



Methods used in sea turtle genetic and molecular studies

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Sea turtles have a tropical and subtropical distribution and can be found in nearly all seas and oceans. They have been the subject of considerable genetic research. However, it does not yet appear that the molecular techniques used for these genetic studies follow a consensus or universal set of tools to be followed for subsequent studies. This is not desirable since it may preclude data exchange and use among studies. Thus, the aim of this review was to survey the main genetic and molecular methods used for sea turtle research worldwide. To achieve this goal, a total of 95 scientific papers were compiled from online databases. We considered articles that used molecular tools for genetic analysis and provided detailed locality data. The following aspects were assessed: species studied, local of sample collection, type of tissue used for molecular studies and type of genetic material used. The seven known sea turtle species have different distribution patterns, with some overlapping of occurrence. *Chelonia mydas* has been studied genetically along the coasts of all continents. Skin is the most common type of tissue used for molecular analyses. From genetic studies on sea turtles, it is possible to verify the occurrence of hybridism. This phenomenon is relevant to the conservation of the species and was reported in six articles on this topic. Therefore, it is considered that genetic and molecular assays in sea turtles are important tools for biological evaluation and protection. The aim was to survey the main methods used in genetic and molecular research on marine turtles.

Keywords: DNA, Chelonian, Testudines, marine turtles

INTRODUCTION

Sea turtles are highly migratory reptiles that are capable of transoceanic migrations (Boyle et al., 2009). They have a relatively high rate of gene flow, complex population structure and show multiple paternity (Bowen & Karl, 2007). Hybridisation in sea turtles has been reported, indicating that genetic introgression occurs across different species (Vilaça et al., 2012). Population genetics studies of sea turtles are challenging, due to the constant modifications in populations and the long and complex life cycle, including the alteration of habitats during life stages, which alters the behaviour of the group (McClellan & Read, 2007; Arthur et al., 2008).

Molecular studies have provided significant insights into sea turtle populations worldwide, including results on genetics, phylogenetics, evolution and conservation (Naro-Maciel et al., 2008; 2010; Barley et al., 2010; Shamblin et al., 2012a; Bradshaw et al., 2018; Camacho-Sánchez et al., 2022). Phylogenetic analyses using nuclear markers reveal a possible pattern of introgression between species of sea turtle populations in Brazil (Vilaça et al., 2012). Furthermore, the combination of nuclear and mitochondrial markers analyses has proven to be effective for evolutionary and genetic studies in the

group (Naro-Maciel et al., 2008; Garofalo et al., 2013; Vilaça et al., 2022) and has allowed the identification of hybrids (Arantes et al., 2020a; 2020b; Brito et al., 2020). In addition, analyses of polymorphic regions and the use of microsatellite markers have provided interesting results that are relevant to the ecology and conservation of the group (Roden & Dutton, 2011; Chow et al., 2019; Zhang et al., 2021; Naguib et al., 2022).

The present study aimed to compile the methodologies and results used from genetic and molecular analyses involving sea turtles worldwide. This information should provide a clear picture of the main gaps in the current knowledge about marine turtle genetics and indicate new approaches to study this complex group.

Sea turtles genetic studies in worldwide

We compiled 95 research papers retrieved from scientific online database that covered studies on population genetics and phylogeography of all seven sea turtle species known worldwide. These articles were published between 2007 and 2022, and all of them specified the use of molecular analyses. Only studies in which the information about species and occurrence location are clear were considered for this evaluation. However, some research did not present clear information on specific

