses. The datasets were divided into three partitions based on a best substitution scheme estimated using the PartitionFinder2 software (Lanfear et al., 2017): 1) the first position of protein-coding sequences, rRNAs, and tRNAs; 2) the second position of protein-coding sequences, control region and origin of replication; and 3) the third position of protein-coding sequences. To account for different rates of heterogeneity among partitions, the best-fitted nucleotide substitution model was selected for each partition using a greedy algorithm and Bayesian information criterion implemented in PartitionFinder2: Partition (1) TRN+I (2) HKY+I, and (3) HKY+I.

Time-calibrated Bayesian phylogeny and the time to the most recent common ancestor (TMRCA) were estimated using: an uncorrelated lognormal relaxed clock model; a Yule speciation process when using multiple species as outgroups; and a coalescent model assuming constant population sizes when using a single species as an outgroup. The relaxed clock rate prior of 0.00667 substitution/site/million years was used (Dornburg et al., 2012) and the tree was calibrated using

the normal distribution prior for Delphinidae TMRCA (mean = 4.83 million years ago (Mya), SD = 0.2) and the genus *Orcaella* TMRCA (Mean = 2.89 Mya, SD = 0.36), according to McGowen et al. (2020). The MCMC analyses in BEAST v2.5 (Bouckaert et al., 2019) were performed based on six independent runs for 100,000,000 iterations, sampled every 10,000 steps and a burn-in of 10,000 iterations was discarded. Convergence of each MCMC run was checked based on an adequate effective sample size > 200 and the stable trace of the log-likelihood plots.

Data availability statement

The mitochondrial DNA sequences from this study have been deposited in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) with accession numbers MZ351459–MZ351463 and MZ351448–MZ351458.

Ethic statements

This study was reviewed and approved by King Mongkut’s University of Technology Thonburi (Approval no: KMUTT-IACUC-2020/012, IBC-2020-033) and the Kasetsart University Ethical Committee (Approval no: ACKU64-VET-031, IBC-64-V02).

Results

Mitochondrial DNA diversity and population differentiation

The mtDNA fragments of the control region from 74 individuals were successfully sequenced (64 and 10 sequences, respectively from Thailand and Indonesian samples). In total, 770 bp with 26 variable sites were obtained and 16 haplotypes were defined, with no diagnostic sites detected that were unique to Indonesian or Thai populations (Table 1). Three haplotypes were detected from Indonesian populations, with none of them shared among the three localities: Mahakam River (OBID01, *n* = 8); Balikpapan Bay (OBID02, *n* = 1); and coastal Panajam Paser Utara (OBID03, *n* = 1). In Thailand, 13 haplotypes were found, some of which were shared between localities: 1) haplotype OBTH11 (*n* = 2), found in both LGT and UGT; and haplotypes OBTH01, OBTH02, OBTH04, OBTH05 (*n* = 32) in both Songkhla Lake (SKL) and nearby LGT (Table 1).

The Indonesian populations (IND) had lower haplotype diversity (*h*), yet higher nucleotide diversity (π) compared to those in SKL, LGT and UGT from Thailand, where the SKL population had the highest number of haplotypes and the UGT population had the highest haplotype diversity (Table 2).

Table 1 Haplotype and variable sites based on 770 bp partial control region of Irrawaddy dolphin

