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Research Article

From genes to conservation: The potential of genetic resources of Amphibian in Malaysia for next generation monitoring and conservation strategies

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Abstract

Genetic monitoring plays a crucial role in the conservation management of amphibians due to their vulnerability to habitat changes. Traditional biodiversity monitoring methods, such as trapping and morphological identification, are being complemented by advanced technologies that enhance species identification sensitivity and monitoring capabilities. Genetic resource availability is essential for effective amphibian biodiversity assessment and monitoring. This study aims to evaluate the current state of genetic resources and their comprehensiveness for amphibian biomonitoring and conservation in Malaysia. The study focused on seven targeted mitochondrial DNA loci, and data regarding these loci were obtained from the National Centre for Biotechnology Information (NCBI) database. The results showed that 69.8% of amphibian species in Malaysia had genetic data available for at least one or more of the targeted genes, with the 16S gene being the most widely represented. However, only 12.1% of threatened species and 61.2% of endemic species had accessible genetic information. Biological utilization was identified as the primary threat to amphibian species in Malaysia, accounting for 27.0% of recorded threats. These findings highlight the need for further improvement in achieving comprehensive genetic resource information for amphibians in Malaysia. Advancements in enpromisesntal DNA (eDNA) analysis and Next Generation Sequencing (NGS) technologies hold promise in enhancing efforts to conserve amphibian diversity. It is crucial to continue expanding genetic resources to support ongoing monitoring and preservation initiatives for amphibians in Malaysia, ensuring effective conservation management.

Keywords: Amphibian, Ecology, Genbank, Mitochondrial DNA, Molecular

Introduction

Malaysia is recognized as one of the mega-diversity countries, ranking 12th globally in terms of biodiversity. This highlights the country's remarkable richness and endemism in various flora and fauna. In the Malaysia National Report to the Convention on Biological Diversity in 2015, it was estimated that approximately 242 species of amphibians have been documented in Peninsular Malaysia, Sabah, and Sarawak. Furthermore, ongoing discoveries continue to contribute to the growing inventory of amphibian species in the country. While Malaysia is often associated with iconic animals like the Malayan tiger, Asian elephant, and Orangutan, it is important not to overlook the diversity of other animal groups found throughout the country. One such group is the Anurans, which includes frogs, toads, and caecilians. These amphibians represent a significant component of Malaysia's diverse fauna.

According to Norhayati (2017), amphibian species in Malaysia belong to seven major families namely Bufonidae, Dicroglossidae, Megophyridae, Microhylidae, Ranidae, Rhacophoridae, Rhacophoridae and Ceratobatrachidae found in both Peninsular Malaysia and Borneo. Amphibians are one of the important and diverse components of many terrestrial and freshwater ecosystems (Collins & Crump, 2009), contributing to a diverse range of ecological functions (Tyler et al., 2007; Collins & Crump, 2009). According to a study carried out by Cortes-Gomez et al. (2015), amphibians contribute to at least five categories of ecological functions including energy flow through ecosystems, pollination, seed dispersal, bioturbation, and nutrient cycling. Frogs are considered as one of the most sensitive animals in the world due to their skin and react adversely to changes in their environments which makes them an indicator species that can indicate the status of the ecosystems (Saber et al., 2017). The presence of several amphibian species in one region might signify a healthy natural ecosystem due to their life cycle requirement being met (Izam et al., 2019). Amphibian species transmit nutrients from aquatic to terrestrial systems and devour detrital and leaf-litter invertebrates on the forest floor because of their biphasic life cycle (Fulton, 2013).

The decline in fauna diversity in Malaysia, including amphibians, is indeed a matter of concern. Anthropogenic activities, as highlighted by Aiken and Leigh (1985), have had a significant impact on the wildlife of the country over the past few decades. Habitat loss and degradation have resulted in the decline of iconic species like the Malayan tiger (Ten et al., 2021) and Asian elephant (Saaban et al., 2020). Despite the implementation of various conservation approaches, such as legal instruments and protected areas, the pressure on Malaysia's biodiversity continues to increase, and forest cover loss persists. This situation is particularly evident in the case of amphibians, which have experienced substantial declines and even extinctions globally (Zhengjun & Yiming, 2004).