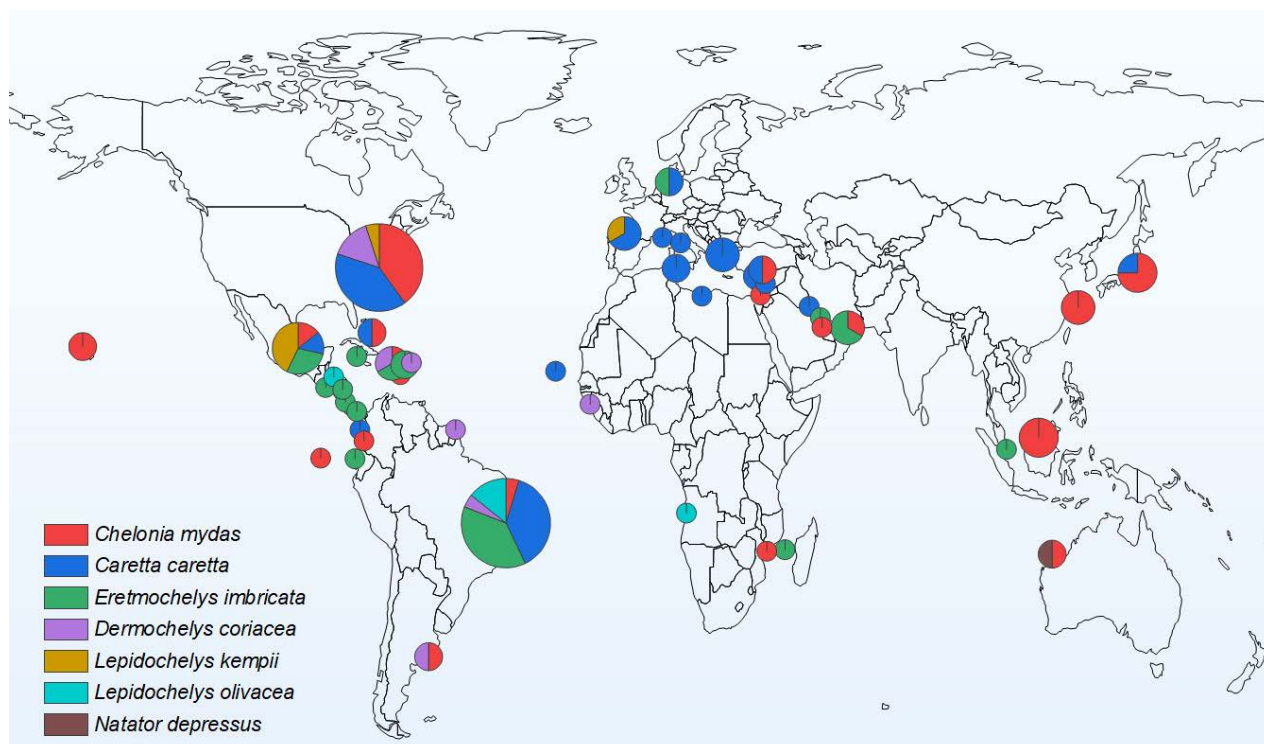


Figure 1. Map of the reporting of sea turtle species, by sampling locations, registered in the literature

points, such as the gene used for the amplification of the genetic material, the exact country in which the samples were collected or the type of tissue used for DNA extraction.

The articles analysed sea turtles recorded in 46 different countries. The continents that were most present in the studies were North America ($n = 27$) and South America ($n = 18$). The USA and Brazil were the countries most approached in the surveys. The analyses indicate a predominance of studies about the genetics and phylogeography of sea turtles in the Atlantic Ocean (Fig. 1).

Sea turtles are distributed worldwide in tropical and subtropical waters. The Atlantic Ocean is an area of intense occurrence of species, in different stages of life and foraging behaviour (Hays et al., 2006; Cantor et al., 2020; Colman et al., 2019). The place is also known as a refuge area for hatchlings during the period following emergence (a period known as the "lost years"). At this stage, the hatchlings are adrift at sea, being observed again only when they are young (Carr, 1987; Putman et al., 2020).

Molecular analyses using mitochondrial DNA (mtDNA) indicate that the genetic structure of populations of *Caretta caretta* in the Atlantic Ocean is modified, requiring follow-up studies in the region. For *C. caretta*, the Atlantic Ocean proves to be a place of high complexity for genetic stocks and a complex network of migration paths (Shamblin et al., 2012b; LaCasella et al., 2014; Naro-Maciel et al., 2014). This complexity promotes high interest in investigative studies on the molecular genetics of Atlantic Sea turtle populations.

