

Cryptic Species Identification in Marine Flatfishes Through Genetic Barcoding

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Abstract

The current paper discusses the growing biological issue of "cryptic" species. For species with substantial genetic variety but little morphological variation, a cryptic species notion was recently put out. In order to identify three new species and illustrate their dual nature, we use special material from a fascinating group of nudibranch molluscs of the genus *Trinchesia* from European waters as a study in itself. Each species in that complex has stable morphological and molecular distinctions, but their overall similarity can be determined as a "cryptic" species complex. This species complex may therefore be referred to as "cryptic," "pseudocryptic," or "non-cryptic." We further link the species problem to epigenetics and provide evidence for a very high rate of speciation in this species complex. When a finer multilayer morphological and molecular scale is employed to uncover more accurately defined species, the "cryptic" addition to "species" is no longer essential because the degree of crypticity is a continuum. This is supported by the broad discussion of the available metazoan-wide data in the current study. Molecular and morphological approaches should be used to create a fine-scale multilevel taxonomic framework. This does not necessarily mean that purely molecular-based "cryptic" species will be a posteriori transformed into morphologically defined "pseudocryptic" ones. The current study's implications are significant for various disciplines, such as fine-scale biodiversity assessments and conservation biology.

Keywords: Cryptic Species (CS), Marine Flatfishes (MF), Genetic Barcoding (GB), Identification (II)

Citation: Zoe, B. 2024. Cryptic Species Identification in Marine Flatfishes Through Genetic Barcoding. *FishTaxa* 33: 31-40.

Introduction

Any species that is different a little bit or completely from all other species that are well known can be described under the category of cryptic species. However, a long history is found, which is completely based on a wide range of confusions. Two or more biological groups that are morphologically different from each other and cannot be bred and are compared on behalf of their physiologic race (Steinke et al., 2009). Let's discuss certain examples that can clarify the concept of cryptic species. A genus of mosquitos is known as a malaria vector, and it is known as *Anopheles*. This is the fungus that causes the disease cryptococcosis, and is considered the sister species of *Bactrocera tryoni* named Queensland fruit fly. All this discussion comes under the category of examples of cryptic species. This pest cannot that stinguished from the other two species considered s,isters (Kochzius et al., 2010). A method that is classically acceptable in which DNA is utilized to detect and recognize the species, is considered the Barcode of life. A very particular piece of DNA taken from the cell's mitochondria is called cytochrome C oxidase I gene named CO1. Cryptic species is a term that is utilized too much, and the representation of taxa is described as a single species because morphological differences can be seen. A method that is common for the identification of cryptic gastropods is the analysis of species that belong to various genera that are different. Mitochondrial DNA is used in this procedure along with other genetic (Francisco et al., 2022).

A method that is traditionally employed is DNA for the detection and determination of species that are then mentioned as the Barcode of life. A very particular piece of DNA that is taken from the mitochondria of the cell

is utilized, and it is named cytochrome C oxidase. Generally, if we describe the term of cryptic species, then it is the representation of two or more species which are the same in representation, but it seems very difficult to recognize them concerning morphology. However, their connection with their origin is unique and well-isolated. Cryptic species have a close relationship with each other, but a fine distinction can also be seen from their ancestors. By look, they are the same, but their evolutionary pathways are completely independent. The cryptic species have an opposite relationship with the adaptive radiations(Pappalardo et al., 2015). The phenomenon of parallelism does the origin of cryptic species. There is no connection of sister taxa found between them. However, on behalf of their genetics, they are separate from each other on that level, and do not fall under the category of symplesiomorphic. But the reality is that they have similar ancestors, simply without respect to morphology. The barcode of DNA for the species of animals, which is well distinguished, can be identified by the 650-bp portion that contains the gene coding of mitochondria for cytochrome C oxidase. The role of DNA barcoding is such that it will enhance the procedure of identification, development of understanding of taxonomy, phylogeny and mapping the distribution of African fishes which are biogeographic(Chan et al., 2024). A methodological method of DNA barcoding is the identification of fish by the application of specific genes or genes. The phenomenon of DNA barcoding is very effective concerning cost and a way which is to be done quickly for the identification of species of fish which are living in aquatic environments. Water samples which are taken from water bodies consist of DNA fragments which will become the source of clues as to whether the fish are found in this water or not(Ward et al., 2005). Here we are going to discuss certain mitochondrial markers which play a significant role in the identification of fish species. One of them is gene which is utilized for cytochrome-c-oxidase subunit. In this biological marker, a segment is found that is 600bp long and it will become the source of for identifying the fishes on the behalf of their taxonomy by the application of DNA barcoding.

