3.1 Methodology

The study on molecular taxonomy with reference to barcoding has a different level of advance technology for species identification. It is highly essential to identify the right kind of methodology for barcode the species and thereof process the data. This chapter mainly deals the description of the study area, collection of fish samples, morphological and morphometric study of fish, extract DNA from the fish and its sequence processes. The sequenced data further analysed through the different procedure for the evaluation, interpretation and thereof the conclusion been made for the studied fish taxonomy.

3.1.1 Study Area

Andaman and Nicobar Islands (92° to 94° East and 06° to 14° North), is an archipelago with 572 Islands/Islets, stretching over 700 kilometre (km) from North to South, in the Bay of Bengal. These islands are nearby to East Asian countries and more than 1400 km from main land India. Andaman Islands are volcanic rock type of land mass surrounded with different sort of endemic flora and fauna (Tikader et al., 1986). The head quarters of Andaman and Nicobar Island is Port Blair town. It has a major sea and air port along with fish landing centres. The economy of the Andaman Group of Islands depends on the exploitation of its marine resources. Exportable fishes of grouper and snapper from the Andaman Groups of Islands is in the range of 75% and 15%, respectively. The remaining percentages covered by emperor, perches and bottom fishes.

The fish samples landing centres of Port Blair is main source for the arrival of fish from the sea after it catches. Five centres were identified for the groupers collection from the fish landings areas. The details of these five fish landing centres (Fig. 3.1) are as follows:

3.1.1.1 Junglighat

The Junglighat fishing harbour is main fishing activity area of south Andaman. The medium to small size fishing crafts are operate from this harbour. From this harbour nearly 50 -80 medium size mechanised boats were operated in 2009 – 2011
periods. Since, this fishing harbour close to major commercial activities of Port Blair, the large amount of landings were observed in this centres compare to others. This landing centre situated in Andaman Sea.

### 3.1.1.2 Dignabad

The Dignabad fish landing centre is another landing centre situated within the city limit of Port Blair. From this landing centre near about 40-45 medium size mechanised fishing boats are operated daily. Here also observed good amount of fish landing for local market. Dignabad landing centre is also situated in Andaman Sea.

### 3.1.1.3 Guptapara

The Guptapara fish landing centre is a natural fishing harbour surrounded in mangrove ecosystem. This natural eco fishing landing centre is operated with 30-35 medium size mechanised fishing boats for fishing activities. This landing centre located in Bay of Bengal.

### 3.1.1.4 Wandoor

The Wandoor fish landing centre is a natural sandy beach fishing harbour. This sandy beach situated in Bay of Bengal side Port Blair. The fisherman engagement their fishing activity using 50-60 mechanised fishing boats.

### 3.1.1.5 Panighat

The Panighat fish landing centre is natural muddy and rocky fishing harbour. This fishing harbour situated in Andaman sea side of the south eastern coastal region of the Port Blair city. The fisherman engagement around 50-60 mechanised fishing boats for their fishing activities.

### 3.2 Sample collection and Preservation

Andaman Islands coral reef environment exhibits around 52 species of Serranidae family and in which 14 species are commercially important for exports to various countries (Rajan, 2001). However, only 33 species belonging to family Serranidae were collected from local five major fish landing sites in Port Blair.
The fish samples were collected during the period 2009 – 2011 continuously, from fish landing centres. Each species five representative specimens were collected, however the rare specimens or not occurring frequent species were collected one specimen only. Out of these six specimens, triplicate specimens used for COI gene sequence analysis and one specimen stored in 10% formaldehyde and boric acid mixture and deposited in Department of Ocean Studies and Marine Biology (DOSMB), Pondicherry University, Port Blair and other one stored in alcohol as a reserve. Over and above, one individual of all the studied species were also kept in deep freezer at -20°C. Once the specimens brought to the

![Fig. 3.1 Study Area](image-url)
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laboratory from the fish landing centres, immediately photographed and morphometric identification were carried out for it traditional taxonomic identification to the species level. Once identified species were reconﬁrmed with the experts from the Zoological Survey of India (ZSI), Port Blair as well as Central Marine Fisheries Research Institute (CMFRI) Mandapam, Tamil Nadu. After its species level conﬁrmation, the particular species were cut into a piece and collected their tissue which had been preserved in 95% Absolute Alcohol, labelled it and store in to a -20°C for further analysis of DNA isolation and barcoding processes.