Our analyses indicate a prevalence of studies that included the species *C. caretta* ($n = 38$), followed by

Chelonia mydas ($n = 34$) and *Eretmochelys imbricata* ($n = 25$) (Fig. 2A). Studies revealed a high complexity of the genetic structure in *C. caretta*, with the occurrence of subpopulations, in addition to a correlation between foraging behaviour and genetic structure (Watanabe et al., 2011).

The population of loggerhead (*C. caretta*) in the French Mediterranean presents a complex diversity, in which different origins have been identified for different groups within this population, obtaining different and complex results with mitochondrial and nuclear analyses (Loisier et al., 2021). Reports indicate hybridisation of the species *C. caretta* and *E. imbricata* (Proietti et al., 2014; Arantes et al., 2020a).

It is possible that the large number of studies on loggerhead (*C. caretta*), green (*C. mydas*) and hawksbill (*E. imbricata*) sea turtles are a consequence of the wide distribution of these species worldwide and of the reports of their hybridisation. There is a well known connection between *C. mydas* foraging areas in Brazil and genetic evidence indicates that turtles from different regions share feeding areas, thus enabling greater connectivity between groups (Naro-Maciel et al., 2007).

The sea turtle species share feeding and nesting areas. Evidence indicates that there is gene flow between species in different locations (Vilaça et al., 2021; Roberts et al., 2004). Genetic analyses of sea turtle populations are essential to fully understanding how gene flow occurs within the group, as well as the occurrence of subpopulations, new haplotypes and hybridisation within and across populations (Vilaça et al., 2012; Roden et al., 2013; Arantes et al., 2020b; Camacho-Sánchez et al., 2022).