Deforestation, as reported by Asad et al. (2020) and Gaveau et al. (2014), plays a significant role in the declination of amphibian populations in Malaysia. The country, including Borneo, has been experiencing one of the highest deforestation rates globally, with logging concessions covering a large portion of the remaining forests. The loss of canopy cover due to logging activities can lead to increased desiccation rates in amphibians, making it challenging for them to maintain optimal moisture content (Semlitsch, 2000). Conversion of natural forests to agricultural areas, such as oil palm plantations, further exacerbates the decline of amphibians and overall biodiversity. The rapid global demand for palm oil has contributed to high levels of carbon emissions and biodiversity loss (Taheripour et al., 2019). Research by Graeme et al. (2012) has shown that oil palm plantations have lower species richness of frogs compared to other habitat types, leading to population fragmentation and extinctions. These landscape changes and habitat loss have had severe consequences for amphibians in Malaysia, highlighting the urgent need for conservation efforts, habitat protection, and sustainable land-use practices.

Many efforts have been taken to protect threatened species and their habitats in order to prevent further declination and this can be done by gathering information about their distribution, diversity and ecology of the species through biodiversity monitoring (Zainal-Abidin & Noor-Adelyna, 2020). Traditionally, biodiversity monitoring on amphibian diversity were done either with active technique or passive techniques sampling. Most of the inventory studies in Malaysia used passive sampling techniques such as drift-fenced traps and visual encounter survey to determine the diversity which needed a lot of times and manpower to be completed (Ehwan et al., 2016; Fatihah-Syafiq et al., 2020). According to Ali et al. (2018), passive sampling techniques is much better method compared to visual searches as the method able to capture rare species which have its own conservation importance. Besides, the species identification process still uses traditional method which is based on morphological characteristics which can discriminates and misidentify species with taxonomy confusion that have various external body colorations especially when specimens are not fresh, museum preserved species and samples showing phenotypic plasticity (Indahsari et al., 2019). With the ongoing reduction in the number of taxonomic experts, the traditional identification-based methodology may be hindered, perhaps might introducing bias in the assessment (Shaw et al., 2016). These commonly practiced in biodiversity monitoring require some improvement in order to protect the species. Therefore, more detailed, and extensive tools must be developed to supplement the limitation of the existing conventional method.

Recent developments in molecular technology have opened a new chapter in the effort to conserve species. With the development of genetic tools and combination of powerful computation, molecular approach had become an alternative for species identification besides morphological characteristics. Approaches such as DNA sequencing allows for identification of parentage, more distant relatives, new populations, unidentified individuals, and others (Haig, 1998). DNA barcoding for amphibian were used in order to help in wildlife management and conservation since the method is able to give the accuracy for species identification especially from unknown samples (Jeong et al., 2013; Hebert & Gregory, 2005). One of the latest approaches for assessing and monitoring amphibian diversity is through environmental DNA. In contrast to conventional survey methods, eDNA offers the advantages of being non-invasive (no organisms are caught, disturbed, or killed during monitoring) and reduces the risk of unintentional secondary dispersal of alien species and diseases (Valentini et al., 2015). Overall, eDNA methods used for species detection demonstrated higher detection capability and cost-effectiveness compared to traditional methods (Darling & Mahon, 2011; Dejean et al., 2012). There are two main approaches using eDNA: eDNA barcoding, which aims at detecting a single species in the environment (Dejean et al., 2012) and eDNA metabarcoding which identifies several taxa simultaneously from an environmental sample without prior knowledge of the species likely to be present in the sampled ecosystem (Taberlet et al., 2012; Li et al., 2021; Sakata et al., 2022). The latter method is based on the use of primers that is specific for a certain taxonomic group along with the latest sequencing technology called Next-Generation Sequencing (NGS).