Haplotype	Gene	<i>n</i>	Control region																										
			L15812 / Dlp8G																										
Primer																													
Position*			15499	15516	15517	15582	15595	15625	15639	15675	15740	15754	15787	15828	15863	15864	15880	15881	15922	15972	15976	15996	16009	16027	16074	16094	16110	16168	
Locality																													
OBID01	MHK	8	T	G	T	G	T	A	T	T	T	T	G	C	C	T	C	G	T	G	A	T	A	T	A	A	A	A	C
OBID02	BBY	1	C	A	T	.	.	T	A	.	A	G	.	G	C	G	.	G	.	
OBID03	PPU	1	C	.	C	.	C	.	.	.	C	C	A	T	.	C	.	.	C	.	G	.	G	.	G	G	.	T	
OBTH01	LGS	2	C	.	C	C	A	T	T	C	.	.	C	.	G	.	G	.	G	G	.	.	
OBTH02	LGS	17	C	.	C	C	A	T	T	C	.	.	C	.	G	.	G	.	G	G	.	.	
OBTH03	SKL	2	C	.	C	C	C	A	T	T	C	.	.	C	.	G	.	G	.	G	G	.	T	
OBTH04	LGS	10	C	.	C	C	C	A	T	T	C	.	.	C	.	G	.	G	.	G	G	.	T	
OBTH05	LGS	3	C	.	C	C	A	T	.	C	.	.	C	.	.	.	G	.	G	.	.		
OBTH06	UGT	1	C	.	C	A	.	.	C	.	.	C	A	T	.	C	.	.	C	.	.	C	G	.	G	.	G	T	
OBTH07	UGT	2	C	.	C	A	.	.	.	C	.	C	A	T	.	C	.	.	C	.	.	C	G	.	G	.	G	.	
OBTH08	UGT	6	C	C	C	A	C	A	T	.	C	.	.	C	.	.	C	G	.	G	G	.	T	
OBTH09	UGT	1	C	.	C	A	.	C	.	.	.	C	A	T	.	C	.	.	C	.	.	C	G	.	G	G	.	T	
OBTH10	SKL	1	C	.	C	.	.	C	.	.	.	C	A	T	T	C	.	.	C	.	G	.	G	.	G	.	G	T	
OBTH11	ULG	2	C	.	C	.	.	.	C	.	.	.	A	T	T	.	.	.	C	.	.	G	.	G	.	G	.	T	
OBTH12	UGT	1	C	.	C	A	C	A	T	.	C	.	.	C	.	.	C	G	.	G	G	.	T	
OBTH13	SKL	16	C	.	C	C	A	T	T	C	.	.	C	.	.	G	.	G	.	G	.	T	

MHK = Mahakam; BBY = Balikpapan Bay; PPU = Panajam Paser Utara; LGS = Lower Gulf of Thailand and Songkhla Lake; SKL = Songkhla Lake; UGT = Upper Gulf of Thailand; ULG = Upper and Lower Gulf of Thailand; *n* = number of individuals with each haplotype from samples used in this study; * = nucleotide sequence position corresponds to complete mitogenome sequence of *Orcaella brevirostris* in NCBI database (Accession number NC019590)

Table 2 MtDNA diversity (mean \pm SE) of four Irrawaddy dolphin populations based on 770 bp control region

Population	H	<i>h</i>	π
IND	3	0.3778 \pm 0.180	0.0064 \pm 0.003
SKL	7	0.6851 \pm 0.079	0.0015 \pm 0.000
LGT	5	0.6147 \pm 0.097	0.0022 \pm 0.001
UGT	6	0.7576 \pm 0.120	0.0038 \pm 0.001

H = number of haplotypes; *h* = haplotype diversity; π = nucleotide diversity; IND = Indonesia; SKL = Songkhla Lake; LGT = Lower Gulf of Thailand; UGT = Upper Gulf of Thailand

Based on the pairwise F_{ST} and G_{ST} values (fixation index and genetic differentiation coefficient) from structure analyses, strong ($F_{ST} = 0.1441$ – 0.7316 , $G_{ST} = 0.1595$ – 0.3506) and significant ($p < 0.0001$) genetic differentiations were detected across all sites, except for SKL-LGT divergence that was low, yet significant ($p < 0.0001$), as shown in Table 3. The highest level of F_{ST} and G_{ST} was detected between the IND and SKL populations ($F_{ST} = 0.7316$, $G_{ST} = 0.3172$, $p < 0.0001$). Consistent with the number of shared haplotypes, the SKL and LGT populations had the lowest differentiation ($F_{ST} = 0.1441$, $G_{ST} = 0.1595$, $p < 0.0001$). The AMOVA for the four populations indicated that the majority of genetic variation was observed among populations (66.21% of the total variance), with smaller variation within populations (33.79%). This result corroborated with the pairwise distance between populations evaluated using G_{ST} and F_{ST} (Table 4).