DNA barcoding is the way that proves helpful for identifying and classifying the fish efficiently. Cytochrome c oxidase subunit is the gene which is a very short and effective fragment that can prove helpful in the barcoding of DNA(IMTIAZ et al., 2017). This phenomenon will prove helpful in the identification of species quickly and accurately. The fish barcode which is found in the life campaign is a collaborative research effort that is acceptable internationally and proves very helpful in the building of a reference Library which consists of DNA barcodes of all species of fish that are taken from the voucher specimens having authoritative identifications on the base of taxonomy (van Loon, 2023). A diverse variety of fauna fish is found in the northern Arabian Sea as that sea is based on such water that is very rich in nutrients. From the coastal waters of Pakistan, 303 specimens of different species were accumulated(Costa et al., 2023). These specimens consist of 100 genera, 61 families 15 orders that are then recognized as the 120 species on behalf of morphology and 18 others which were not given the status of any well taxa on the behalf of morphology. The origin of the DNA barcode is 557 mesopelagic and upper bathypelagic teleost specimens which were collected from the waters of Atlantic Canada (Kenchington et al., 2017). DNA barcoding is a tool which plays a significant role in the identification of species however according to certain studies this method can be utilized to observe the diversity of eggs of fish in the seascape of low latitude. According to recent studies, DNA barcodes are the sources which can be utilized for accessing the vast variety of eggs of fishes in the region of equatorial southwestern Atlantic, which is a region which is very rich concerning today's nutrients but is still under study (Amir et al., 2022; Matthew et al., 2023).

Research Objective

The main purpose of this research is to understand the topic of Cryptic Species Identification in Marine Flatfishes through Genetic Barcoding. It elaborates on the importance of genetic barcoding in the identification of cryptic species.

Literature Review

Researchers claim that distribution patterns of cryptic species are observed using the barcoding approach. The barcoding techniques identify the presence of *Anaxipha* species in the larvae of *Olixon*. the diversity found in the larvae of *Olixon* is due to genetic factors. Studies suggest that barcoding uniquely differentiates species based on their taxonomic features(Armstrong et al., 2024).studies explain that morphological features of larvae species provide basis for their classification. the larvae stage is different for each species and is the diagnostic way of determining the taxonomic background of species. specification of fish larvae is possible using DNA barcoding(Chan et al., 2024).studies explain that the existence of species on Earth is still unknown. The information gained through various research studies reveals that the number of species on earth is almost two billion, of which 3.1 percent possess cryptic diversity(Cheng et al., 2024).studies explain that identifying fish species through their larvae is possible using the barcoding method.in the southwestern Atlantic Ocean, the identification process of fish species through DNA barcodes provides information about the cryptic diversity found in fish eggs(Costa et al., 2023).studies explain that the demand for fish products is increasing around the world which emphasizes the need for improving the fish-based trading process The whole process of production of fish-based products is monitored using technology-based equipment. for making the labeling of fish products a reliable process the use of molecular barding methods is preferred (Filonzi et al., 2023).studies conclude that species belonging to the Carangidae family are classified as species having wide diversity.to study the diversity of Carangidae species the COI-based barcoding system is employed for phenotypic analysis(GAMAGE et al., 2024) Studies scholars suggest that during the early embryonic or developmental stage, the larvae of fish species are found at the upper layer of marine ecosystem .these larvae are dispersed into different habitats through water currents. when a larvae reach a particular habitat, it adjusts itself to the circumstances of that habitat. studies on ichthyoplankton reveal great about the diversity shown by aquatic species in different habitats (Guerchon et al., 2023).studies show that the determination of genetic features of fish species provides data about their ability to survive in different habitats. the DNA of *C. lingua* species was sequenced to determine its genetic behavior(Jena et al., 2023).studies provide evidence-based data regarding the evolutionary history of *Psettodes* flatfishes. globally the diversity of *Psettodes* flatfishes is the result of their genetic variation(Kundu et al., 2023) Studies made on carbs reveal that the rise in demand for carbs has increased their commercial importance .carbs are popular seafood that are consumed by many people around the world. the mining of edible carbs with non-edible one's results in serious health outcomes in human.to avoid this problem the genetic testing of carb species is done before they are sold in the market. after genetic testing, the carbs are packed in packing labeled with DNA barcodes(Mazumder & Ghosh, 2024).studies reveal that fish population is introduced in artificially developed reef aquatic system to understand the spatial distribution patterns of species. underwater drones provide information about the management of artificial reefs. The fish species present in the coral reefs are labeled with DNA barcodes to ease the process of underwater analysis(Miyajima-Taga et al., 2024) Studies on terrapins provide details about their phylogenetic linkages. Also, the putative cryptic species are conserved using the approach of mitochondrial-based barcoding. To identify the diversity of terrapins in the southern river the DNA barcodes act as an efficient tool(Mohd Salleh et al., 2023).studies about jellyfish and their related species reveal that they are the most common and popular species found in the aquatic ecosystem. The popular form of jellyfish is moon jellies. *Aurelia* is a jellyfish species that has been taxonomically classified into thirty types using DNA barcodes (Moura et al., 2023).studies provide evidence that more knowledge is valuable regarding the species of mesopelagic regions then specie of marine ecosystems. information regarding the marine ecosystem is obtained by studying the otolith's taxonomic behavior. moreover, the cryptic species that are studied using the barcoding approach provide more knowledge related to species of mesopelagic regions (Quigley et al., 2023).studies show that morphological studies relate to the zooplankton species provide insight about their

