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Gut content analysis based on DNA barcoding and visual identification: A case study on *Priacanthus hamrur* (Forsskal) (Priacanthidae) from Kerala coast, India

Suvarna Devi, S.* and Soorya Hareendran

Department of Aquatic Biology and Fisheries, University of Kerala, Thiruvananthapuram 695581, Kerala, India
**E.mail: suvarnaraja1995@gmail.com*

ABSTRACT

Deep sea fishes coming under the family Priacanthidae are increasing in the commercial landings along the Indian coast and are rapidly gaining popularity as food fish. The food and feeding habits of Moontail bullseye, *Priacanthus hamrur* (Fabricius, 1775), has not been investigated in detail from the Indian waters. Using an integrated approach of visual identification and DNA barcoding, the gut contents of *P. hamrur* obtained from the trawlers of Shakthikulangara and Neendakara landing centres of the Kollam district were analysed. Gut content analyses revealed that the fish is a demersal carnivore feeding largely on benthic shrimps, teleosts, polychaetes, lobsters and octopuses. From diet analysis, shrimps and fish were suggested to be the main food items of *P. hamrur*. Seven unidentifiable samples from the gut of the fish were subjected to DNA barcoding, and identified as fishes *Bregmaceros* sp. and *Callionymus kaianus*; shrimp *Plesionika narval*; stomatopod *Oratosquilla oratoria;* octopus *Amphioctopus membranaceus* and squat lobster *Paramunida lophia*. The study suggests that *P. hamrur* is a carnivorous species that prefers shrimps and fish over other items as food.

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1. Introduction

Priacanthids are found along the southwest and east coasts of India, found in a 50-400m depth zone (Premalatha, 1997), and have been identified as a major demersal resource suitable for exploitation (Anjanayappa, 2007). Fishes coming under the family Priacanthidae, commonly called bullseye or big eye, have started emerging as a vital fishery resource in the trawl landings of India (Sivakami et al., 2001). The species known from Indian waters include *Priacanthus hamrur* (Forskal, 1775), *P. tyaenus* (Richardson, 1846), *P. macracanthus*, *P. blochi* (Bleeker, 1853), *Priacanthus sagittarius* Starnes, 1988 and *P. cruentatus* (Naik, 1990). Of these, *Priacanthus harmur* is the most common species supporting the fishery, and stock structure analyses were done to delineate the stocks of the species (Mallik et al., 2020).

One of the economically important deep-water fishes in Kerala, *Priacanthus hamrur* (Forsskal), occurs on the outer shelf of the EEZ of India (Mandy and Inasu, 2002). The fish is rich in protein (17.8%) and low in lipids (5.1%) (John and Sudarsan, 1988). The food value of bullseye was analysed from the southwest coast of India, and the fish is as good and tasty as any common table fish (Dhananjaya et al., 1984). The yield of edible meat from *P. harmur* varied from 35-40 % of the wet raw fish; moreover, the fish powders had a balanced amino acid composition and were shown to have a high nutritional value (Nair et al. 1990). Commercial trawling to deeper fishing grounds captured many of these non-conventional fish. The bullseye fishery is considered a looming demersal fishery resource (Sivakami et al., 2005). *P. harmur* along the west coast of India contributed an average of 16,870 t during 2000-2004 and increased subsequently to 30,000 tons (Anon, 2017).

Food and feeding habits change with the season in the fish's history and the kind of food (Chakraborti, 1998). The importance of the knowledge of food and feeding habits of the fishes in understanding their biology has been well established. It helps recognise the fish's trophic interactions within a community (Blaber, 2000) which is a prerequisite for the successful management of any fishery. Studies based on gut or stomach contents rely on the taxonomic identification of partially digested prey fragments, requiring an exhaustive prior knowledge of prey morphological diversity. With more technologically advanced approaches, diet analysis in recent years includes analysis of fatty acid, stable-isotope and DNA-based diet determination techniques (Schmidt et al., 2009; Corse et al., 2010; Hardy et al., 2010). DNA-based dietary analysis was first used by Asahida et al. (1997) to examine predation by sand shrimps (*Crangon affinis*) on stone flounder (*Kareius bicoloratus*). DNA-based approaches potentially provide more accurate methods for dietary studies (Pompanon et al., 2012). DNA-based techniques have successfully identified prey items in stomach, gut or faeces samples (Symondson, 2002). A similar approach was employed to identify prey fish from the stomach contents of twelve species of eastern North Pacific groundfish and the stomach contents of piscivorous catfishes and *Lutjanus campechanus* (Paquin et al., 2014; Moran et al., 2015; Szedlmayer and Brewton, 2019; Spanik et al., 2021). However, DNA degradation in dietary samples limits the length of fragments that can be successfully amplified by PCR (Symondson, 2002; Deagle et al., 2006; Troedsson et al., 2009). This study documents the gut content of *P. hamrur* collected from the Kerala coast of India using conventional and DNA-based approaches.