3.3 SYSTEMATIC CATALOGUE

The collected fishes through different landing centres were brought to the laboratory and identiﬁed based on the following merismatic characters provide by Food and Agriculture Organisation (Randall and Heemstra, 1991; Heemstra and Randall, 1993) to evaluate its taxonomy.

**Domain:** Eukaryota  
**Kingdom:** Animalia  
**Phylum:** Chordata  
**Subphylum:** Vertebrata  
**Superclass:** Gnathostomata  
**Division:** Teleostei  
**Subdivision:** Euteleostei  
**Superorder:** Cyclosquamata  
**Order:** Aulopiformes  
**Suborder:** Percoidei  
**Family:** Serranidae  
**Subfamily:** Epinephelinae  
**Tribe:** Epinephelini  
**Genus:** Aethaloperca, Cephalopholis, Epinephelus, Plectropomus and Variola
The morphometric characters for the identified species were presented in this section.

### 3.3.1 Groupers diagnostic features

According to Food and Agriculture Organisation (1993 and 2011) identification key the following morphometric characters were used for the identification of Family Serranidae, Sub Family Epinephelinae, Genus *Aethaloperca, Cephalopholis, Epinephelus, Plectropomus* and *Variola* and their species. The body is robust or somewhat compressed, oblong-oval to rather elongate. Mouth with small, slender, depressible teeth on jaws, vomer and palatines (*Anpyerodon* lacks palatine teeth); distinct canine teeth present at front of mouth in some species; no molars teeth; maxilla exposed when mouth is closed; supra-maxilla well developed. A single dorsal fin with VII to XI spines and 10 to 21 rays; anal fin with III spines (inconspicuous in *Plectropomus*) and 7 to 13 rays (the last dorsal- and anal-fin rays usually split to their base but counted as a single ray); caudal fin with 13 to 15 branched rays; pelvic fins I spine and 5 branched rays; pelvic-fin insertion under or a little behind pectoral-fin base; no scaly process at base of pelvic fins. Edge of pre-opercle serrate (serrae reduced in adult *Cephalopholis* and *Plectropomus*); opercle with 3 flat spines upper and lowermost spines often covered by skin and scales; upper edge of operculum free; gill membranes separate, joined to isthmus far forward, with 7 branchio-stegal rays. Anterior and posterior nostrils close together. Lateral line single, lateral-line scales separated, inconspicuous, smaller than surrounding scales and mostly covered by them; Scales small, more than 78 oblique series from upper end of gill opening to caudal-fin base; soft dorsal, anal and caudal fins scaly. Vertebrae has 10 pre-caudal and 14 caudal; first dorsal-fin pterygiophore with one supernumerary fin spine and no separate distal element; supra-neural bones 2 numbers (*Plectropomus* genus have only one supra-neural present).

The colour patterns are one of the basic tools for the identification of grouper species. Most of the adult species, except some species, exhibit distinct colour pattern enough to identify the particular species. Most of the cases, the colour pattern differ from juveniles to adults stages. The species grow in adult or large size dark spots became smaller and numerous. As well as, their changing colour pattern depend
upon the mood of the fish, within short time and also has a white blotches or bars as representation of a “fright” or “stress” pattern. Similarly, in the same species, habitant in shallow water not shows much reddish colour but the deep water catch exhibit more reddish. The bright yellow or orange-yellow colour represents a xanthic colour morph for few species. The normal colour pattern of live fish obscured during the post catch period. The fish, after its death the skin bleaches its colour. The lateral line count was carried out right side with its head pointing left.

The close similarity of the morphological character identification of *Epinephelus* species in the field is cumbersome. The best effort to be made for differentiation based on the different head structure, caudal fin shape and number of fin rays (Heemstra, 1991).