taxonomic history. to identify each species of zooplankton the use of molecular met barcodes is made by researchers. the presence of zooplankton species across the whole aquatic ecosystem is determined through this molecular barcoding strategy (Stepien et al., 2023). scholars conclude that an accurate assessment technique for fish species identification is DNA barcoding. The barcodes are especially design to reveal the presence of cryptic species in the aquatic environment. PCR-based primers are used for developing the approach of barcodes. the primers provide amplification of the DNA barcodes to make the process of DNA sequencing efficient (Takenaka et al., 2023). studies reveal that the ability of aquatic species to resist the change in climate factors is influenced by intraspecific features. The barcoding system is an efficient system in classifying the bio-diversified aquatic species of marine ecosystem In the sea in Japan the sample of species having barcodes was taken for evaluation of AVS in marine species (Turanov et al., 2024). scholar studies provide the basis for identifying the composition of species present in large aquatic environments. The species from ECSHZ were taken for analyzing the sequencing of COI in species of the East China Sea. the two approaches were used to assess the identified species from ECSHZ. these two approaches are BPP as well as ABGD These approaches provide evidence that no variation is found in intraspecific species. also, the use of DNA barcodes for constructing a species phylogenetic trees was made to understand the evolutionary relation among species belonging to ECSHZ (Wang et al., 2024). The latest technology-based technique has been developed recently to understand the diversity of cryptic species in numerous aquatic environments This modern technology approach is regarded as eDNA barcodes These barcodes are developed to monitor marine species by studying their DNA sequences. In South California, DNA barcoding technology is used as a conservation strategy for saving marine species (Waters, 2023). scholars' studies provide suggestions that the PCR amplification process is implemented for sequencing the mitochondrial-based DNA of *Sillago* species. PCR techniques amplify the DNA making the understanding of the sequencing process easier for species classification (Xiao et al., 2023).

Cryptic Species Identification in Marine Flatfishes

Achieving sustainable anthropogenic development and comprehending and satisfying the needs of biodiversity are also becoming pressing research issues, as are challenges like global climate change, population and ecological environment issues, and the production of energy and food. Accurate and quick species identification is highly desired worldwide for the preservation and sustainable use of biodiversity resources. A fundamental need for biological study is the identification and categorization of species. Classical taxonomy has greatly aided in the categorization of species based on morphological traits; yet, because of morphological plasticity, it is unable to reliably differentiate between all species, especially those that are similar and related. As a result, new techniques are required to facilitate species identification using traditional taxonomic approaches. Using DNA sequencing, namely DNA taxonomy, as the primary platform for biological classification was initially proposed by the researchers. The idea of DNA barcoding was then presented by Professor Paul Hebert of the University of Guelph in Canada. He stressed the importance of this technique for biological taxonomy and species identification, and he recommended using the mitochondrial cytochrome C oxidase subunit I (COI) gene as the foundation for animal DNA barcoding. By employing a brief segment of around 650 bp from the mitochondrial COI gene to identify species based on sequence differences, it has been demonstrated that DNA barcoding may be used to identify both freshwater and marine fish species. Numerous studies have demonstrated the widespread usage of DNA barcodes in phylogenetic analysis, cryptic species discovery, animal species identification and categorization, and other fields. Additionally, barcode databases like the Barcode of Life Data Systems (BOLD), which barcoded around 96,425 fish specimens from 10,267 species, have been created using DNA barcodes. By automating and standardizing the specimen identification process, DNA barcoding can supplement conventional species identification and lessen reliance on taxonomists' expertise. Known as "the roof of the world," the Qinghai-Tibet Plateau (QTP) is a relatively unique region with a high diversity of indigenous species. The three