2. Materials and Methods

Specimens of *Priacanthus harmur* (Forsskal, 1775) were obtained for the study from the trawl landings of Sakthikulangara and Neendakara fishing harbours (lat 08º30'N, long 76º53.3'E) Kollam district, Kerala state, southwest coast of India from May 2016 to April 2017. A total of seventy-eight fishes were collected and analysed for the present study. Samples were frozen and transported to the laborotary Department of Aquatic Biology and

Fisheries, where measurements such as Standard length (SL) and Total weight (TW) were taken and stomachs were extracted. During stomach content analysis, the gut was dissected, and parts of the contents were washed and preserved in 95% ethanol for barcoding analysis, and the rest of the contents were preserved with 10% formalin. Food items were categorized under taxonomic groups, and subsequently, items were identified to the lowest taxon possible, depending on the state of prey digestion (Pillai, 1952). Gastro somatic index was found using the formula following Khan et al. (1988).

GSI= (weight of gut/weight of fish) ×100

Several prey items in the gut which could not be identified were subjected to DNA analysis to recognise them as higher taxa. Total DNA was extracted from 100-500 mg of tissue by standardised salting out protocol adopted for precise and quick DNA isolation from the tissues (Miller et al., 1988). The ethanol-preserved tissues were thoroughly washed with 0.75% saturated NaCl 3-4 times. The finely cut tissues were then placed in a 1.5 ml microcentrifuge tube (Eppendorf) and 500 µL solution I [50 m MTris-HCl (Dissolve 3.028 g of Tris base in 40 ml of distilled water and pH adjusted to 8.0 using HCl and the volume was made to 50 ml, then autoclaved and stored at 4 ºC); 20 m M EDTA (Dissolve 9.31 g of EDTA in 40 ml distilled water and pH adjusted to 8.0, using NaOH, made up the volume to 50 ml, then autoclaved, and stored at 4 ºC) and 10 µl of 10 % SDS] was added. The tissue was mixed with 5 µl of Proteinase K [20 mg ml-1 (10 mg Proteinase K dissolved in 500 ml distilled water and stored at -20 ºC)] and quickly vortexed. The sample was incubated at 55 °C in a water bath for 2 h with occasional mixing. Following incubation, the sample was chilled over ice for 10 min, and 250 µl of solution II (6 M NaCl) was added and inverted several times for thorough mixing. The tube was chilled on ice for 5 minutes and centrifuged at 8000 rpm for 15 minutes. About 500 µl of supernatant was carefully collected into a newly labelled 1.5 ml microcentrifuge tube, and 1 ml of 100 % AR grade ethanol was added to precipitate the DNA. The samples were frozen at -20 °C overnight. The next day, the samples were centrifuged at 11,000 rpm for 15 minutes. The supernatant was removed, and the DNA pellet was rinsed with 500 µl of 70 % cold ethanol and centrifuged at 11,000 rpm for 5 min. The supernatant was carefully removed and allowed to dry partially with the lid off at room temperature. The pellet was re-suspended with 50 ml of 1X TE buffer [mix 2 ml (10 m M) stock solution of 0.5 M Tris HCl (pH-8.0) with 0.2 ml (1 mm) stock solution of 0.5 M EDTA (pH-8.0) and made up the solution to 100 ml with distilled water, then autoclaved and stored at 4º C] depending on the size of the pellet. This dissolved DNA was a template for Polymerase Chain Reaction (PCR). The quality and concentration of DNA were checked on 1 % agarose gel (dissolve 0.3 g of agarose in 30 ml 1 X TAE).