### 3.3.2 Key to Genera level identification

Identify the groupers at the generic level the following systematic was followed:

#### 3.3.3 Diagnostic Features of *Aethaloperca* Fowler, 1904:

Body deep and compressed, the depth greater than the head length and contained 2.1 to 2.4 times in standard length, the body width contained 2.3 to 2.8 times in the depth. The soft portion of dorsal fin and spinous are completely or partially separated by a deep notch. Canine teeth present in jaws; head normal invariably with a convex profile; hind nostril normal, round; dorsal fin with 6 to 11 spines. Scale large, 40 to 55 along lateral line. Anal fin with 9 soft rays; no depressible teeth in upper jaw................. *Aethaloperca*.

The genus *Cephalopholis* and *Gracila* are closely related to the genus *Aethaloperca* based on its IX dorsal-fin spines and several trisegmental pterygiophores in the dorsal and anal fin. Further, it differs from *Cephalopholis* and *Gracila* in the configuration of the pectoral and median fins and in some cranial features such as the anteriorly converging parietal crests and the well-developed median crest on the frontals that extends to the rear edge of the ethmoidal depression. *Aethaloperca* also differs from *Gracila* in the shape of the maxilla and in having a larger head and deeper body, with a colour of dark brown to black.
Gill rakers 8 to 10 on upper limb, 15 to 17 on lower limb, the longest gill raker, slightly longer than longest gill filaments. Dorsal fin with IX spines and 17 or 18 rays, the fin origin over opercle; the third or fourth spine longest; anal fin with III spines and 8 or 9 rays; pectoral fins asymmetric, with 17 to 19 rays, the fifth or sixth ray longest. Caudal fin truncate, with 8 branched rays, 9 pro-current rays in upper part; 7 branched rays and 8 procurent rays in lower part. Scale shapes on body ctenoid, with auxiliary scales; lateral-line scales 48 -54 and lateral-scale series 94 -104. Dark brown to black in colour with occasionally with an orange cast, usually with a pale vertical bar on side of abdomen; distal part of spinous dorsal fin dark orange to brownish red: inside of mouth, gill cavity and upper jaw membranes reddish orange. Juveniles with a broad white posterior margin on caudal fin and a narrow white margin on soft dorsal fin...........Aethaloperca rogaa

3.3.4 Key to Species of Cephalopholis genus

The soft portion of dorsal fin and spinous are completely or partially separated by a deep notch. Canine teeth present in jaws; head normal invariably with a convex profile; hind nostril normal, round; 9 to 11 dorsal, fin spines; lower edge of pre-operculum without antrorse spines. 2 to 3 curved canines on each side of lower jaw. No distinct enlarged canines on each side of lower jaw; caudal fin rounded, truncate or emarginated. 9 dorsal fin spine are noticed.................Cephalopholis.

Caudal fin rounded; head length 2.2 to 2.7 times in standard length; Body depth 2.3 to 3.2 times in standard length; body width 1.9 to 2.4 times in body depth; Pelvic-fin length 1.5 to 2.35 times in head length; pectoral-fin rays 15 to 20 (rarely 15); anal-fin rays 8 to 10; Anal fin rays usually 8; colour generally brown to dark brown. Small dark spots or dark-edged pale blue spots on head. Dorsal-fin rays usually 15; lateral-scale series 84 to 98; pectoral-fin length contained 1.4 to 1.6 times in head length; dark-edged blue spots only on head and anteriorly on body (distribution Andaman Sea, Philippines, and Indonesia to New Caledonia and Great Barrier Reef) .......................................................... C. microprion

Caudal fin rounded; head length 2.2 to 2.7 times in standard length; Body depth 2.3 to 3.2 times in standard length; body width 1.9 to 2.4 times in body depth;
Pelvic-fin length 1.5 to 2.35 times in head length; pectoral-fin rays 15 to 20 (rarely 15); anal-fin rays 8 to 10; Anal fin rays usually 8; colour generally brown to dark brown. Small dark spots or dark-edged pale blue spots on head. Dorsal-fin rays usually 16; lateral-scale series 92 to 106; pectoral-fin length 1.5 to 1.8 times in head length; blue ocelli on head, body, and basally on median fins (distribution Andaman Sea, Gulf of Thailand, Indonesia, Philippines, northern Australia, and New Britain) ................................................................. \textit{C. cyanostigma}