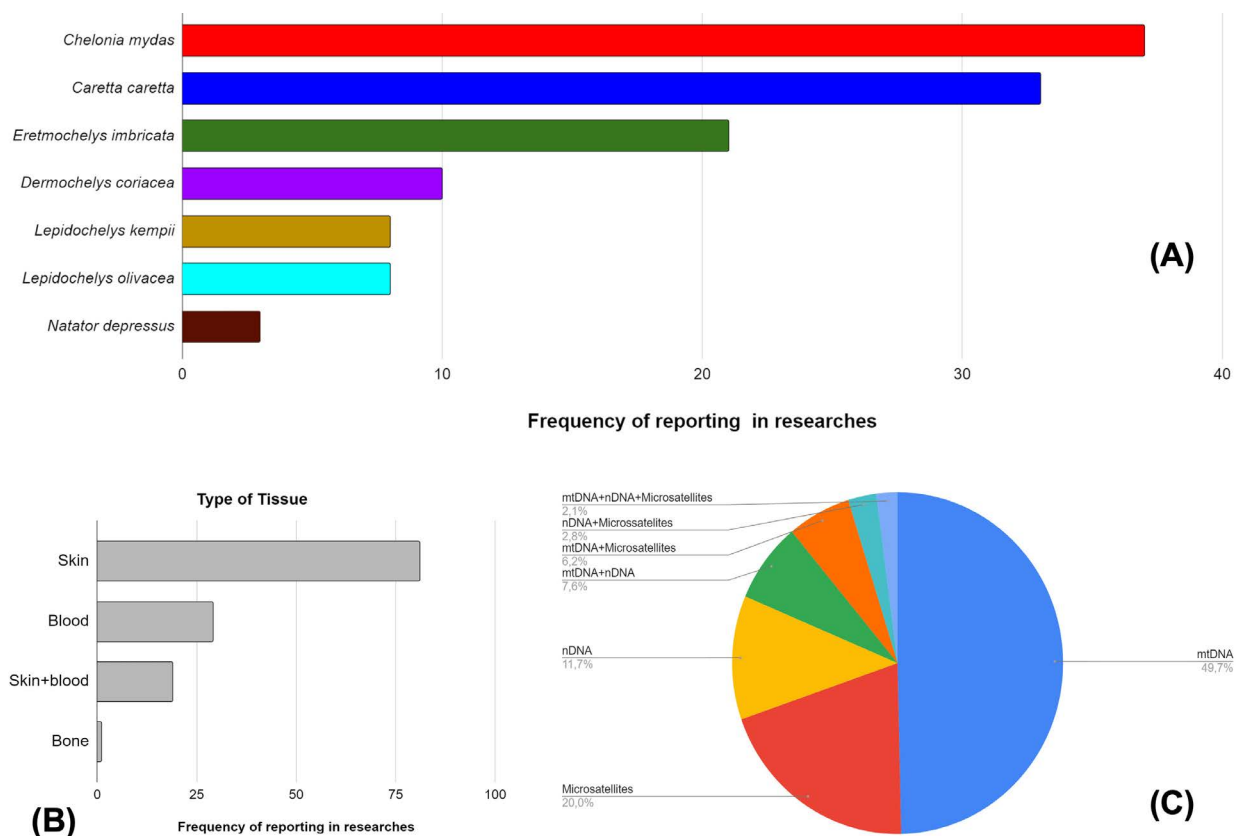


Figure 2. Data summary panel- **(A)** frequency of reporting of sea turtles species according to literature, **(B)** frequency of reporting of type of sea turtle tissue used for molecular analyses, **(C)** percentage of reporting of type of genetic material used on molecular analyses

Methods for molecular analysis

Tissues used for DNA extraction

Four different tissues were reported for the extraction of DNA. Skin was the most frequent, as observed in Alstad et al. (2011), Arantes et al. (2020a), Brito et al. (2020) and Zhang et al. (2021) (Fig. 2B). “Muscle” was not frequently mentioned, but possibly some authors categorised this type of tissue as “skin/tissue”, not making it clear exactly which type of tissue was used. Blood was also used for the analyses, although much less frequently. Blood samples were used for the characterisation of the complete mitochondrial genome, studies on hybridisation impacts and characterisation of molecular markers (Drosopoulou et al., 2012; Dutton et al., 2013; Soares et al., 2018). Blood samples were not generally used for molecular analyses. This may be due to the fact that blood collections require laborious handling of live animals, and that some analyses with blood tissue must be performed promptly after collection to avoid cell degradation. After collection, hematocrits are obtained by centrifuging the blood and are stored at 4 °C (van de Merwe et al., 2010), which is not always possible during field activities.

A study with *Lepidochelys kempii* compared the efficacy of blood samples, cheek swabs, cloacal swabs, tissue and dermal scales. The results showed a very similar quality of DNA obtained from blood and tissue samples, with both techniques indicated for genetic studies (Stephens et al., 2010). Collecting other tissue samples is generally more practical in the field than collecting blood, which requires specific training and more sampling material.

Krestoff et al. (2021) reported the use of bones for extracting DNA. The authors used bones from juvenile *L. kempii* between 1 and 2 years old and obtained high quality mtDNA sequences. This fact suggests an alternative for molecular studies when there is no possibility of obtaining another type of tissue.

Non-invasive sampling has been used in various instances as a source for sea turtle DNA. Lanci et al. (2012) reports the use of swabs with buccal and cloacal content to obtain samples for DNA extraction. Even though the authors obtained good quality DNA, they still recommend extraction from tissues such as blood and skin to obtain high quality DNA. DNA extraction and analysis of microsatellite markers from eggshell samples of non-viable *C. caretta* hatchlings was efficient in terms of quantity and quality of genetic material (Shamblin et al., 2011). Environmental DNA samples from sea turtles can also be extracted from water and sand samples in nesting areas (Farrell et al., 2022). The literature presents a good range of tissue possibilities for DNA extraction from sea turtles, all of them producing reliable results (Roden et al., 2013; Proietti et al., 2014; Bradshaw et al., 2018). However, blood samples require greater storage and collection criteria, while bone samples demand a more complex methodology for cleaning and purifying the DNA after extraction. Skin seems to be the most versatile source of DNA, since it can be obtained from animals, at all life stages, with minimal animal discomfort.