The amplification of the mitochondrial gene, cytochrome c oxidase 1 (CO1), was done using universal primer LCO (5'-GGTCAACAAATCATAAAGATATTGG-3"). The fragment of CO1 with an average length of 658 bp was amplified with the following thermo-profile: initial denaturation at 95 °C for 2 min, 35 cycles at 94 °C for 20 s, annealing at 56 °C for 40 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min, followed by the indefinite hold at 4 °C. Purification of PCR products was done by Bionteq Gel Elution Kit and sequenced in both forward and reverse directions using PCR primers. The forward and reverse strands were edited, aligned and assembled using BioEditv.7.0.9.0 Sequences were verified for integrity by MEGABLAST searches using the BLAST tool. Sequences generated were deposited in Gen Bank in Sequin format.

3. Results

Conventional gut content analysis

Gut content analysis revealed the qualitative and quantitative food spectrum of *P. hamrur*. Traditional morphological characterization led to species-level identification of several gut contents; those unsure were identified up to the genus level. Digested prey that has lost most of its physical characteristics was identified with the help of the molecular technique of DNA barcoding. Fishes of smaller length groups were not available in the trawl bycatch and therefore, could not be included in the analysis.

The diet comprised 9 prey items (species/taxa), including shrimps, fishes, octopuses, lobsters, stomatopods, polychaetes, algae, phytoplankton, zooplankton and digested matter. Among fishes, *Grammoplites scaber, Solea elongata, Cyanoglossus macrostomus, Sacura boulengeri, Bregmaceros* sp, *Callionymus kaianus* and *Nemipterus japonicus* were identified. The shrimps *Solenocera choprai* and *Plesionika narval*; Octopus, *Callistoctopus* sp and *Amphioctopus membranaceus*; Stomatopod, *Kempella stridulans* and lobsters *Petrarctus* sp; and *Nephropsis* sp were also recognized from gut content of *P. hamrur* (Table 1).

It is clearly understood that *P. hamrur* is mainly a carnivore feeding on shrimps forming the highest percentage (33.31%) closely followed by digested matter (32.39%); teleosts (19.30%) represented by a variety of species, followed by polychaetes (5.84%), zooplankton (2.61%), and octopus (2.42%) were the next preferred items. Ranking next were the algae (1.83%) and stomatopods (1.23%), represented by the alima larva and squilla; lobster (0.52%) and phytoplankton (0.13%) were the least for the study period (Fig.1).

From the present study, we can infer that the fish *P. harmur* prefers shrimp over other items. In most cases, food was in a digested condition, possibly due to the delay in landing the catch. Hence only a few items were identified up to the species level through the conventional method.

Gut content analysis using DNA barcoding

Six food items from the gut were subjected to DNA analysis, of which 5 yielded readable sequences. The read lengths range between 612 bp and 629 bp for teleosts, 612 bp and 628 bp for shrimps, 601 bp for Octopus, 628 bp for lobsters and 615 bp for stomatopod. The sequences were compared to the reference library in the Barcode of Life Database (BOLD). Agarose Gel Electrophoresis was carried out to confirm the amplified region of CO1 of specimens.