Table 3 Values of pairwise population genetic differentiation based on G_{ST} (above diagonal) and F_{ST} (below diagonal) estimates for populations of Irrawaddy dolphin collected from four geographic regions based on 770 bp control region sequences

$F_{ST} \backslash G_{ST}$	IND	SKL	LGT	UGT
IND	-	0.3172*	0.3506*	0.2970*
SKL	0.7316*	-	0.1595*	0.1774*
LGT	0.6936*	0.1441*	-	0.2016*
UGT	0.6913*	0.6557*	0.6278*	-

IND = Indonesia; SKL = Songkhla Lake; LGT = Lower Gulf of Thailand; UGT = Upper Gulf of Thailand

* = significant ($p < 0.0001$) result after Bonferroni correction

Table 4 Analysis of molecular variance (AMOVA) based on 770 bp control region of Irrawaddy dolphin from four locations

Source of Variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	3	109.8770	2.0526 Va	66.21
Within populations	70	73.3120	1.0473 Vb	33.79
Total	73	183.1890	0.5000	

df = degree of freedom

Phylogenetic and haplotype network reconstruction

Phylogenetic reconstruction supports recent radiation and a complex evolutionary history with rapid diversification of the Irrawaddy dolphin. Paraphyletic relationships based on the 770 bp control region were detected among haplotypes from the Indonesian clade with respect to those from Thailand (Fig. 2). Compared to their conspecifics, there is a closer genetic affinity between the haplotype OBID03 from PPU, Indonesia and OBTH04 in both SKL and LGT, and Hap7 from SKL, Thailand (Beasley et al., 2005). Similarly, the haplotype Obr13 from the Andaman coast in western Thailand (Dai et al., 2021) was clustered within the strongly supported MHK clade, The Obr4 haplotype (Dai et al., 2021) from LGT, Thailand was also more closely related to the Chilika population in India compared to their Thailand conspecifics. Overall, the current results do not support reciprocal monophyly of both the Thailand and Indonesian haplotypes in relation to those from other populations. The TCS haplotype network of all Irrawaddy dolphin populations was consistent with the rapid diversification patterns observed from the tree topology. Genetic differences among haplotypes were only by 1–3 mutational steps and clustering based on sample localities was not observed.

Time-calibrated phylogeny and divergence dating

The current mitogenome-based estimation of divergence time among the 12 haplotypes of the Irrawaddy dolphin (Fig. 3) showed consistent branching patterns observed in the family Delphinidae, with recent radiation with rapid diversification in the Pleistocene era (< 2.5 Mya). The genus *Orcaella* separated from *Orcinus orca* since 8.596 Mya (95% CI: 8.481 – 8.717 Mya). The splitting between *O. brevirostris* and *O. heinsohni* occurred around 3.072 Mya (95% CI: 2.4516–3.7023 Mya). The Irrawaddy dolphin diverged into two strongly supported clades since 501.2 Ka (95% CI: 303.3–784.6 Ka). The first clade consisted of MHK and BBY populations, both of which were subsequently isolated from each other around 304.4 Ka (95% CI: 157.8–505.3 Ka). The second clade consisted of the PPU, Indonesia and Thailand haplotypes. During the Middle Pleistocene (~MIS 7), two haplotypes unique to the UGT (OBTH08 and OBTH09) started to be separated from the rest of the Thailand haplotypes around 234.6 Ka (95% CI: 117.8–401.2 Ka).