In Malaysia, genetic studies of amphibians started around 2005 which can be considered late compared to other countries which may be due to the lack of expertise and the high cost of genetic sequencing in the past twenty years (Chan et al., 2022). Genetic studies can improve the availability of genetic resources because of their capability to determine the species complexes within populations that can complement traditional taxonomy in a shorter time (Iwatsuki et al., 2015). Because the samples for eDNA metabarcoding do not comprise the entire organism, in situ comparisons are not possible (Deiner et al., 2017). The only way to determine taxonomic affinities is to compare the retrieved sequence to taxonomically annotated sequences, such as those in the NCBI Genbank nucleotide database (Benson et al., 2013). Therefore, developing genetic resources for amphibians is crucial so it can be used as references in order to implement eDNA metabarcoding and monitoring. In this study, we focus on determining the current genetic resources and its comprehensiveness along with reviewing the conservation status, endemicity and threats to amphibian diversity in Malaysia. The findings of the study can be used for future application of next generation monitoring and conservation on amphibians to prevent from extinction risk.

Material and methods

In this study, several information was collected and analysed. A full checklist of amphibian in Malaysia from Amphibian Species of the World (https://amphibiansoftheworld.amnh.org/) were retrieved. The species listed were screened and verified before the final checklist was finalized. Next, the genetic resources of seven targeted locus of mitochondrial DNA (mtDNA) which is 12S Ribosomal RNA (12S), 16S Ribosomal RNA (16S), Cytochrome b (Cytb), Cytochrome oxidase subunit I (COI), Nicotinamide adenine dinucleotide dehydrogenase (NADH), Displacement loop (D-loop) and Transfer Ribonucleic acid (tRNA) were retrieved from a public genetic repository National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Each species was searched using its latest species name and to verify the availability of the locus that was conducted in July 2022.

Then, the conservation status of each amphibian species was assessed based on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, which classifies species into various conservation categories: Not Evaluated (NE), Data Deficient (DD), Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), and Critically Endangered (CR). Furthermore, additional information regarding the level of threats and endemicity for each species was obtained from the IUCN and Amphibian Species of the World, respectively. Then, the acquired data were organized and sorted using Microsoft Excel, facilitating the representation of the findings through data visualization techniques involving charts and graphs.

Results

A total of 288 species of amphibian in Malaysia were assessed through Genbank and there were 686 records of genetic information on the seven targeted genes have been recorded. Among the seven targeted genes, information on 16S rRNA was available for most of the species, which is 200 species (29.2%), followed by the 12S ribosomal RNA gene (12S) with 175 species (25.5%), and the transfer ribonucleic acid (tRNA) with 120 species (17.5%). The D-loop gene is the least information available with only 13 species (2.1%) (Fig. 1).

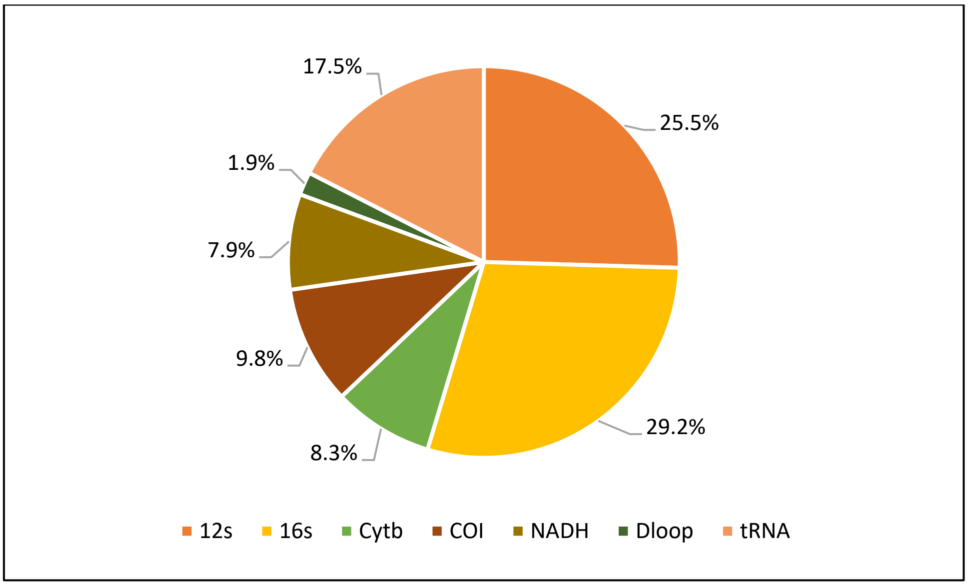

Figure 1 The distribution of amphibian species in Malaysia based on seven targeted genes of Mitochondrial DNA (mtDNA).