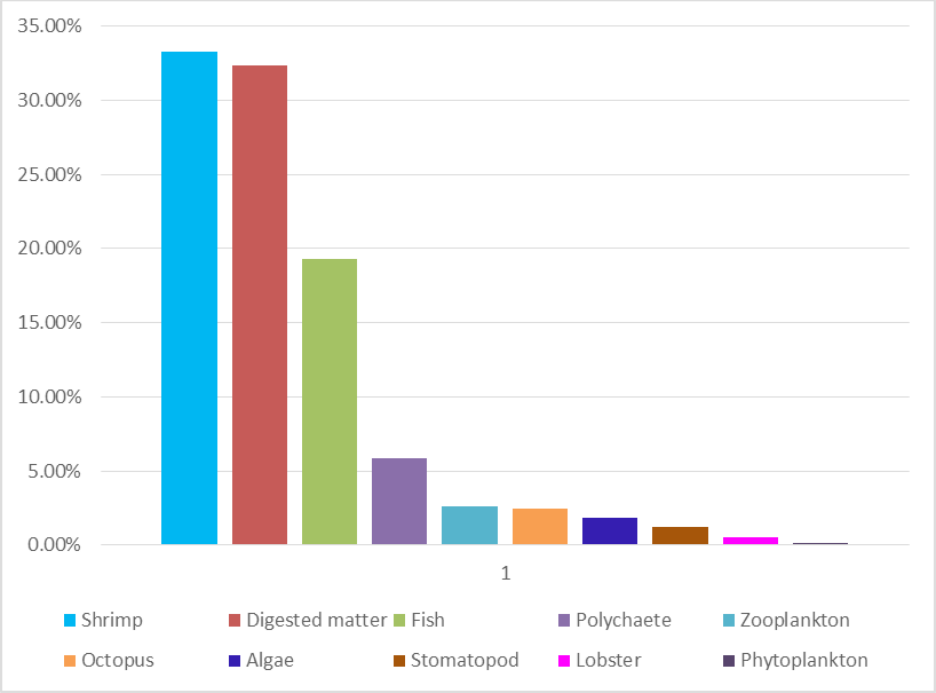

Fig. 1. Percentage composition of food items in the gut of *Priacanthus harmur*

4. Discussion

Deep sea fish are rapidly gaining importance as a potential fishery resource, as the inshore fishery alone can no longer satisfy the growing demand for fish (Khan et al., 1996). Non-conventional deepwater fish *Priacanthus hamrur*, an emerging species in India, is one of the economically important. In the seventies, these fishes were caught in trawls and were discarded in the sea since there were other species of choice available as table fishes. But, over the years, the landing of quality fishes dwindled, and *P. hamrur* was found to be highly comparable with other popular table fishes. The experiments conducted (CIFT, 1990) have indicated that deep-sea finfish resources could also be utilized for various fishery products. Therefore, exploitation of deep sea finfish resources on a larger scale from the Exclusive Economic Zone can significantly change the scenario of the Indian fishery sector; hence there is vast scope for exploitation of this non-conventional fishery resource.

The analyses of stomach contents during the study revealed that *P. hamrur* is carnivorous and feeds mainly on shrimps and fish. The fish also consumed polychaete, zooplankton, octopus, algae, stomatopods, lobsters, and phytoplankton. Similar results were obtained by Anjanayappa (2007) on *P. hamrur* from off the Mangalore coast. Philip (1994, 1998) observed that *P. hamrur* is a carnivorous species feeding on crustaceans, teleost fishes and occasionally on other organisms like cephalopods, polychaetes and gastropods and young fish fed on smaller crustaceans like Acetes spp., megalopa, alima, copepods, amphipods etc. showing a marked preference for crustaceans. Zacharia et al. (1991) reported that *P. harmur* is highly carnivorous and found squids and lizard fish in gut contents. Mandy and Inasu

Table 1. Samples of dietary items with percentage similarity with species available in the NCBI database and samples identified through morphology

Samples identified through DNA barcoding				Samples identified by their morphology
Species	**Seq: length (bp)**	**Similarity (%)**	**Acc: number**	
Bregmaceros nectabanus	612	98% *Bregmaceros* sp	OL512867	*Bregmaceros* sp, *Nemipterus japonicas, Callionymus kaianus*
Callionymus kaianus	629	99% *Callionymus kaianus*	KF265033	*Grammoplites scaber, Solea elongata, Cyanoglossus macrostomus, Sacura boulengeri*
Plesionika narval	612	97% *Plesionika narval*	KP398864	*Solenocera choprai, Plesionika narval*
Leptochela gracilis	628	97% *Leptochela gracilis*	OP741018	
Amphioctopus membranaceus	601	99% *Amphioctopus neglectus*	MT784165	*Callistoctopus* sp, *Amphioctopus membranaceus*
Oratosquilla oratoria	615	89% *Oratosquilla oratoria*	MF173560	*Kempella stridulans*

(1999, 2003) recorded deep sea prawns, prawn tissues, bristles of annelids, invertebrate eggs, animal tissues, fat droplets, pieces and tentacles of coelenterates from the gut of *P. hamrur*. Premalatha (1997), while working on the food and feeding habits of *P. hamrur* from the south-west coast of India, reported that this species had no preferential feeding and anchovies, small crustaceans, and parts of cephalopods were the commonly found food items. According to Sivakami (2001), this species feeds on pelagic crustaceans, followed by fishes and smaller molluscs. Kizhakudan and Zala (2006) stated that squids and crabs ranked lowest in the order of preference food items and indicated the tendency of this carnivorous fish to feed more on pelagic and mesopelagic forms, especially *Acetes* spp., than on benthic forms.