Caudal fin rounded; head length 2.2 to 2.7 times in standard length; Body depth 2.3 to 3.2 times in standard length; body width 1.9 to 2.4 times in body depth; Pelvic-fin length 1.5 to 2.35 times in head length; pectoral-fin rays 15 to 20 (rarely 15); anal-fin rays 8 to 10; Anal-fin rays usually 8; Dorsal-fin rays 15 to 17; lower-limb gill rakers 17 to 19; auxiliary scales present on body; colour dark brown, covered with small dark-edged blue ocelli; 5 or 6 pale bars often visible on rear half of body (Red Sea and Indo-Pacific) ................. \textit{C. argus}.

Caudal fin rounded; head length 2.2 to 2.7 times in standard length; body width 1.9 to 2.4 times in body depth; Pelvic-fin length 1.5 to 2.35 times in head length; anal-fin rays 8 to 10; Anal-fin rays usually 8; Lateral-line scales 66 to 80; lateral-scale series 115 to 134; pectoral-fin rays 18 to 20; body depth 2.3 to 2.8 times in standard length; colour generally red to reddish brown (juveniles and some adults may be dark purple or brown) with widely scattered whitish blotches (Indian Ocean) or generally brownish, covered with small dark red to reddish brown spots and irregular white blotches (tropical Indian Ocean and Pacific)............\textit{C. sonnerati}.

Caudal fin rounded; head length 2.2 to 2.7 times in standard length; body width 1.9 to 2.4 times in body depth; Pelvic-fin length 1.5 to 2.35 times in head length; anal-fin rays 8 to 10; Anal-fin rays usually 8; lateral-line scales 54 to 68; lateral-scale series 79 to 121; pectoral-fin rays 16 to 19; body depth 2.6 to 3.5 times in standard length; caudal fin blackish red, the corners broadly red, each set off by an oblique white stripe; pectoral fins red, shading to orange-yellow distally (Pacific); or caudal and pectoral fins uniformly blackish (Indian Ocean)......................................................... \textit{C. urodeta}

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Caudal fin rounded; body depth 2.6 to 3.5 times in standard length; body width 1.9 to 2.4 times in body depth; Pelvic-fin length 1.5 to 2.35 times in head length; pectoral-fin rays 15 to 20 (rarely 15); anal-fin rays 8 to 10; Anal-fin rays usually 8; lateral-line scales 45 to 56; lateral-scale series 79 to 88; head length 2.2 to 2.4 times in standard length; dark brown saddle spot on caudal peduncle, followed by a smaller spot; oblique dark streak on caudal fin (Indo-Pacific) C. leopardus

Caudal fin rounded; body depth 2.6 to 3.5 times in standard length; body width 1.9 to 2.4 times in body depth; Pelvic-fin length 1.5 to 2.35 times in head length; pectoral-fin rays 15 to 20 (rarely 15); anal-fin rays 8 to 10; Anal-fin rays usually 8; Lateral-line scales 45 to 56; Lateral-scale series 90 to 121; head length 2.3 to 2.6 times in standard length; Head, body, and fins covered with small blue ocelli; No dark blotches dorsally on body; no blue lines radiating from eyes (Red Sea and Indo-Pacific) C. miniata

3.3.5 Key to the Species of Epinephelus spp.

The soft portion of dorsal fin and spinous are completely or partially separated by a deep notch. Canine teeth present in jaws; head normal invariably with a convex profile; hind nostril normal, round; 7 to 9 soft anal fin rays; pre-operculum edge rounded or with slight angle only; body oblong and relatively less compressed laterally; caudal fin mostly rounded, sometimes truncate; colour not uniformly dark brown. Dorsal fin spines more or less equal in length to soft rays, with median ones the longest..................Epinephelus.