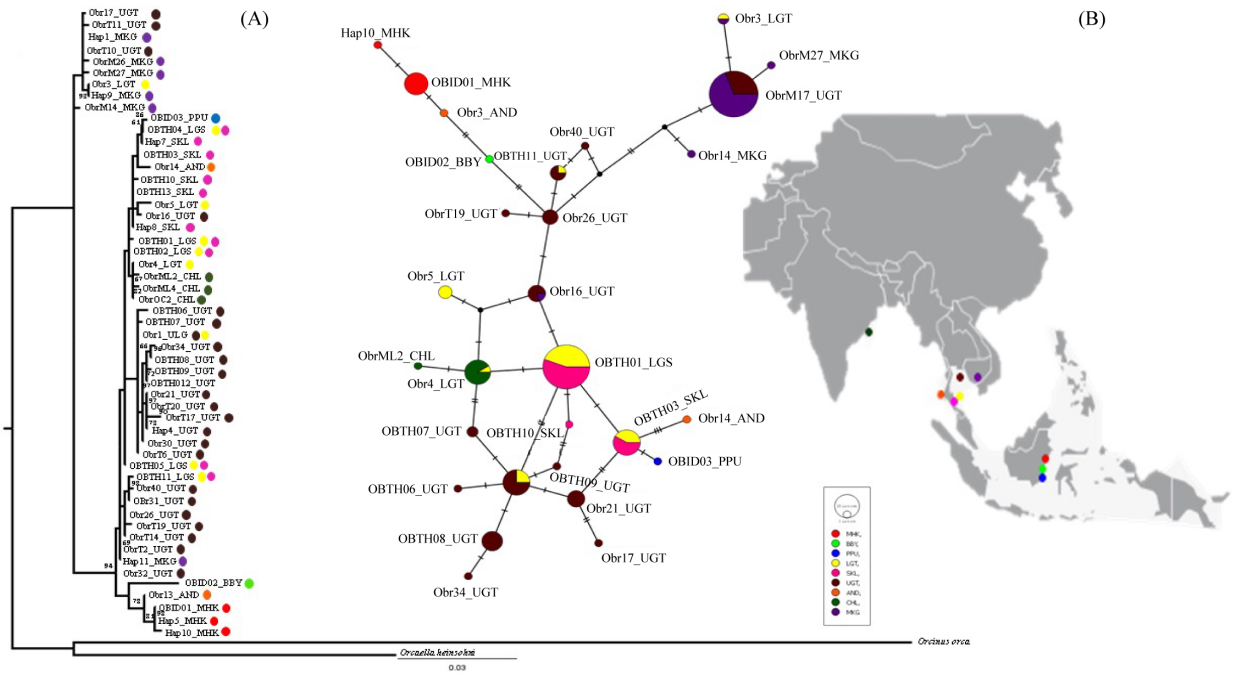


Fig. 2 (A) Phylogenetic reconstruction of Irrawaddy dolphin haplotypes based on partial control region sequences (770 bp) using maximum-likelihood methods with 1,000 bootstrap replicates, where circle on node represents bootstrap values > 60 and *Orcinus orca* and *O. heinsohni* represent outgroup. Haplotype abbreviation refers to Table 1 and Table S2. ; (B) Templeton-Crandall-Sing (TCS) network of Irrawaddy dolphin based on partial control region of mitochondrial DNA (770 bp), where size of each circle represents sample size and length of each line represents number of mutational steps between haplotypes, MHK = Mahakam population, BBY = Balikpapan Bay population, PPU = Panajam Paser Utara population, SKL = Songkhla Lake population, LGT = Lower Gulf of Thailand population, UGT = Upper Gulf of Thailand population, AND = Andaman Sea population, CHL = Chilika Lagoon population and MKG = Mekong population

