

## MITOCHONDRIAL HETEROPLASMY IN CRUSTACEANS AND ITS IMPLICATIONS TO SPECIES DELIMITATION: A MINI-REVIEW

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**Abstract:** Mitochondrial DNA sequences are commonly used as molecular markers for species identification and phylogenetic studies due to their uniparental inheritance among other features. This uniparental transmission typically results in organisms having a single type of mitochondrial DNA (homoplasmy). However, there are instances where organisms with different mitochondrial haplotypes (heteroplasmy) are found, and this phenomenon complicates the function of the mitochondrial DNA region as a tool for species identification and evolutionary inference. Given the economic importance of the crustacean species, this review investigates the prevalence of heteroplasmy in crustaceans and its potential impact. To date, heteroplasmy has been reported in 12 crustacean species, with most cases (66.7%) occurring at the dual tRNA alanine/valine position. Heteroplasmy can be detected using digestive enzymes and various polymerase chain reaction-based methods. The presence of mitochondrial heteroplasmy in crustaceans affects the species delimitation process. This review highlights the presence of heteroplasmy in crustaceans and the importance of identifying populations with heteroplasmic organisms, especially economically important crustaceans in fisheries and aquaculture. Future efforts in developing diagnostic markers, conducting long-term, large-scale monitoring, and using next-generation sequencing technology for mitochondrial genome sequencing will aid in characterising heteroplasmy in crustaceans.

Keywords: Uniparental inheritance, heteroplasmy, crustaceans.

### Introduction

The DNA of eukaryotes is found in two main organelles, the nucleus and the mitochondria, with most genes tightly packed into chromosomes in the nucleus. Genes in the nucleus are biparentally inherited, with half coming from each parent. In contrast, it is widely accepted that mitochondrial DNA is exclusively maternally inherited (Lee *et al.*, 2023). This notion—maternal inheritance of mitochondrial DNA—forms the basis of many scientific applications, including population genetics and evolution (Wang *et al.*, 2019; Sun *et al.*, 2021a), species identification and validation (Waiho *et al.*, 2017), forensic science (Amorim *et al.*, 2019), and medical genetics related to mitochondrial disorders (Wallace, 2018).

Uniparental transmission or inheritance of mitochondrial DNA in most animals is thought to be a preventive measure against the spread

of selfish or fast-replicating mutations within the population (Greiner *et al.*, 2015). However, the homoplasmic, uniparental, and non-recombining universal features of mitochondrial genomes are being challenged. Researchers, using new methods such as next-generation sequencing technologies have documented heteroplasmy (the occurrence of more than one type of mitochondrial DNA in an individual) in different groups of organisms, including birds (Gandolfi *et al.*, 2017; Päckert *et al.*, 2019), frogs (Radojčić *et al.*, 2015), fishes (Shigenobu *et al.*, 2005), molluscs (Ghiselli *et al.*, 2019), humans (Stefano & Kream, 2016), cetaceans (Vollmer *et al.*, 2011), and crustaceans (Chow *et al.*, 2021).

Among the various groups of organisms with documented heteroplasmy, crustaceans are one of the most diverse groups and can be

found in all environments—marine, freshwater, and terrestrial. There are approximately 17,229 species within 203 families (De Grave *et al.*, 2023). Species within this subphylum are morphologically diverse and most species have complex life cycles that involve contrasting environments and/or distinctive lifestyle (Olesen, 2018). The subphylum Crustacea also encompasses numerous species of high value to the global fisheries and aquaculture sectors (Raupach & Radulovici, 2015).

The availability and applicability of DNA sequencing technology, along with the abundance of deposited sequences in public databases for comparison, facilitate the easy identification of specimens with complex and morphologically distinct life stages such as the larval and immature stages of many crustaceans (Raupach & Radulovici, 2015; Marco-Herrero *et al.*, 2021; Xu *et al.*, 2022). Specifically, researchers are focusing on the use of mitochondrial DNA for molecular species identification due to the lack of recombination, introns, high substitution rates, large copy numbers, and maternal inheritance (Bernt *et al.*, 2013). In addition to species identification, mitochondrial DNA also serves as the backbone for various fields in the fisheries and aquaculture sectors of crustaceans, including understanding genetic structure and phylogenetic relationships (McMillen-Jackson & Bert, 2004; Mondal *et al.*, 2020), stock identification and assessment (Shaklee & Bentzen, 1998; Zhao *et al.*, 2021), and crustacean product traceability (Sun *et al.*, 2021b).