Based on the three genes that have the most information among the seven targeted genes, 69.4% of amphibian species in Malaysia had genetic sequences for 16S gene with 30.6% of the remaining species having not been assessed yet. For 12S gene, there is about 60.8% of amphibian species have data sequences available in Genbank with the remaining 39.2% of species having no genetic information available. For the tRNA gene, the genetic sequence for 41.7% of the species are available. For the remaining targeted genes including Cytb, COI, NADH and D-loop, the data sequence available ranged from 5% to 25% which is relatively few compared to the number of species that have not been accessed yet.

Only 9 species (3.1%) have completed genetic information on seven targeted genes out of total 288 species while 200 species (69.8%) had information at least one or more genetic information out of the seven targeted genes. The remaining 79 species (27.4%) lacked any information on their mtDNA. The species which have data sequence for all the seven targeted genes including two species from the genus *Microhyla* (*Microhyla butleri*, and *Microhyla heymonsi*) and genus *Fejervarya* (*Fejervarya cancrivora* and *Fejervarya limnocharis*). The other remaining five species that have sequences for all the seven targeted genes belonged to different genera including *Duttaphrynus*, *Haplobatrachus*, *Kaloula*, *Lithobates* and *Odorrana*. Among the 79 species with no data available in Genbank, 10 species were from the genus *Leptobrachella* that were the least studied species concerning genetic information followed by eight species from the genus *Leptomantis* and *Kalophrynus*.

There are eight families of amphibians in Malaysia based on the total 288 species that had been examined. Among the eight families, Ranidae had the most genetic information recorded with 163 (23.7%) data sequences, followed by the family Bufonidae with 132 (19.2%) data sequences for at least one of seven genes of interest and Microhylidae with 115 (16.7%) data records (Fig. 2).

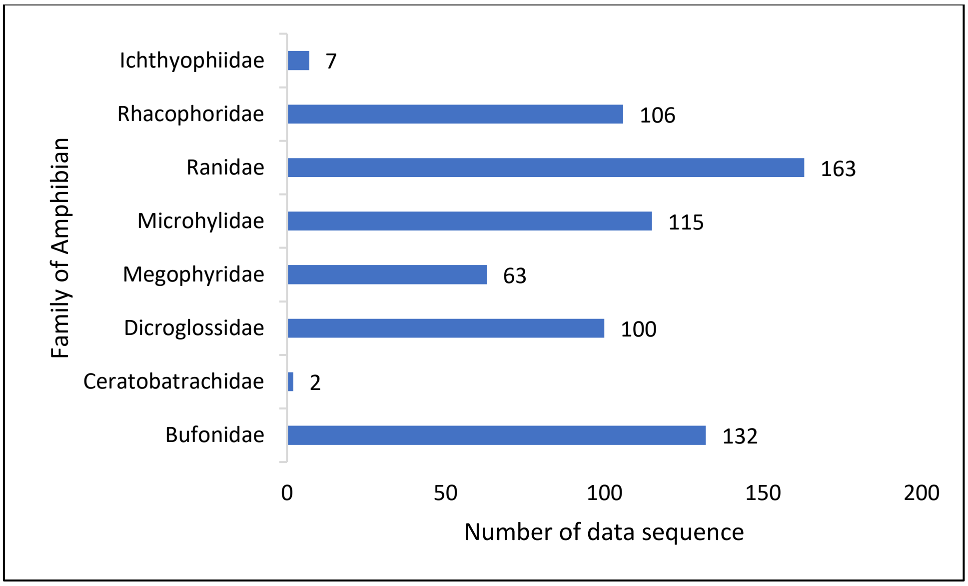

Figure 2 The availability of genetic resources of amphibians in Malaysia based on the family.

Among the eight families that had been screened, the genetic information of four families which are Ceratobatrachidae, Megophyridae, Rhacophoridae and Ichthyophiidae were less than 25%. The least studied family were Ceratobatrachidae which has a singular species (*Alcalus baluensis)* that had been genetically studied and the family of Ichthyophiidae with only three out of nine species had genetic information available related to the species *Ichthyophis asplenius*, *Ichthyophis biangularis* and *Ichthyophis larutensis*.