orders of fish that are indigenous to the Qinghai-Tibet area are Salmoniformes, Siluriformes, and Cypriniformes. Triplophysa, a member of the Nemacheilinae (Cypriniformes) family, is extensively spread on the QTP and in the surrounding areas. It is a unique group that has adapted to the QTP's unique climate, which includes low temperatures and oxygen shortages. Thirty-three Triplophysa species were recognised in 1992. But as time has gone on, many new species have been named, and 140 legitimate species have been found. This research demonstrated that there is a significant amount of undiscovered biodiversity in the genus Triplophysa, and many species have not been identified or published, despite the possibility of some synonymous species. The existence of cryptic species or the absence of a thorough categorization review are the primary causes of the phenomena wherein several new species are discovered. Limited gene exchange between populations has resulted from the plateau loach fish's straightforward body form, rather conservative morphological evolution, and poor movement capacity brought on by the water system's constraints. Many hidden taxa may have gone unnoticed because of the gradual process of species development, which includes reproductive isolation and genetic structural differentiation, even though it is morphologically undetectable. Thus, while studying cryptic diversity, the genus Triplophysa should be taken into account. Despite its drawbacks, traditional morphological categorization has long been a major factor in species identification. Morphological distinctions are difficult to discern in fish of the genus Triplophysa, in particular, since morphological plasticity is shown and the phenotypic is readily influenced by biological variables and the external environment. In addition, several species were initially recognised many years ago with very simplistic morphological descriptions. All of these issues have hindered later taxonomic research and species identification. Because it is difficult to acquire comprehensive data for comparisons, the distribution of certain species may be incorrectly enlarged and divided into many regional populations. The Triplophysa species classification is a perfect example of this extensive and complicated classification history. Fish biodiversity must be identified and documented in order to safeguard species and forecast how biodiversity will adapt to climate change. Although DNA barcoding has some limitations, such as introgression, pseudogenes, molecule definition, and incorrect software models or parameters that result in excessive species division or clustering, it has been proposed as an important tool for closing the large gap in our current understanding of biological diversity. Several reasonably automated species division algorithms have been created employing DNA barcoding data sets, with the potential to boost efficiency and reduce subjectivity. There has been no investigation into employing DNA barcoding to identify or assess cryptic biodiversity in the genus Triplophysa in the northeastern QTP. The DNA barcoding approach was utilized to assess the richness of plateau loach in the northeastern QTP, a biodiversity hotspot, using a large sample collection from this area. A database of plateau loaches in this region was also created using DNA barcoding. Examining the links between morphological species and molecular operational taxonomic units (MOTUs) revealed the genus's hidden diversity. This study will help to conserve this vital fish species while also expanding our understanding of plateau loach biodiversity.