However, the recently discovered notion of paternal inheritance of mitochondrial DNA in various species, including crustaceans, presents a complication to its potential usage in species with mitochondrial heteroplasmy. Mitochondrial heteroplasmy could lead to polymerase chain reaction (PCR) amplification bias, consequently causing misidentification of species (Martínez *et al.*, 2023) and complicating any downstream applications, including the overestimation of population diversity and species frequency (Chow *et al.*, 2021).

Thus, this mini-review aims to summarise the currently known crustacean species documented with heteroplasmy, the existing heteroplasmy detection methods, and the potential impact on species delimitation of crustaceans. The information brought together by this mini-review highlights the need for further exploration of different crustacean species with possible mitochondrial heteroplasmy. Additionally, it underscores the importance of considering paternal inheritance in studies of mitochondrial DNA, particularly in species where this phenomenon has been observed. The current status of heteroplasmic occurrence in crustaceans outlined in this review serves as a valuable baseline reference for researchers by consolidating the current knowledge, methodologies, and advancements related to the presence of heteroplasmy in crustaceans, thereby ensuring the accuracy of the species delimitation process. Aside from highlighting the need for long-term and large-scale monitoring efforts and detecting heteroplasmic occurrence in a wider range of crustaceans, especially those of economic importance, this review also provides insights to inform conservation and fishery management efforts. Ignoring the presence of heteroplasmy in crustaceans could lead to inaccurate species identification and has serious implications, including mismanagement in the conservation of crustacean populations due to the over- or underestimation of the effective population size, and jeopardising the sustainability of the crustacean fishery sector, ultimately causing significant losses in biodiversity and adverse effects on food security (Rodríguez-Pena *et al.*, 2020; Martínez *et al.*, 2023).

### ***General Functions and Genome Structure of Mitochondria***

Mitochondria are present in the cells of all eukaryotes and these essential organelles are involved in the production of ATP, which serves as the main energy source for most biological, physiological, and biochemical processes, ranging from general homeostasis

to movement and development (Brand *et al.*, 2013). In addition to their involvement in oxidative phosphorylation for ATP generation, mitochondria are essential for ion homeostasis, cell apoptosis, various metabolic pathways such as lipid and amino acid metabolisms, and the production and removal of reactive oxygen species (ROS) (Tirichen *et al.*, 2021).

In general, the circular mitochondrial DNA of animals are approximately 16 kb in size and typically comprises 37 genes (two rRNA genes, 13 protein-coding genes, and 22 tRNA genes) (Table 1) (Boore, 1999). Additionally, the mitochondrial DNA genome includes a variable region, known as the control region, which is the longest non-coding region within the mitochondrial DNA and is the most polymorphic.

The unique features of the control region—its fast evolutionary rate, polymorphic nature, lack of recombination, maternal inheritance, and presumed selective neutrality—make it a valuable target for selecting favourable markers in the identification and phylogenetic studies of animals, including crustaceans (Bronstein *et al.*, 2018). However, segmental duplications and pseudogene formation of the control regions have been reported in some species, complicating the downstream PCR amplification process (Cadahía *et al.*, 2009). Thus, researchers often resort to use the cytochrome c oxidase subunit 1 gene (*COI*) or the 16S ribosomal RNA

coding gene (*16S*) is the common barcoding gene in most species, including various families of crustaceans (Marco-Herrero *et al.*, 2021; Xu *et al.*, 2022).

The gene arrangements of mitochondrial sequences often remain the same or exhibit only minor variations over long evolutionary periods, despite the rapid evolution of mitochondrial sequences. Thus, the stable gene arrangements within major groups, but variation among groups facilitate the resolution of their phylogeny and evolution (Boore, 1999).

### **Maternal Inheritance of Mitochondrial DNA**

Uniparental inheritance of mitochondrial DNA is responsible for offspring homoplasmy, where all offsprings have genetically identical mitochondrial genomes. Although uniparental inheritance of mitochondrial genomes is risky as it excludes the benefit of sexual recombination and thus exacerbates the accumulation of deleterious mutations, it can prevent intragenomic conflict between competing mitochondrial DNAs from two parents. Uniparental inheritance ensures that any unfavourable mitochondrial mutations are confined to a single lineage (Munasinghe & Ågren, 2023).