Off 288 species of amphibian, 46 (16.0%) species had its conservation status of Not Evaluated (NE). More than half of the total species examined were categorized as Least Concern (LC) (60.1%). There were nine species (3.1%) of amphibian species were listed as Critically Endangered (CR). The remaining 60 species (20.8%) were categorized as Endangered (EN), Vulnerable (VU), Near Threatened (NT) and Data Deficient (DD) (Fig. 3).

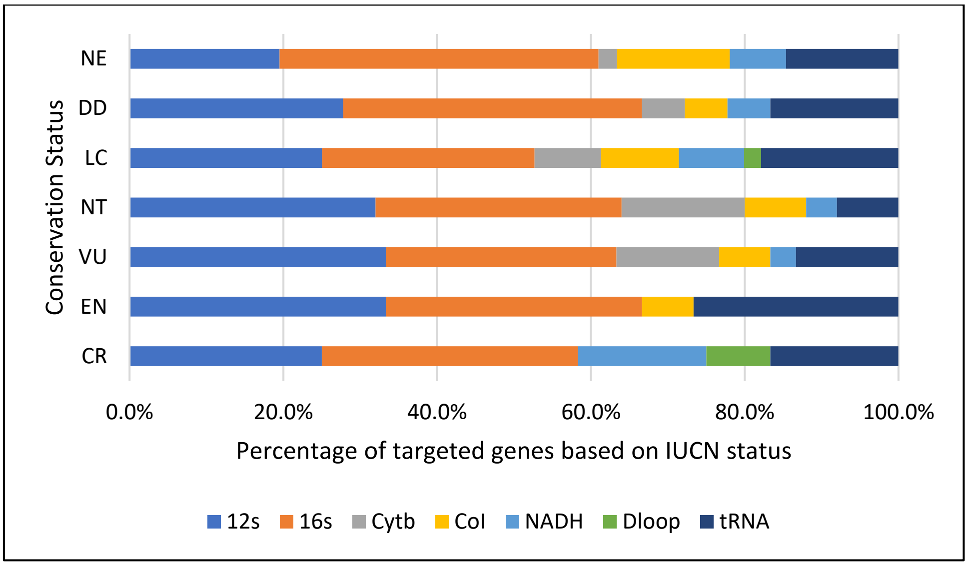

Figure 3 The availability of genetic information based on conservation status.

There were 46 species categorized under NE and only 12.7% of the list had genetic information related to the targeted genes. Similarly, only 12.9% (20) of species which classified as DD had their mtDNA gene record available. Of 173 species of amphibians under category LC, only 44.8% had their genetic information recorded. For those species categorized as NT, out of 13 species, only 25.5% of them had genes of interest recorded. For category VU, 33.0% (13) species from the species listed had been genetically studied and had data sequences available in Genbank. Meanwhile, only 16.5% (13) of species categorized as EN had their data sequences recorded. Then, for those species under the category CR only had little recorded genetic information with only 19.0% of 9 species had their information available. There are 152 (52.8%) out of the total species number are endemic to Malaysia. 93 (61.2%) out of 151 endemic species had their genetic information available while the remaining 59 (38.8%) species have no sequence data recorded. Species categorized as LC were the most studied among the endemic species, with 57 out of the 73 species having genetic sequences recorded while the least studied species were those species categorized as DD with only six species out of 17 species had been studied and had data sequences available in Genbank (Fig. 4).