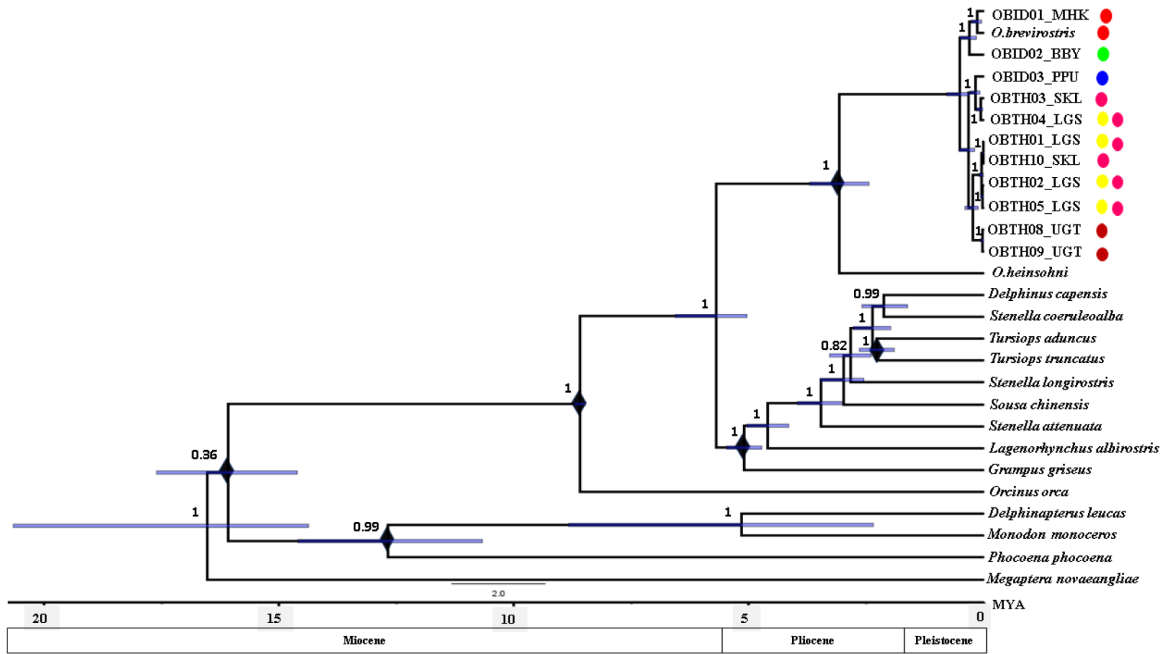


Fig. 3 Divergence tree reconstruction of Irrawaddy dolphin haplotypes based on complete mitogenome sequences (16,387 bp) using Bayesian methods with 100000000 MCMC chain, where node bar represents 95% highest posterior density (HPD) of divergence time estimation, species names in italic denotes outgroups, black diamond shape indicates point of calibration and number above node represent Bayesian posterior probability, MHK = Mahakam population, BBY = Balikpapan Bay population, PPU = Panajam Paser Utara population, SKL = Songkhla Lake population, LGS = Lower Gulf of Thailand population and Songkhla Lake, and UGT = Upper Gulf of Thailand population; MYA = Million years ago

Discussion

The current study provided support on recent lineage diversification of the Irrawaddy dolphin, close evolutionary relationships between Thailand and Indonesia haplotypes and a major genetic structure among the Upper Gulf, Lower Gulf-Songkhla Lake, Thailand and Indonesia populations.

Mitochondrial DNA diversity and population differentiation

The number of variable sites based on the 770 bp control region sequences used in this study was higher than for previous studies (Krützen et al., 2018; Caballero et al., 2019) due to longer sequences. Therefore, higher SNPs allow the detection of phylogenetic signals, particularly those with recent, rapid diversification (Taylor et al., 2017). The numbers of haplotypes detected from Thailand (13 haplotypes) were higher than those reported from the Mekong and Gulf of Thailand populations (Krützen et al., 2018; Caballero et al., 2019). Of the three haplotypes detected in Indonesia, one haplotype differed from other studies (Beasley et al., 2005; Yusmalinda et al., 2017). A larger sample sizes and number of variable sites, together with broader geographic coverage within Thailand and Indonesia, potentially contributed to the higher mtDNA diversity detected from each country compared to the previous population genetics and phylogeographic studies (Krützen et al., 2018; Caballero et al., 2019; Dai et al., 2021).

Discrepancy between nucleotide diversity and haplotype diversity might be the result of population substructure and highly diverged haplotypes detected within the assumed population (Fu, 1995; Verity and Nichols, 2016). Compared to SKL, the LGT and UGT populations of the Thailand dolphin and higher nucleotide diversity in Indonesia might have resulted from the combination of the highly divergent OBID03 haplotype from PPU to the more closely related OBID01 from MHK and OBID02 from BBY. The high level of mtDNA diversity in UGT was consistent with the level reported in other mtDNA studies (Caballero et al., 2019; Dai et al., 2021) and in a demographic study estimating the population sizes around 400 individuals (Hines et al., 2015). Maintenance of metapopulation dynamics across fragmented habitats in the Upper Gulf of Thailand and Cambodia might explain the higher genetic diversity of UGT populations compared to the other isolated populations.