Although it is still unclear why maternal inheritance is preferred over paternal inheritance, researchers suggest that it

Table 1: The genes typically found in the mitochondrial genomes of animals and their abbreviations

<b>Encoded Protein</b>	<b>Abbreviations</b>
Cytochrome oxidase subunit I, II, III	<i>COI, COII, COIII</i>
Cytochrome <i>b</i>	<i>Cytb</i>
NADH dehydrogenase subunits 1-6, 4L	<i>ND1-6, 4L</i>
ATP synthase subunits 6, 8	<i>ATP6, ATP8</i>
Large ribosomal subunit RNA	<i>lrRNA</i>
Small ribosomal subunit RNA	<i>srRNA</i>
18 Transfer RNAs	Corresponding one-letter amino acid code
Two transfer RNAs specifying leucine	<i>L1, L2</i> , or differentiated by codon recognised
Two transfer RNAs specifying serine	<i>S1, S2</i> , or differentiated by codon recognised

may be due to the higher copy numbers of mitochondrial DNA and lower mutation rates in female gametes compared with male gametes, including in crustaceans (Xu *et al.*, 2017). Various mechanisms for eliminating paternal mitochondria have been proposed (Sato & Sato, 2013; Munasinghe & Ågren, 2023). For example, in vertebrates, ubiquitin-mediated degradation of paternal mitochondria has been observed in mammals (Onishi *et al.*, 2021) while partial degradation of paternal mitochondrial DNA nucleoids occurs during spermatogenesis and complete destruction of paternal mitochondrial DNA structures happens after fertilisation in the fish *Oryzias latipes* (Nishimura *et al.*, 2006). In invertebrates, autophagic elimination of paternal mitochondria is exhibited by the nematode *Caenorhabditis elegans* (Molina *et al.*, 2019). Additionally, in arthropods, *Drosophila melanogaster* eliminates paternal mitochondrial DNA during spermatogenesis (DeLuca & O’Farrell, 2012). While various mechanisms of paternal mitochondria elimination have been identified, it is important to note that these mechanisms are potentially species-specific, and to date, little is known about the mechanisms ensuring maternal mitochondrial DNA inheritance in crustaceans.

**Heteroplasmy in Crustaceans**

Heteroplasmy of mitochondrial DNA can arise from somatic mutations, leakage of paternal mitochondrial DNA during fertilisation, or maternal inheritance from a heteroplasmic egg (Figure 1) (Parakatselaki & Ladoukakis, 2021). While heteroplasmy in crustaceans is rare, it is increasingly being documented as researchers gain a better understanding of the molecular sequence changes following heteroplasmy. Table 2 summarises the currently known crustacean species that exhibit paternal inheritance.

In homoplasmic inheritance, only the mitochondrial DNA from the female parent is passed on to the offspring (maternal inheritance). In contrast, heteroplasmic inheritance occurs when mitochondrial DNA from both male and female parents is inherited by the offspring. In both mitochondrial homoplasmic and heteroplasmic inheritance modes, the nuclear DNA is inherited equally from both parents.

**Heteroplasmy Detection Methods**

Mitochondrial heteroplasmy was first reported in *Drosophila mauritiana* before the development of PCR. At the time, researchers relied on