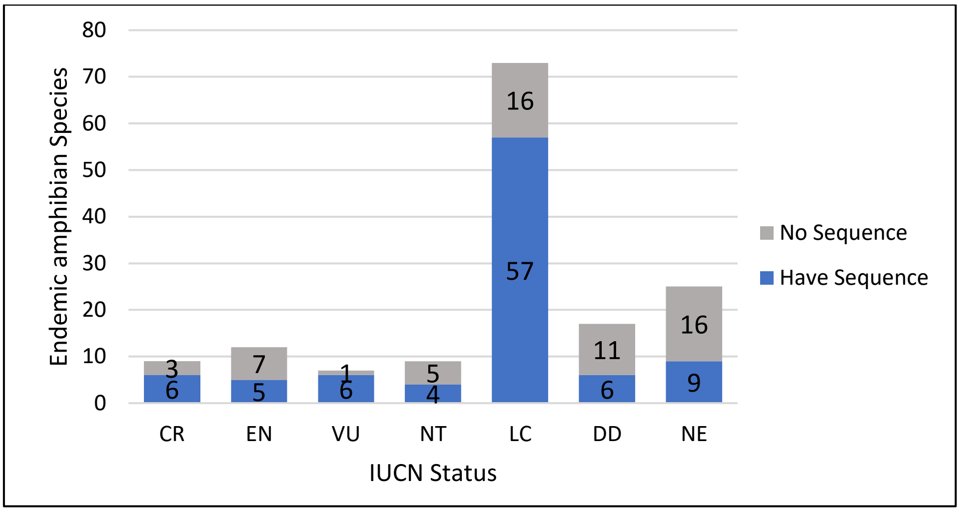

Figure 4 The availability of genetic information of endemic amphibian species with IUCN status.

Threats faced by amphibian species in Malaysia were assessed based on the category of threats on IUCN and from 288 amphibian species in Malaysia, 149 (27.0%) were under the threat of biological resource use which makes this the highest threat for the amphibian species in Malaysia. The next highest threat faced by those amphibian species is agriculture and aquaculture with 129 (23.4%) out of 288 species while the threats of residential and commercial development become the third highest threat faced by amphibian species in Malaysia with 61 (11.1%) species. There are also 107 (19.4%) species that do not have the information on their threats and have been categorized as Not Evaluated. For the remaining threats including pollution, climate change, transportation, energy production, natural systems modification, human intrusion, geological events and invasive species, the number of species ranged from 1% to 9% (Fig. 5).

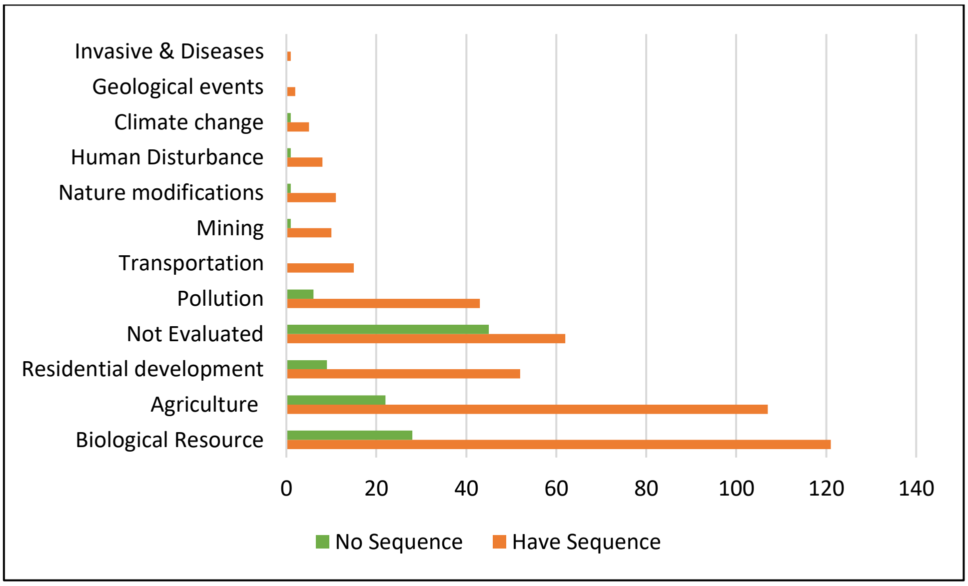

Figure 5 Threats faced by amphibians in Malaysia.

There 81.2% of species that are threatened by biological resources use had data sequence for at least one or more seven genes of interest. Followed by threats coming from agricultural activities with 82.9% species from the total 129 species having their genetic information available. For those species that were threatened by residential development, 85.2% of species had their data sequence recorded. For those species that are not evaluated, more than 50% of the total number of species which is 107 had genetic sequence available. For threats including pollution to climate change, all the threats ranging from 50% to 90% of the amphibian species had genetic resources available in Genbank. Meanwhile, only one species was threatened by invasive and disease problems and that one species had their genetic information for one or more seven of the targeted genes.