Contrasting levels between the relatively high mtDNA diversity and low population size within Songkhla Lake might be due to high historical connectivity with the coastal

population. Although rigorous estimates of population sizes are not available, the SKL population sizes in the early 2000s was thought to be below 20 (Smith et al., 2004, 2007). Intensive fishing activities, fixed fish traps and dam construction have driven the dolphin population to the northern part of the lake (Beasley et al., 2002; Smith et al., 2007), while the southern habitat has become shallower with limited prey availability (Brownell et al., 2019). However, placement of dispersal barriers between the lake and coastal populations occurred within a recent timeframe (< 100 years, or 3–4 generations). For species with a long generation time, there will be a lag between barrier placement and mtDNA-based detection of strong genetic structuring. Therefore, historical factors, including large and stable population sizes, ancestral haplotype retention and high connectivity (Wan et al., 2004; Frankham et al., 2009) between the lake and coastal habitats might account for the relatively high genetic diversity within Songkhla Lake. Detection of shared haplotypes and weak genetic differentiation among the SKL and LGT populations also support the historical connectivity hypothesis. Alternatively, the continued occurrence of dolphins within the Lake might imply underestimated population sizes and/or undetected migrants and genetic exchange with the adjacent LGT population along the coast (Brownell et al., 2019). Combined demographic and genetic monitoring is needed for population status assessment to provide more accurate demographic estimates.

Compared to the SKL population, the larger MHK population in East Kalimantan, Indonesia is expected to retain a higher level of genetic diversity. However, the current study detected no mtDNA variation from samples obtained from Mahakam River (Table 1). The limited sample sizes, high genetic stochasticity linked to a population bottleneck and the high site fidelity and/or social structure in group-living mammals might explain the observed genetic patterns. Along the 240 km stretch of the River and its tributaries, the current sample size ($n = 8$) represented 10% of the population estimate (< 80 individuals; Krieb personal communication) but may not represent an overall population genetic diversity. Therefore, a broader geographic coverage and intensive sampling efforts are needed to assess MHK genetic diversity and differentiation from BBY and PPU in estuarine and coastal ecosystems.

Historic range expansion and founder events followed by genetic isolation are also important factors leading to low genetic diversity of the edge population (Eckert et al., 2008; Romiguier et al., 2014; Ellegren and Galtier, 2016). Indonesian populations are distributed the easternmost area of Irrawaddy

dolphin. In other delphinids, genetic diversity of the peripheral populations has been shown to be lower than those at the center, or those genetically connected to the others in metapopulation dynamics (Parra et al., 2018a; Faria et al., 2020). Indonesian founders might come from a few individuals that originated from the same sources and subsequently became isolated from the ancestral population along the expansion front. To provide support to this hypothesis, intensive sampling efforts are needed from the Indonesian population to develop inferences of the long-term demographic trend.

Foraging specialization, high natal site fidelity and the social structure of group-living marine mammals could have influenced the fine-scale population structure and low genetic diversity within each locality (Louis et al., 2014; Bowen et al., 2016; Passadore et al., 2018; Vachon et al., 2018). Residency or natal site fidelity observed in other delphinids, including the bottlenose dolphin (*Tursiops truncatus*), might be relevant to explain the low genetic diversity of Indonesian populations. Strong site fidelity to the riverine or coastal areas where they were born, coupled with small and nonoverlapping core home ranges in the Australian humpback dolphin (Parra et al., 2018a), bottlenose dolphin (Barragán-Barrera et al., 2017; Passadore et al., 2018) and Boto (Gravena et al., 2019) have been shown to influence the fine-scale social structure and local philopatry. Even though the suitable habitats of MHK, BBY, and PPU appear to be contiguous, natal site fidelity, matrilineal social structure and resource specialization might increase genetic differentiation among the three localities.