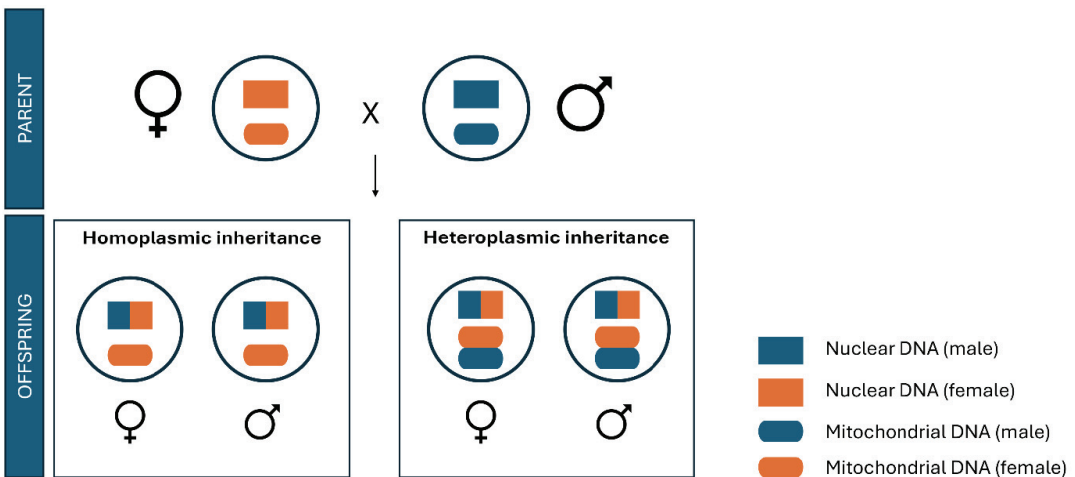


Figure 1: The concept of homoplasmy and heteroplasmy. In homoplasmic inheritance, only the mitochondrial DNA from the female parent is inherited by the offspring (maternal inheritance). In contrast, heteroplasmic inheritance occurs when the mitochondrial DNA from the male parent is also inherited by the offspring. The nuclear DNA is inherited equally from both parents in both mitochondrial homoplasmic and heteroplasmic inheritance modes

Table 2: Crustaceans exhibiting heteroplasmy

Family	Species	Heteroplasmy Information	Reference
Armadiillidae	<i>Cubaris murina</i>	Heteroplasmy at the dual tRNA alanine/valine	Doublet <i>et al.</i> , 2012
Armadillidiidae	<i>Armadillidium vulgare</i> <i>Armadillidium maculatum</i> <i>Armadillidium nasatum</i>	Heteroplasmy at the dual tRNA alanine/valine	Doublet <i>et al.</i> , 2012
Cylisticidae	<i>Cylisticus convexus</i>	Heteroplasmy at the dual tRNA alanine/valine	Doublet <i>et al.</i> , 2012
Majidae	<i>Maja brachydactyla</i>	Heteroplasmy of <i>COI</i> and <i>16S</i>	Rodríguez-Pena <i>et al.</i> , 2020
Palaemonidae	<i>Macrobrachium amazonicum</i>	Nuclear insertions of mitochondrial DNA and heteroplasmy of <i>COI</i>	Iketani <i>et al.</i> , 2020
Palinuridae	<i>Panulirus japonicus</i>	Nuclear mitochondrial pseudogene and heteroplasmy of <i>COI</i>	Chow <i>et al.</i> , 2021
Platyarthridae	<i>Platyarthrus hoffmannseggii</i> <i>Platyarthrus caudatus</i>	Heteroplasmy at the dual tRNA alanine/valine	Doublet <i>et al.</i> , 2012
Porcellionidae	<i>Porcellio gallicus</i>	Heteroplasmy at the dual tRNA alanine/valine	Doublet <i>et al.</i> , 2012
Portunidae	<i>Callinectes sapidus</i>	Heteroplasmy of <i>nad2</i> , <i>nad4</i> , <i>COI</i>	Williams <i>et al.</i> , 2017

restriction enzymes to characterise the two mitochondrial DNA variants found in eggs of single females, based on their differential digestion patterns (Solignac *et al.*, 1983). The advent of PCR enabled more precious detection of heteroplasmy, owing to its ability to identify individual sequence variations. Commonly used PCR-based methods for detecting

heteroplasmy include PCR/restriction fragment length polymorphism (RFLP) (Moraes *et al.*, 1992), real-time PCR (qPCR) based on allelic refractory mutation detection system (ARMS-qPCR) (Machado *et al.*, 2015), and the more recent digital PCR (dPCR) (Shoop *et al.*, 2022). A general comparison of these three methods is summarised in Table 3.

Table 3: Comparison between the three heteroplasmy detection methods

	PCR/RFLP	ARMS-qPCR	dPCR
Sensitivity	Moderate	High	Very high
Quantification	Limited quantification capabilities	Precise quantification of heteroplasmy occurrence	Precise quantification of heteroplasmy occurrence
Throughput	Low	Moderate	High
Complexity	Relatively simple	Moderate	Complex
Cost	Moderate	Moderate to high	High
Equipment needed	Standard laboratory equipment	Specialised qPCR equipment	Specialised dPCR equipment



PCR/RFLP combines the use of restriction enzymes to digest and produce distinguishable DNA fragments of different size and numbers, along with the amplification strength of PCR, thereby generating sufficient characteristic banding patterns to detect mutations within the mitochondrial genome (Wilding *et al.*, 1997). However, the effectiveness of PCR/RFLP is often hampered by the high formation of heteroduplexes during the PCR process (Moraes *et al.*, 1992). In addition to PCR, RFLP is often coupled with Sanger sequencing to characterise mitochondrial DNA fragment sequences, allowing for the detection of heteroplasmy as low as 15%. However, Sanger sequencing is not favoured by researchers for heteroplasmy detection due to the imprecise peak height calling at polymorphic nucleotide locations (Shoop *et al.*, 2022).