Shrimps constituted the main food item, which occurred in all the months with considerable variations. These variations may be due to factors such as the relative abundance of shrimps. The shrimp *Solenocera choprai* was detected in the gut content in the present study. The stomach contents of *P. harmur* from the east coast of India collected from shallow water recorded *Penaeus* spp and *Metapenaeus* spp, whereas stomachs of fishes from deeper waters contained the deep-sea prawn *Solenocera* sp., which is reported to be abundant in 50-200 m depth (Philip, 1994). *Penaeus* spp., *Metapenaeus* spp. and *Solenocera* spp. were recorded as the most important food items among crustaceans (Anjanayappa, 2007).

Naik (1990) studied the food and feeding habits of Priacanthids (*P. tyaenus, P. hamrur, P. cruentatus,* and *P. blochi*) and also reported that bullseye is a carnivorous species, feeding on fishes, crustaceans and polychaetes. Studies carried out on the food and feeding habits of species like *P. tayenus,* and *P. macracanthus* in southeast Asian regions like Malaysia (Ambak et al., 1987), Hong Kong (Lester,1968), Thailand (Chomjurai, 1970; Wetchagarun, 1971) and Panay Islands (Senta, 1978) have also indicated that crustaceans were the more preferred food items by priacanthids in those regions. The digested matter was abundant among the food items and occurred in all the months with considerable variations. Anjanayappa (2007) also made similar observations stating that semi digested food materials encountered in most of the months, mainly constituted by shrimps, crustacean remains and other food organisms. Philip (1994, 1998) reported that the semi-digested matter comprised more than 50% of the stomach contents of *P. hamrur* from the upper-east coast of India.

The occurrence of fish in the gut contents was accounted for in all the months with remarkable variations. Teleosts formed another important group of food items, comprising *Grammoplites scaber, Solea elongata, Cyanoglossus macrostomus, Sacura boulengeri, Bregmaceros* sp, *Callionymus kaianus* and *Nemipterus japonicus*. Of these, flatfishes and *Nemipterus japonicus* were dominant. Anjanayappa (2007) recorded the dominant food items in the gut of *P. hamrur* as flat-heads, flatfishes, *Saurida* spp., *Stolephorus* spp., *Leiognathus* spp. and *Nemipterus* spp. Philip (1998) observed teleost fishes in the stomachs of individuals having a length of 100 mm and above. Sivakami et al. (2001) reported a variety of species of fish in the diet of *P. hamrur,* such as *Stolephorus* spp., silver bellies, *Saurida* spp., flatfishes and flatheads. *Bregmaceros* sp. was dominant in the food during January to March of both years (Philip, 1994).

Amphioctopus neglectus and *Callistoctopus* sp, stomatopod: *Kempella stridulans* and lobsters; *Petrarctus* sp and *Nephropsis* sp were also detected from the gut content of *P. hamrur* in the present study. According to Anjanayappa (2007), food items like juveniles of squid, octopus, crab, and cuttlefish were recorded in lesser quantities. In the present study, there were no seasonal variations in the preferred food items. Seasonality was noticed in respect of some food items of *P. hamrur* by Philip (1994), and the changes in the food items during different months could have been influenced by the samplings made from different depths and areas, where the relative abundance of different food organisms shows variation.

In the present study, most of the food items from the gut content were seen in a depth below 50m. Most of these specimens were obtained mainly from January, February and March, during which mature gonads were noticed. Similar observations were made by Philip (1994) in *P. harmur* that most of the food items identified from the stomach were present in samples collected from depth zones below 50 m and 51-100m. He also stated that the abundance of mature *P. hamrur* of both sexes in the deeper waters could be taken as an indication that the fish spawns in deeper waters. But Vijayakumaran and Naik (1989) claimed that the fish is mainly a deep-water inhabitant exhibiting migration towards shallow water during pre-monsoon, which appears to be for breeding purposes.