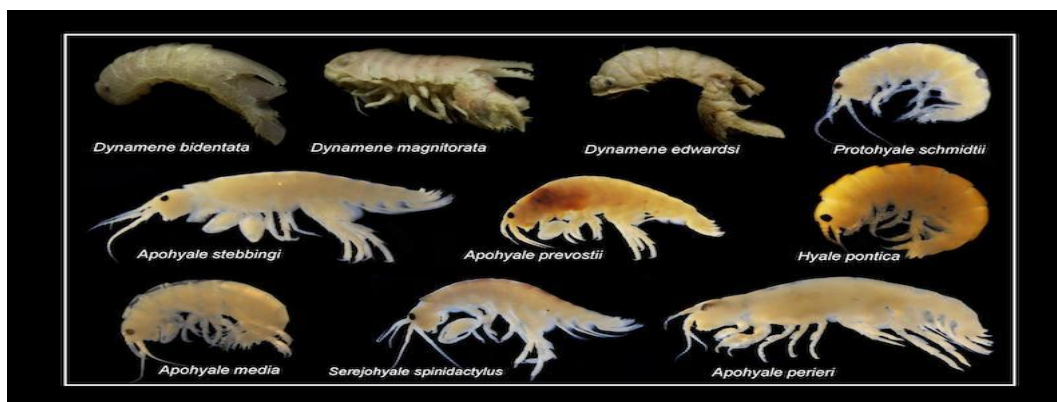


Figure 1: Cryptic Species

Cryptic Species

Cryptic Species Term Use

Despite a lengthy history of widespread misunderstanding, the term "cryptic species" is commonly used to refer to any species that is discovered to vary morphologically from other recognized species by a little amount or not at all. As early as 1718, distinct biological "kinds" with little physical distinctions were identified in birds. In this context, the English term "cryptic species" was used to describe species that remain genetically isolated owing to mating choice, but do not alter their appearance. The German and French words that originally referred to many related species that are frequently confused for one species due to their indistinguishability were translated into English as the synonymous term "sibling species." Since then, the terms "cryptic species" and "sibling species," which refer to two or more related species that are morphologically difficult to distinguish from one another, have been used largely interchangeably. "Cryptic species" typically refers to a species that has few or no external diagnostic features, making them difficult to identify. The terms "pseudocryptic species" (previously morphologically unidentifiable species that are discovered to possess certain distinctive characteristics) and related expressions like "cryptic speciation" (processes leading to cryptic species) and "cryptic diversity" (the existence of numerous cryptic species in a study group) have also been employed.



Figure 2: Marine Flatfishes

Marine Flatfishes

Members of other families are harvested in small amounts, but Pleuronectidae flatfishes are commercially significant in northern seas. No other flatfish are used to the same degree as Pleuronectidae, but some Bothidae and Soleidae (soles) are in tropical and temperate waters. Although certain species can be found in the Arctic, flatfishes are mostly found in temperate and tropical waters. The largest Atlantic halibut may grow to a length of over 2 meters (almost 7 feet) and weigh over 325 kg (716 pounds), while the smallest size is around 100 mm (4 inches). Some species live entirely or in part in freshwater, although the majority are marine. Although flatfish may be found at depths of up to 1,000 meters (3,300 feet), the majority of them inhabit water that is less than 200 meters (660 feet) deep on the continental shelf.

Reproduction

Some flatfishes spawn in estuaries, but most spawn offshore. Females often release several hundred thousand eggs, indicating great fecundity (big female halibut have between 2,500,000 and 3,000,000 eggs). The tiny eggs, with or without oil globules, can sink to the bottom (demersal) or float freely (pelagic). The length of newly

born larvae is 1.5 to 3 mm, or 0.06 to 0.12 inches. Newly born larvae have a very high mortality rate, and active feeding starts soon after hatching. Until they undergo metamorphosis or soon after, larvae float with the currents (planktonic) and then sink to the bottom to become adults that live on the bottom (benthic). While quick swimming is achieved by undulating the body and caudal fin, slow swimming is achieved by undulating the dorsal and anal fins.

Feeding Behavior

Only their eyes are visible, while flatfishes lie on the bottom, usually covered in mud or sand. The eyeballs are individually movable and may be lifted or lowered. Crustaceans, various bottom invertebrates, and tiny fish are the main food sources for flounders. They stay still while eating until their prey approaches too closely, at which point they practically jump off the bottom to go after them. Humans are the main predator of many flatfishes; however, they are also preyed upon by a range of huge fish and cetaceans, including whales and porpoises.

Form and Purpose

Many animals exhibit sexual dimorphism, which means that the male has one or more of the following characteristics: longer pectoral fin rays, bigger interorbital bones, tentacles on the eyes, spines on the skull, and more complex coloration. The majority of flounder species have a large dorsal fin that extends from the head to the caudal (tail) fin, as well as an anal fin that runs from the vent (anus) to the tail fin. Pectoral fins are found in all flatfish larvae, although they are missing or decreased in adults from the Soleidae and Cynoglossidae families. Caudal fin rays and the structures that support them change throughout time. Scales can be smooth (cycloid) or rough-edged (ctenoid). Different animals have unique dentitions that reflect their eating patterns. Active predators have huge, well-developed teeth in both jaws, whereas species that live mostly in muck and eat bottom invertebrates have teeth on the lower jaw's blind side. The ovaries extend posteriorly from the body cavity between the epidermis and a thin layer of muscle just above the muscles of the anal fin rays, making it easy to distinguish between the sexes. The testes cannot be examined without dissection. As they spiral into the body cavity, the stomach and intestines form a loop. The primary aspect of metamorphosis is the movement of the eye through or around the skull. This is performed by travelling across the middorsal ridge, in a dip between the supraorbital bars (above the eye) and the ventral edge of the dorsal fin, or over the skull. The supraorbital bars extend forward from the cranium to the ethmoid area of the skull, or the region in front of the eye, and then proceed ventrally until they rest next to each other. As the eye migrates, the dorsal border of the supraorbital bar is reabsorbed to allow the eye to pass through the skull. Once the eye has finished its migration, the supraorbital bars ossify to produce the interorbital bone. The blind (bottom) side frontal bone transfers to the ocular (upper) side, forming a piece of the upper eye's optic capsule floor. The torsion (twisting) of the frontals, ethmoid, and mouthparts is the flatfish skull's most distinctive characteristic.