Table 1. Sizes of fragments (bp) generated by PCR amplifications with corresponding mitochondrial targets, primers and molecular analyses

Region	Species*					
	CM	CC	EI	DC	LO	LK
COI						
L-turtCOI/H-turtCOIc	815					
VF/VR						624
CONTROL REGION						
LTCM2/HDCM2	457–481			496		
LTCM1/HDCM1	486–489					
LCM15382/H950	740–818	683–859	740–880	496–832	800–890	800
LCM15382/CC443		241–817				
LCM15382/H599		380				
TCR5/TCR6	380–396	380				
CMD1 F/CMD5R	200					
LTEi9/H950	800		740			
TCR5/TCR6	360					
TCR6/L15926			486			
LTEi3/HDEi1			550			
L71/H599		510				
CMMTF1/TCR	520					
LTCM2/HDCM2	481			496		
HDCM-1/TCR5		306				
LCM15382/H950	740–800	723–830	740–850	740	830–890	
Ei9F/H95R	800					
TCR5/TCR6	360					
16S						
16SAR/16SBR	550					
CYT-B						
MT-NAD4F/MT-NAD4R						371
STR						
CM-D-1/CM-D-5	400					

*CM: *Chelonia mydas*; CC: *Caretta caretta*; EI: *Eretmochelys imbricata*; DC: *Dermochelys coreacea*; LO: *Lepidochelys olivacea*; LK: *Lepidochelys kempii*

mtDNA and nDNA gene analyses

A high percentage of articles (65%) analysed mtDNA (Table 1) as the main methodology for genetic analysis. Of these, combinations of mtDNA and nuclear DNA analyses were used in 7% of the articles, and 6% combined mitochondrial and microsatellite markers, indicating interest in research about polymorphisms found in different species of sea turtles (Fig. 2C).

Mitochondrial sequences provide information on maternal lineages with no recombination and are useful for determining the evolutionary histories of different groups (Otálora et al., 2018). They are also useful for species identification through barcode analysis (Camacho-Sánchez et al., 2021).

The main amplified mitochondrial regions and the size of the respective fragments are shown in the table above. The only region that has been analysed in all species is the Control Region, specifically the fragment amplified by LCM15382/H950, which makes this region a strong candidate for identification and genetic comparison between species. Only one study carried out

the complete sequencing of the mitochondrial genome, corresponding to *C. caretta*.

We observed a smaller variety of regions and primers for nuclear genes (Table 2), with no simultaneity of use between species for any of them. Specific data on the size of the fragment amplified with each gene in relation to different species was not provided. However, the CMOS, R35, RAG1, RAG2 and BDNF genes were used to amplify *C. mydas* (CM), *E. imbricata* (EI), *C. caretta* (CC) and *Lepidochelys olivacea* (LO) samples (Vilaça et al., 2012; Soares et al., 2017; Brito et al., 2020).

Use of highly polymorphic short tandem repeat (STR) markers for *C. mydas* revealed a genetic structure not previously found for the Mediterranean population, in addition to indicating novel migratory routes for the population (Tikochinski et al., 2018). A study of sea turtle phylogenetics based on mitochondrial and nuclear genes shows similar results for both sets of markers (Naro-Maciel et al., 2008). In contrast, a recent study indicated that *E. imbricata* individuals that had been identified both morphologically and by mtDNA analysis, showed

Table 2. Sizes of fragments (bp) generated by PCR amplifications with corresponding nuclear targets, primers and molecular analyses and the respective species used in the studies

Region	Species*						
	CM	CC	EI	DC	LO	LK	ND
MHC	162	162					
CMOS	x	601	601		x		
RAG1	x	x	368		x	624	
RAG2	x	x	620		x		
R35	x	x	439		x		
BDNF	x	x	559		x		
Other techniques							
Microsatellite	x	x	x	x	x	x	x
SNP	x						
RFLP	x						
ddRAD						x	

*CM: *Chelonia mydas*; CC: *Caretta caretta*; EI: *Eretmochelys imbricata*; DC: *Dermochelys coreacea*; LO: *Lepidochelys olivacea*; LK: *Lepidochelys kempii*; ND: *Natator depressus*

evidence of hybridisation with *C. caretta* when nuclear sequences were analysed (Brito et al., 2020).