ARMS-qPCR functions by selectively targeting point mutations (i.e., single nucleotide polymorphism, SNP) in human mitochondrial DNA, and due to its increased oligonucleotide specificity, this method allows the detection of heteroplasmy at levels below 1% (Machado *et al.*, 2015; Ryan *et al.*, 2016). However, the use of qPCR requires conducting two separate assays, one for each haplotype (i.e., mutant and wild type) (Shoop *et al.*, 2022).

dPCR is similar to qPCR, but does not require a standard curve. Instead, it employs a massive sample partitioning strategy and Poisson statistics to provide real-time absolute quantification of the targeted DNA sequence within a sample. Due to its high sensitivity (Belmonte *et al.*, 2016), dPCR is particularly useful for detecting low-abundance targeted sequences such as SNPs, including heteroplasmic mitochondrial DNA mutations (Tytgat *et al.*, 2021; Shoop *et al.*, 2022). Characterisation of a specific SNP using dPCR can involve either two different fluorophore probes to mark and calculate the relative frequency of two distinct alleles or a single probe targeting the mutant haplotype. Shoop *et al.* (2022) reported that using the single-probe strategy with dPCR is feasible, with the wild-type mitochondrial DNA haplotype still being amplified, albeit

at a lower efficiency. Matsumoto *et al.* (2023) successfully used droplet dPCR (ddPCR) to detect mitochondrial DNA heteroplasmy of < 10% in humans. Although dPCR has not yet been used in crustaceans, it holds promise as a valuable tool for the future characterisation of mitochondrial DNA heteroplasmy in other organisms, including crustaceans.

## Impact of Heteroplasmy on Species Delimitation

### *Species Identification*

Delineating and delimiting species is the cornerstone of understanding the biodiversity and evolution processes. Due to the distinct maternal inheritance feature of mitochondrial DNA, the use of standard reference DNA sequences based on different fragments of mitochondrial genes aids in species identification, as well as in subsequent phylogeny and phylogeography studies (DeSalle & Goldstein, 2019). However, while nuclear mitochondrial pseudogenes (Numts) can lead to incorrect species delimitation (the detection of still unknown species based on the deviations from known sequences), and misinterpretation of their subsequent phylogenetic relationships (Kim *et al.*, 2013), reports on the misinterpretation of species delimitation due to mitochondrial heteroplasmy are limited. The almost similar pattern of heteroplasmy with other noisy sequences in the chromatograms of the targeted mitochondrial DNA sequences also complicates identification (Figure 2).

Nonetheless, Martínez *et al.* (2023) evaluated mitochondrial heteroplasmy as a potential confounding factor in phylogenetic and population genetic studies using bivalves with doubly uniparental inheritance (DUI) mitochondria, specifically the South American and Antarctic marine bivalve *Aequiyoldia eightsii* species complex. Compared with nuclear SNPs, mitochondrial sequences of the *A. eightsii* species complex within Antarctic populations, including *COI*, yielded ambiguous base calls and led to amplification bias, thereby overestimating species richness with high confidence. A significant percentage of Antarctic

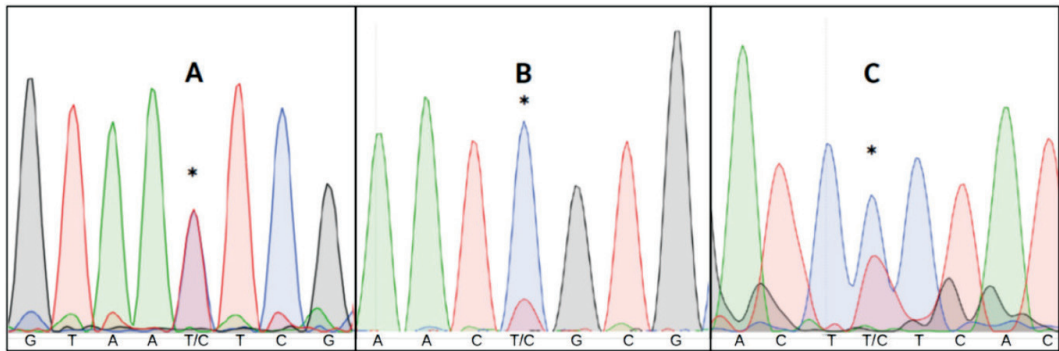


Figure 2: Examples of point heteroplasmy, where (A) represents likely heteroplasmy based on the completely overlapping fluorescent peaks; (B) represents likely background noise due to the lower secondary peak; and (C) represents putative heteroplasmy with background noise. Putative heteroplasmy are marked with \*. From Menéndez *et al.* (2023), used under Creative Commons CC-BY license (<https://doi.org/10.7717/peerj.16028>)