Figure 3: Structure Diversity

Structure Diversity

Fish have seen several evolutionary modifications during their more than 450-million-year existence in order to adapt to almost every type of aquatic habitat. Terrestrial vertebrates are really just well adapted fishes because when fishes migrated to land, they evolved into tetrapods, or four-legged fishes. Although many fish fit to the traditional image of a sleek, slippery aquatic creature with fins and gills for respiration, many others do the reverse. Fins can be reduced or even lost, or they can be elaborately extended and form complex shapes; the mouth, eyes, nostrils, and as well as gill openings can vary greatly in position; the body can be elongated in many forms and drastically shortened in others; and flattened in some (mostly in bottom-dwelling fish) and laterally compressed in many others. Many evolutionary lineages have evolved air breathers. Fish exhibit a wide spectrum of hues, often with extraordinary intensity, on a single individual, making them among the most brilliant species. The colors and shapes of other fishes are cryptically matched to their particular surroundings. The pigments may appear to glow because of the structure of the fish's surface, which intensifies their brightness. It's true that some unrelated fish have light-producing organs. Many fish can alter their colour, some for camouflage and others to enhance their behavioral signs.

Distribution and Abundance

Except for exceptionally hot thermal fishponds and salt-alkaline lakes such as the Dead Sea in Asia and the Great Salt Lake in North America, fish may be found in nearly any natural body of water. The present distribution of fishes is influenced by the Earth's geological history and development, as well as the aptitude of fishes to familiarize to their habitat and experience evolutionary change. It is evident that fishes are dispersed geographically and in accordance with their surroundings. Habitats in freshwater and saltwater are very different. Although some, like salmon, migrate between freshwater and marine habitats, fishes in these ecosystems are frequently distinct, even in close proximity. Freshwater environments come in a wide variety. The overall structure and physiological traits of fishes found in temperate streams, tropical rivers, Arctic lakes, mountain torrents, and tropical lakes clearly differ from one another. Even in almost similar environments, as where a steamy mountain inundation meets a lowland stream, the fish species will differ. Muddy coastlines, bays, estuaries, rocky and sandy coasts, deep ocean floors (benthic), mid-water marine (bathypelagic), and surface marine (pelagic) are among the several types of marine ecosystems. For example, the fish faunas of tropical and temperate regions might differ because of their rocky coastal shorelines, even if both habitats are present along the same coastline.

Conclusion

The biology results about cryptic species are inextricably linked to methodological issues. More than 70% of the species in our collection of shelled marine gastropods are visually distinctive (not cryptic), and many of them are conchological unique, making them suitable for exploration in the fossil record. In order to synthesize various reports for a given group, a detailed evaluation with clear descriptions and criteria to appraise these species is required. Authors have differing degrees of confidence in their ability to identify cryptic species, which frequently depends on the data and methodology available. Intermittent group-specific surveys would be useful to determine the prevalence of cryptic species and link their occurrences to possible biological or evolutionary causes. For a more complete sympathetic of cryptic species, whether to calibrate phylogenies or find potentially exclusive morphological information to comparability with present species, it will be necessary to integrate life history, ecological and evolutionary knowledge, and fossils of the study taxa. All of these facts may be thoroughly examined by using an interdisciplinary and inclusive method to studying cryptic species, which may help us better understand how cryptic species impact both extinct and existing biodiversity. Although

very suggestive, our findings are not yet definitive. Thus, we advise that further research on cryptic species:

1. Clearly define the word "cryptic species" or cite one to illustrate the presumptions and standards applied when classifying research taxa;
2. reference taxonomic everything, such as those that recognize unidentified or described cryptic species within the research taxonomy, to ensure that these occurrences are assessed after they are first published and remain relevant;
3. To make it evident what evidence supports the species being identified, incorporate remarks on morphologic (or other phenotypic) evaluations in genetic-based inquiries;
4. When evaluating the reliability of species designations, phylogenetic studies, or ecological results, further integrate with fields like ecology, biogeography, and palaeontology.

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