Morphological identification led to species-level identification of five fish prey items, stomatopod *Kempella stridulans* and shrimp *S. choprai*. Direct sequencing of 6 prey samples that could not be classified to a lower taxonomic level through visual examination, when subjected to DNA barcoding analysis further improved the identification accuracy rate of gut contents of three samples and revealed the presence of shrimp *Plesionika narval* (98%), *Leptochela gracilis* (97%); octopus *Amphioctopus neglectus* (99%); fish *Callionymus kaianus* (99%) upto species level and Bregmaceros spp (98%) to genus level. Sequence assignment to a taxon is a critical step in the analysis and is done by comparing each sequence to a reference database (Jones et al., 2011). Although one sample was identified as *Oratosquilla oratoria* (89% similarity) through barcoding, the species was *Kempella stridulans* as identified by an expert; hence the sample identified only up to its higher taxonomic level. According to Pompanon et al. (2012), the barcode region's taxonomic resolution (i.e., resolution capacity) needs to be considered, and some barcode regions will only identify taxa above the species level. Different sequencing platforms may also preferentially sequence specific amplicons (Dohm et al.2008) or fail to sequence particular amplicons (Deagle et al. 2009) completely. This may also lead to some target sequences being unrepresented in the final data set. A species can be identified in a complex substrate using

broad coverage primers when its DNA represents a low proportion of the target DNA (Pegard et al., 2009).

This work shows that molecular genetic techniques can identify prey fish species after much longer digestion than possible with conventional methods. In cases where the proportion of stomachs with unknown material and unidentified fishes is present in most diet studies (Legler, 2009; Mullowney, 2001), molecular genetic techniques would have proven helpful in identifying prey species, especially on piscivorous fishes (Szedlmayer and Brewton, 2019). DNA barcoding can identify species with fragments as short as 100 bp with at least 90% efficiency (Meusnier et al., 2008). This opens an outstanding possibility to obtain sequences from short DNA fragments quickly and cheaply (Hajibabaei et al., 2006).

DNA degradation in dietary samples limits the length of fragments PCR can successfully amplify (Symondson, 2002; Deagle et al., 2006; Troedsson et al., 2009). For this reason, the length of barcoding regions used for dietary analysis is generally in the 100–250 bp range, which inevitably reduces taxonomic resolution. It is possible in some situations to use barcodes as long as 585 bp (Juen & Traugott 2005), 650 bp (the COI marker defined by Folmer et al. 1994; Blankenship & Yayanos 2005) or even more when DNA is extracted from intact prey remains (Clare et al. 2009, 2011). Here bp ranged between 601 and 629, but most sequences did not show 99% similarity except two. Gut contents are complex sources of DNA consisting of a mix of DNA from the predator itself, its inherent gut flora and potential prey items. Not all DNA needs to be equally amplified when using PCR to amplify DNA from complex sources. This can be true if the amplification efficiency is equal for all DNA molecules in the template (Wintzingerode et al. 1997). While using universal primers, the DNA from taxa having precise complementary sequences will be preferentially amplified (Blankenship and Yayanos, 2005). According to Pompanon et al. (2012), the low amount of target DNA in many dietary samples, combined with the extreme sensitivity of PCR and the ability to recover thousands of sequences per sample, means that even minor contamination will be represented in the final data set.

Though our study had limitations with a short sampling period and small sample size, this study suggests the food preference of *P. hamrur*. More importantly, this study demonstrates the potential application of molecular genetic techniques for identifying semi-digested food remains in the gut. Both conventional methods, and diet analysis through barcoding, suggested that *P. hamrur* is a carnivorous species that prefers shrimps and fish over other items as food. Recently, there has been a considerable increase in the landings of bullseye fish in India, and in 2021, the landing was around 31,117 tonnes (FRAD, CMFRI, 2022). This demands further monitoring of their food and feeding behaviour and understanding their impact on the food web structure involving commercial and non-commercial species.

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