The combination of mtDNA and nDNA was also used to characterise the genetic diversity of different sea turtle populations in the Mediterranean, Turkey and Brazil (Yilmaz et al., 2011; Garofalo et al., 2013; Vilaça & Santos, 2013). This approach has similarly been used to investigate multiple paternity in *L. olivacea* in Honduras (Duran et al., 2015). Thus, in complex genetic structures, with the occurrence of hybridism, gene flow and migratory behaviours, such as those found among sea turtles, it seems highly advisable to use methodologies that encompass both mitochondrial and nuclear regions, ensuring that no relevant genetic information is missing. The combination of both genetic markers has proven important for unravelling phylogenetic relationships and provided new perspectives on relationships between species, for example, on the position of *Dermochelys coriacea* within the genetic relationships of turtles (Deef, 2023).

The use of microsatellite markers was notably prevalent in sea turtle studies. This approach consists of genotyping mainly anonymous nuclear loci characterised by the presence of STR of two, three or four nucleotides. Specific primers that allow amplification of 12 microsatellite loci in *C. mydas* from Hawaii have been described (Dutton & Frey, 2009). Microsatellite markers have also been described for *L. olivacea* (Aggarwal et al., 2008), *E. imbricata* (Lin et al., 2008; Miro-Herrans et al., 2008) and *C. caretta* (Shamblin et al., 2007).

The identification of microsatellite markers enables studies of population, kinship, reproductive and migratory behaviour of sea turtles. Inbreeding identification and cross-amplification tests were successfully performed for *C. mydas* using microsatellite markers (Dolfo et al., 2023).

The genetic structure of populations of *E. imbricata* (Mostafavi et al., 2011), *C. mydas* (Roden et al., 2013) and *D. coriacea* (Dutton et al., 2013), were also studied using microsatellite analyses. Tetranucleotide microsatellite loci have been described for *C. caretta* (Shamblin et al., 2007; 2009) and *D. coriacea* (Alstad et al., 2011). Co-dominant and selectively neutral molecular markers, such as microsatellites, are effective in studies on the level of variability between species in temporal and geographic contexts, including the philopatric behaviour of the group (Bradshaw et al., 2018).

Hybrid identification

Hybridism in sea turtles has not been widely studied yet. On the Brazilian coast, there is a high level of hybridisation between hawksbill (*E. imbricata*) and loggerhead (*C. caretta*) turtles (Lara-Ruiz et al., 2006; Proietti et al., 2014; Arantes et al., 2020a). Crossings between loggerhead and olive ridley (*L. olivacea*) also occur frequently (Soares et al., 2017). It is possible that there is significant gene exchange between *C. caretta* and *E. imbricata* in the region (Arantes et al., 2020b) which is an important migration, foraging and spawning route for five species of sea turtles. The high rates of hybridism observed to date indicate an important complexity of gene flow between species.

Molecular investigations based on mtDNA alone are insufficient to identify hybridisation, given that information on paternal DNA is lacking. The maternal genetic signature may coincide with the morphology of the animal, masking a possible hybrid occurrence. Thus, studies with a combination of mtDNA and nDNA are indicated to obtain a more complete information about the genetics of populations (Brito et al., 2020).

The behaviour of hybrid individuals may be different from their parent species, likely causing some kind of ecological imbalance in the dynamics of groups (Proietti et al., 2014). Hybrid hatchlings have been shown to be viable, with no indication of a decline in eclosion success (Soares et al., 2018). A study identified turtle hybrids in Abrolhos (Brazil), an area where this phenomenon was not previously reported, likely leading to a decrease in the reproductive success of the group (Arantes et al., 2020a).

As these are complex populations that are in constant gene flow and migration, the authors encourage further studies on sea turtle populations, requiring a complete approach with mtDNA and nDNA. Monitoring and government conservation actions are also necessary, in order to instigate investigations of the problem around the world.

Next generation sequencing (NGS) and Restriction site associated DNA sequencing (RADseq)

Phylogeny and characterisation studies of hybrid animals has been carried out using NGS, indicating that the technique brings significant advances in solving genetic problems in the group.