The population differentiation pattern observed between SKL, LGT and UGT in Thailand likely followed an isolation by distance scheme as proposed in other studies on the Irrawaddy dolphin (Caballero et al., 2019; Dai et al., 2021) and other cetaceans, including the Australian snubfin dolphin (Parra et al., 2018b), harbour porpoise (Fontaine et al., 2007), and killer whale (Moura et al., 2015). Strong differentiation among populations based on AMOVA analysis was corroborated with high pairwise distance between population evaluated by G_{ST} and F_{ST} . In addition to physical distance in isolation based on the distance model, habitat heterogeneity might promote local adaptation and niche specializations (Hoelzel, 1998). Study on the Franciscan dolphin and long-finned pilot whale suggested that genetic divergence was correlated with differences in habitat preference (Fullard et al., 2000; Mendez et al., 2010). Chlorophyll concentration, turbidity and surface temperature can also affect marine productivity, prey availability and the subsequent habitat occupancy of dolphins (Mendez et al., 2010). Habitat modelling study (Jackson-Ricketts et al., 2020; Sahri et al., 2021) suggested that Irrawaddy dolphin presence

was tightly related to a specific water level and surface temperature, creating a soft dispersal barrier and therefore genetic divergence among localities.

Phylogenetic reconstruction and historical dispersal patterns

Rapid diversification of the Irrawaddy dolphin and the lack of reciprocal monophyly in their Mekong, Thailand and Indonesian haplotypes in relation to other populations were supported by the phylogenetic analysis and Caballero et al. (2019), with an increase in sample size, geographic range and longer sequence of the control region. Haplotype network reconstruction was in agreement with the patterns observed from tree topology (Fig. 2B). Except for the MHK and Chilika populations, there was no strong clustering based on the localities. Each haplotype was separated by 1–3 mutational steps with a star-like network pattern. The observed genetic patterns could be explained by the complex climatic and geological history in this region. During the Pleistocene (2.58 Mya and 11.8 Ka), repeated glacial periods, sea level fluctuation and exposed Sunda Shelf stretching from the Thai-Malay Peninsula to Java, Sumatra and Borneo formed an extensive landmass (Voris, 2000) and potentially facilitate widespread movement in various taxa (Woodruff, 2010; Wurster and Bird, 2016; Krützen et al., 2018; Husson et al., 2020). Unlike the highly mobile delphinids, ocean currents serve as a barrier to the Irrawaddy dolphin which inhabits shallow riverine, estuarine and coastal areas (Minton et al., 2018). Repeated habitat discontinuity during sea level rises, the loss of freshwater corridors and reduced prey availability potentially enabled the rapid diversification observed from the phylogenetic tree and haplotype network in the current study.

Our phylogenetic analyses provide strong statistical support (bootstrap values > 80%) for the Mahakam clade. This pattern could imply that the upstream MHK population has been isolated from its coastal relative (BBY and PPU), or alternatively, this observed clustering arose due to the bias from limited sample sizes. With intensive sampling efforts, it might be possible to detect either the shared or closely related haplotypes between the MHK and BBY populations. Historical dispersal and gene flow during the Pleistocene when widespread palaeoriver drainages could possibly have been present across Sundaland and connected to those in riverine and coastal areas. This hypothesis is supported by phylogeographic studies of Sundaic freshwater fish (Sholihah et al., 2020, 2021). Due to recent demographic and the more restricted movement in the recent timeframe, genetic drift might lead to the loss of genetic diversity from extant populations. Ancient or historical DNA

are needed to provide insight into past population connectivity. A phylogenomic approach based on genome-wide variation will also enable inference of historical gene flow, colonization patterns and demographic changes associated with fluctuating climate and changing dispersal corridors during glacial-interglacial cycles.

Prolonged genetic isolation during the Last Glacial Maxima (~21 Ka) might allow sufficient time for each population to independently evolve and exhibit morphological and socio-behavioral differences between MHK, PPU and BBY (Kreb, 2004). Demographic monitoring based on photographic capture-recapture surveys did not detect interactions among fragmented MHK, PPU and BBY populations (Kreb, 2004). Long-term studies confirmed site fidelity within the river flow; the MHK population is expected to be fully adapted to freshwater habitats. Although some cetaceans are capable of extensive dispersal, daily movement from the Mahakam River to coastal areas is less likely (Kreb, 2004; Noor et al., 2013).