individuals from Potter Cove (40.4%) and Hangar Cove (6.3%) exhibited the co-existence of two haplotypes within a single organism. Martínez *et al.* (2023) suggested using several genetic sources, including nuclear regions, and implementing stringent quality control measures to prevent bias. Additionally, the future development of haplotype-specific and mitochondrial heteroplasmy-specific primers could be useful for ensuring accurate species delimitation.

### Phylogenetics and Evolution

The presence of heteroplasmy can obscure population and phylogeographic information about a species. This is evident in the bumblebee *Bombus morio*, where specimens from Teodoro Sampaio showed an average mitochondrial sequence divergence of 1.8% compared with that of other locations in Brazil (Cseri Ricardo *et al.*, 2020). However, Cseri Ricardo *et al.* (2020) also noted the presence of heteroplasmic haplotypes in Teodoro Sampaio and other sampling locations, resulting in a clustered topology. In contrast, earlier studies indicated the separating clade clustering of the individuals of Teodoro Sampaio, and that the mitochondrial haplotypes from this region could represent a new subspecies of *B. morio* or a new species (Françoso *et al.*, 2016).

### Future Perspectives and Directions

This review highlights the prevailing presence of heteroplasmy in crustaceans and the importance of investigating such phenomenon in commercially important species. Heteroplasmy in crustaceans can have both positive and negative impacts on populations; selective pressures may favour certain mitochondrial DNA variants, potentially leading to the persistence or elimination of specific variant populations over generations (Parakatselaki & Ladoukakis, 2021). Thus, understanding the underlying mechanism and documenting the occurrence of mitogenomic heteroplasmy, especially in crustaceans is crucial for species delimitation—the basis for various biological and conservational research, including sustainable fishery management and aquaculture production of commercially important crustacean species.

Future research should focus on developing diagnostic markers based on documented crustacean populations with mitogenomic heteroplasmic variants. By identifying consistent heteroplasmic patterns, researchers will be able to accurately identify and distinguish closely related populations or species (Tikochinski *et al.*, 2020). There is also an urgent need for long-term, large-scale sampling, and the application of high-throughput sequencing techniques

to amplify the complete mitochondrial genome such as ultra-deep next generation mitochondrial genome sequencing (Kelly *et al.*, 2017) or mitochondrial DNA analysis using Rolling circle amplification and Sequencing (MitoRS) (Marquis *et al.*, 2017) to ensure successful documentation of heteroplasmic variants present in crustacean populations of interest. For example, in blue swimming crabs (*Portunus pelagicus*), 92 heteroplasmic variants were identified from seven samples across three targeted mitochondrial DNA regions: The control region, *COI*, and NADH dehydrogenase subunit 2 (Koolkarnkhai *et al.*, 2019). Long-term monitoring of natural populations will facilitate the observation of temporal variations in heteroplasmic patterns and their correlation with fluctuating environmental factors and population dynamics (Wang *et al.*, 2023).

### Conclusions

Heteroplasmy is present in crustaceans and may interfere with various analyses and approaches involving mitochondrial DNA sequences. Its presence is not always detected without detailed characterisation and investigation, making the identification of heteroplasmy in crustacean species becomes relevant. The use of mitochondrial DNA sequences for DNA barcoding and phylogenetic analyses should be approached with caution, and heteroplasmy should not be dismissed, if present. The inadvertent inclusion of heteroplasmic individuals can hinder accurate delineation of species boundaries, leading to misinterpretation and overestimation of mitochondrial lineages, ultimately resulting in incorrect taxonomic conclusions. Such confusion can have serious implications for conservation, fishery management, and aquaculture of crustaceans. Therefore, it is recommended that future efforts focus on developing an online database containing known sequences and primers for heteroplasmy detection in crustaceans across various geographical populations to ease the identification and reassessment of heteroplasmy status.

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### Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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