Discussion

The genetic resources of amphibian species in Malaysia still require significant attention to fully utilize them in the latest technological approaches. The availability of genetic resources plays a crucial role in applying advanced methods like eDNA metabarcoding. These approaches offer numerous benefits, including the identification of parentage, distant relatives, relative abundance, unidentified individuals, and the detection of rare and invasive species (Haig, 1998; Deiner et al., 2017). Therefore, the lack of genetic information on amphibian species in Malaysia is a concerning issue, particularly for species categorized as Vulnerable (VU), Endangered (EN), and Critically Endangered (CR). Improving genetic information is crucial for preserving the future of amphibian diversity in the country.

This study highlights the 16S rRNA gene as the most widely used locus in amphibian genetic studies, a consensus also supported by Chan et al. (2022), who suggested that 16S rRNA is a highly conserved mitochondrial DNA gene. Vences et al. (2005) conducted a study demonstrating that 16S rRNA exhibited a higher amplification success rate (100%) compared to the usage of various combinations of COI primers. Consequently, due to its efficacy in generating genetic databases, 16S rRNA has been preferred by previous researchers, making significant contributions to the field of amphibian genetic resources. Additionally, the 12S rRNA gene and tRNA gene have also contributed to numerous sequences of amphibian species. These genes have proven to be efficient genetic markers for distinguishing closely related amphibian species, as demonstrated by studies conducted by Matsui et al. (2014), Matsui (2019), Mahmoodi et al. (2018), and Novoa et al. (2012).

Conversely, the D-loop gene has been reported as the least sequenced gene in amphibians, potentially due to its primary function in different studies. The D-loop gene is known to evolve more rapidly than other mitochondrial DNA genes (Brown, 1985), making it an ideal marker for addressing genetic questions at the population level (Hoelzel et al., 1991; Hurzaid et al., 2014), particularly for evolutionary analyses. Consequently, researchers often prefer to use gene markers that are more universal, provide more information, and have higher success rates by utilizing readily available markers specifically established for certain taxonomic levels. Therefore, the selection of suitable gene markers is crucial for generating comprehensive genetic resources for the amphibian species database in Malaysia.

The available genetic sequence data for amphibians reveals that the Ranidae family possesses the highest number of genetic resources, while the Ceratobatrachidae family remains relatively unexplored genetically. This disparity can be attributed to variations in the number of species studied and the specific research objectives. The Ranidae family, also known as True Frogs, has undergone extensive taxonomic reorganization and phylogenetic studies. Presently, an increasing number of new species within this family are being recorded in Malaysia, contributing to the abundance of genetic resources (Che et al., 2007; Stuart, 2008; Chan et al., 2014; Shimada et al., 2015; Yuan et al., 2016; Chan et al., 2018). Additionally, the Rachophoridae family has also amassed a significant number of genetic resources due to its high species diversity in Malaysia (Norhayati, 2017). However, it is worth noting that even within the Ranidae family, certain species such as *Abavorana Nazgul* (Quah et al., 2017) lack available genetic information. Conversely, for the Ceratobatrachidae family, which currently has the least amount of genetic data, *Alcalus baluensis* has been sequenced. This particular species garners more attention due to its endemic status in Borneo (Pui et al., 2016), emphasizing the need for its protection. There is a positive trend in documenting genetic information for endemic amphibian species in Malaysia, as more than half of the species now have at least one genetic sequence. This suggests that endemicity has played a significant role in accelerating the development of the amphibian DNA database in Malaysia.

Moreover, the population status of endemic amphibian species, as determined by their IUCN Red List status, also impacts the availability of genetic resources. The abundance of data is primarily driven by species classified as "Least Concern," while there is a concerning lack of genetic information for species categorized as "Critically Endangered" and "Endangered." The limited genetic data for threatened and endemic species can be attributed to the challenges of assessing these populations. Populations classified as "Critically Endangered" typically have estimates of fewer than 250 individuals, while "Endangered" status applies to populations ranging below 2500 individuals (Mace et al., 2008). Geographically restricted and rare species have become focal points for ecological and conservation studies (Pompa et al., 2011; Enquist et al., 2019). Rare species are of particular concern due to their high risk of extinction, and in some cases, they may face a "double jeopardy" as they often have low abundance and narrow distributions (Gaston, 1998; Hughes et al., 2014), which further constrains efforts to study and generate genetic references for these species. Information on threatened and endemic species is vital as it provides crucial insights into population size, relative abundance, and gene flow, which are invaluable for conservation and management programs.