Divergence dating and rapid diversification

Recent radiation with rapid diversification of the Irrawaddy dolphin during the Pleistocene is supported by the current study's time-calibrated phylogenetic analysis, divergence time estimation and other phylogenetic studies of cetaceans. The mitogenome-based divergence time and mutation rate estimates from the current study were consistent with the split between the genus *Orcaella-Orcinus* and *O. brevirostris-O. heinsohni* based on the mitogenome (Vilstrup et al., 2011), the combination of the nuclear gene, transposons and the mitogenome (McGowen, 2011) and genome-wide SNP data (McGowen et al., 2020). In contrast to the deep divergence between the freshwater and marine populations of other species, the shallow split (< 500 Ka) of the MHK population from the others implied high historical connectivity, followed by the more recent placement of dispersal barriers during the “mid-Middle” Pleistocene beginning around 500–450 Ka. The river dolphins from the genera *Inia*, *Pontoporia* and *Lipotes* have been estimated to have become fully adapted to freshwater during the Late Oligocene (23 Mya) according to McGowen et al. (2020). The deep divergence around 2 Mya during the Pleistocene was estimated from the riverine versus coastal populations of the *Sotalia* dolphin (Cunha et al., 2005). Based on palynological and geological data, the Mahakam River was predicted to have formed during the early Miocene (de Bruyn et al., 2014). The divergence time of the MHK haplotypes was

estimated to have occurred later during the middle Pleistocene when environmental changes, including in surface temperature and sea level, occurs. Cetacean diversification is thought to be related to lower ocean temperatures and increased upwelling of nutrient resources (Berger, 2007; Carstens and Knowles, 2007). The exposed landmass during the low sea level created a paleoriver system that interconnected freshwater watersheds around Sundaland and might have acted as a dispersal corridor (de Bruyn et al., 2014; Sholihah et al., 2020). Prolonged disruption of the palaeoriver system and gene flow would promote diversification among populations of facultative river dolphins.

Water depth, salinity and surface temperature were proposed to influence diversification patterns in Orca (Parsons et al., 2010; Moura et al., 2015) and mammals, as reviewed in Steeman et al. (2009), Pinheiro et al. (2017) and Sholihah et al. (2020). Habitat modelling of the Irrawaddy dolphin supported that these factors are significantly correlated to nutrient-rich effluent and productivity and therefore, to the presence of dolphins and their prey species (Minton et al., 2013; Jackson-Ricketts et al., 2020; Verutes et al., 2021). The “species pump” era, when most freshwater diversification occurred, coincided with divergence time estimates and the Pleistocene environmental fluctuation (de Bruyn et al., 2012).

Due to a few mitogenome data from the UGT populations in Thailand and those from other populations of the Irrawaddy dolphin, phylogeographic inference based on time-scale phylogeny has remained limited. There is insufficient evidence to infer historical connectivity between SKL and the coastal LGT-UGT populations. Therefore, more mitogenome data across the species range are needed to elucidate the origin, the time of colonization events and subsequent isolation. The current study confirmed that phylogenetic analyses based on the mitogenome still have the bias of a single gene tree; therefore, future phylogenomic studies should provide more conclusive evidence on the evolutionary history of the Irrawaddy dolphin.

Implications for conservation management

The current study provided evidence that the Irrawaddy population in the Mahakam River, Indonesia has low genetic diversity and might be less likely to adapt to the changing environment and human disturbance, compared to those populations in Songkhla Lake and the Upper and Lower Gulf of Thailand. Without proper management strategies to reduce mortality and promote connectivity among fragmented

habitats, these populations are at risk of extinction. It is proposed that a conservation action plan should include integrative demographic-genetic approaches to monitor the impacts of environmental and human disturbance on population genetic diversity and connectivity. Mapping spatial genetic diversity across the population range is also important to delineate management units and assess the effectiveness of ongoing conservation practices.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was funded by the Asahi Glass Foundation Grant 2020, Kasetsart University Research and Development Institute (KURDI, Bangkok, Thailand research code FF (KU)12.64) and Petchra Prajom Klao Master's Degree Research Scholarship (Agreement No. 19/2562), King Mongkut's University of Technology Thonburi, Thailand. Associate Professor Worawidh Wajjwalku and Assistant Professor Manakorn Sukmak provided suggestions and laboratory facilities in Thailand. Ms Nongnid Kaolim helped with laboratory work. Fakultas Teknobiologi, Universitas Atma Jaya Yogyakarta provided laboratory access in Indonesia. Muh Iqbal Rais helped with map production.

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