NGS has also made important contributions to the ecological studies of sea turtle populations. NGS was

used to address aspects related to population studies of hybrid animals and investigation into paternity of multiple species, including fitness analyses. One study did not indicate negative impacts when they compared hatchling production rate per nest and viability of hybrid hatchling and parental species, when they investigated only the first generation of hybrids (F1) (Soares et al., 2018).

Arantes et al. (2020b), analysing sea turtles population structure, identified polymorphic sites and inter and intraspecific relationships. This study indicates that there is a male-mediated gene flow that connects populations of loggerhead and hawksbill turtles on the Brazilian coast, with the occurrence of second-generation hybrids (F2). However, the authors conclude that F2 hatchling female hybrids may not survive to adulthood or be less fertile.

The ddRAD-seq technique is a powerful tool that does not require a reference genome and provides sequences from a sample of loci from across the genome. Microsatellites were identified in ddRAD sequences from *L. kempii*, which provides additional material to be used in genetics and conservation research of the group (Hurtado et al., 2016). Furthermore, ddRAD has been used in assays investigating hybridisation and introgression patterns, in which the results indicate cytonuclear incompatibility, resulting in a population decline in future generations (Vilaça et al., 2023).

Arantes et al. (2020a) used the 3RAD (triple-enzyme RADseq) protocol to investigate sea turtles from the Brazilian coast and identified the backcrossing of F1 hybrids and loggerhead turtles. This protocol works as efficiently as other RADseq analyses. However, a lower concentration of DNA is required, with lower initial costs, and can be pooled with other Illumina libraries (Bayona-Vásquez et al., 2019).

Final considerations

In the past 15 years, studies on the population genetics and phylogeography of sea turtles have provided significant advances in the knowledge of these endangered species, promoting a better understanding of their biology and providing a basis for the implementation of effective conservation measures. Although numerous articles have mostly made use of mtDNA sequences, recent studies highlight the importance of assessing nuclear as well as mitochondrial genetic information. Aside from genomic studies, highly variable co-dominant markers, such as microsatellites, seem able to provide the greatest amount of information. This is a good strategy for various purposes in population genetics, relatively practical and more affordable because it is well established.

Molecular assays to detect the genetic inheritance of two parental species of sea turtles were recently performed using RAD-seq and next generation sequencing (NGS) analysis. The results show a possible reduction in the success of hybrid offspring, caused by nuclear incompatibility (Vilaça et al., 2023). These new molecular techniques will be used to answer more detailed questions about the future of sea turtle species.

The reviewed studies report important discoveries about the genetics of sea turtle populations, especially in the Atlantic Ocean. The high complexity of the genetic composition of the Atlantic populations, the high rates of gene flow and the diversity of migratory routes, point to the need for continued studies in the area, including the combination of different techniques using mitochondrial and nuclear genes. Careful attention should be paid to the Mediterranean, which has been the subject of fewer sea turtle studies.

The occurrence of hybrids must be approached with the highest attention and urgency due to the appearance of hybrid individuals in regions where they were not found before. Efforts need to be made to minimise the impacts suffered by the sea turtles, which are quite vulnerable to the environmental changes that have occurred at a rapid rate in recent years. Countries that register sea turtles and their hybrids must have policies that value the preservation of their coasts and consider strategies to mitigate the problem of hybridism, a problem for which the causes are yet to be identified and fully understood.

Finally, we reinforce the importance of using methodologies that are less invasive for the animals. Knowledge about more effective methodologies in chelonian studies will help foster molecular biology studies aimed at obtaining results that can be directly applied to the conservation of all sea turtles.

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Author Contributions

Cibele Castro Monteiro: Conceptualisation, data curation, writing, original draft, review and editing, supervision. Jhonatas Teixeira Viana, Karen Karoline Abreu da Silva, Rafael dos Santos Rocha: Data curation, writing original draft, review and editing. Vicente Vieira Faria, Rodrigo Maggioni: Data curation, writing original draft, review and editing, supervision.

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