Amphibian species possess several characteristics that make them more vulnerable to environmental disturbances compared to other animal groups (Rowe et al., 2003). They face numerous challenges such as habitat degradation, pollution, and unregulated land conversion (Chan & Grismer, 2021; Razali et al., 2018; Rozimah & Khairulmaini, 2016). According to the IUCN Red List, the primary threats to amphibians include the biological use category. Biological resource use in the IUCN context refers to the consumptive use of wild biological resources, encompassing both deliberate and unintentional effects, as well as the persecution or control of specific species (Salafsky et al., 2008). Species threatened by biological resource use often face challenges related to logging and wood harvesting, which involve the extraction of trees or woody vegetation for purposes such as fibre, timber, and fuel (Salafsky et al., 2008). There is a growing global demand for timber products, leading to increased logging activities for various tropical and temperate tree species (Shearman et al., 2012). With the globalization of the timber trade, the negative environmental impacts have also escalated, and the primary cause of concern in the timber industry is forest degradation (Putz et al., 2012). Consequently, these activities directly affect forest ecosystems, including the habitats of amphibian species. Given these challenges, it becomes crucial to generate genetic information for endangered species, as genetics plays a significant role in conservation biology. Genetic data help determine the evolutionary context of endangered species and assist in developing improved management and conservation strategies (Hedrick & Hurt, 2012).

Anthropogenic activities have led to widespread environmental degradation, necessitating urgent assessments of biodiversity (Bellard et al., 2012). Traditional methods of species identification based on direct observation or voucher specimens are increasingly being complemented or replaced by DNA-based techniques. One such approach is environmental DNA (eDNA) metabarcoding, which involves sequencing multiple DNA sequences from a single environmental sample. It is gaining popularity in biodiversity assessments across various habitats, including freshwater and marine environments (Creer et al., 2016; Adamowicz et al., 2019). The DNA sequences obtained from eDNA samples are typically compared to genetic databases such as GenBank for organism identification (Benson et al., 2013). However, concerns have been raised regarding the use of genetic approaches in biodiversity monitoring. Metabarcoding of eDNA aims to detect DNA from multiple species simultaneously to assess community diversity. Universal primers are often employed to amplify barcode regions that can anneal to target DNA regions of multiple taxa (Freeland, 2017). However, these universal primers may not be suitable for eDNA assays due to issues such as mismatches in the primer-binding regions, which limit their effectiveness in specific taxonomic groups. Additionally, the generation of large amplicons from degraded DNA may pose challenges for retrieval (Thomsen et al., 2012; Deagle et al., 2014; Barnes et al., 2014).

Indeed, the choice of primers is crucial for amplifying the target region in amphibian families. The COI region has proven to be reliable for species identification in certain amphibian species, although 16S is still considered superior for barcoding purposes (Che et al., 2012; Hawlitschek et al., 2015). Therefore, a complementary approach utilizing both COI and 16S primers is suggested to increase success rates, considering the high variability observed in amphibians (Vences et al., 2005). Ensuring sufficient coverage between these markers is important to capture sequence variation and improve amplification success. Currently, most studies focus on partial sequences that are specific to the target group, as they provide more reliable and comprehensive species identification. Additionally, it is recommended to develop comprehensive species identification approaches for amphibians. By conducting extensive studies on amphibians, we can identify species that play a significant role in environmental changes. Amphibians are valuable bioindicators that help assess the health and quality of ecosystems (West, 2018). Understanding these species through a molecular approach can enhance conservation efforts and promote effective conservation strategies.

Conclusion

The application of genetics to the management of biodiversity and its habitat is still limited. Knowledge of genetics contributes to conservation efforts and it can result in reducing the loss of genetic diversity, lowering the extinction risk, identifying new and cryptid species, resolving taxonomic problems and many other gaps in conservation management. However, despite the challenges that have arisen with the growth of technology in genetic approach, the opportunities, and benefits of these technologies have proven fruitful. The advancement of technology has provided a main instrument to collect abundance and better data to improve the monitoring of wildlife, their habitats and also their threats which then can be utilized in assisting the management and stakeholder's decisions. With the growth of available data and resources in the next few years, new and amazing discoveries will continue to be made.

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