Caudal fin of adults emarginate to truncate (slightly rounded on juveniles and some specimens of E. bleekeri, and convex in adults if broadly spread); inter-spinous membranes of dorsal fin not incised; Gill rakers elongate (no rudiments), 12 to 16 on upper limb and 20 to 23 on lower limb; dorsal-fin rays 17 to 19; colour purplish to brownish grey with yellowish brown dots on head and longitudinal brown lines on dorsal part of body (lines usually lost on large adults)................................................. E. undulosus
Caudal fin of adults emarginated to truncate (slightly rounded on juveniles and some specimens of E. bleekeri, and convex in adults if broadly spread); inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); dorsal-fin rays modally 17; Gill rakers elongate (no rudiments), gill rakers 24 to 28; body depth 2.3 to 2.9 times in standard length; Colour in life usually dark blue or grey, but without black or white spots, the caudal peduncle (at least posteriorly) and fins bright yellow; large adults lose yellow coloration and become chestnut brown, dark blue, grey, or nearly black and have numerous small, irregular pale blue spots that are more visible underwater; (Indian Ocean but not in Red Sea)..........................E. flavocaeruleus

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); gill rakers 24 to 28; body depth 3.0 to 3.5 times in standard length; dorsal-fin rays 16 to 18 (rarely 15 in E. areolatus); dorsal-fin rays modally 17; lateral-line scales 48 to 54; head and at least front of body with small spots, either yellow (pale in preservative) or brown; serrate at corner of pre-opercle about 3 to 5 times longer than those above corner; head, body, dorsal fin, and upper third of caudal fin with small orange-yellow spots, the lower two-thirds of caudal fin dark grey; (India to Taiwan, Hong Kong and Philippines)................ E. bleekeri

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); lower gill rakers 14 to 16; body depth 3.0 to 3.5 times in standard length; dorsal-fin rays 15 to 17; lateral-line scales 48 to 54; margin of anal fin of adults rounded to slightly angular, the longest ray 2.0 to 2.6 times in head length; pyloriccaeca 11 to 17; dark spots on body of adults sub equal to pupil; Head and body with small close-set yellowish brown to dark brown spots (dark in preservative); fins with small dark spots; (Red Sea to western Pacific).......... E. areolatus

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); lower gill rakers 15 to 18; body depth 3.0 to 3.5 times in standard length; dorsal-fin rays 16 to 18; anal fin of adults angular or pointed, the longest ray 1.9 to 2.3 times in
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head length; pyloric caeca 26 to 52; largest dark spots on body of adults distinctly smaller than pupil (Red Sea to western Pacific)................. *E. chlorostigma*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); lower gill rakers 15 to 18; body depth 3.0 to 3.5 times in standard length; dorsal-fin rays 16 to 18; lateral-line scales with a single tubule (except anterior scales of large *E. brunneus, E. coioides* and *E. malabaricus*); pelvic-fin length 2.2 to 2.6 times in head length; eye diameter contained less than 7 times in head length of 20 cm fish and less than 8 times in head length for 35 cm fish; maximum total length less than 1.5 m (except *E. tukula*, which may reach 2 m); Numerous distinct dark spots (not dots) over most of head and body (spots brownish red to black in life and distinct in preservative); dorsal profile of head convex; spots on head, body, and fins dark brown to black; black blotches on body at base of dorsal fin present or absent; blackish maxillary streak usually present; dark spots on pectoral fins progressively smaller distally; (India, Sri Lanka and Indonesia) ....................... *E. faveatus*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); lower gill rakers 15 to 18; body depth 3.0 to 3.5 times in standard length; dorsal-fin rays 15 to 17; pelvic-fin length 1.9 to 2.3 times in head length (at standard length less than 25 cm); pectoral-fin rays 18 or 19 (often 19); total gill rakers 21 to 24 (modally 23); body width 1.4 to 1.8 times in body depth; lower jaw strongly projecting no dark spots on distal part of pectoral fins; No large blackish blotch at base of posterior dorsal-fin spines (though 1 to 3 spots at this location are usually darker than adjacent spots); dark spots on head, body, and fins less numerous, 5 to 7 on soft dorsal fin of a 11 cm fish and 20 to 21 dark spots on the fin of a 29 cm fish (Indo-Pacific but not in Red Sea or Persian Gulf) ................................................. *E. macrospilos*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); lower gill rakers 15 to 18; body depth 3.0 to 3.5 times in standard length; dorsal-fin rays 15 to 17; pelvic-fin length 1.9 to 2.3 times in head length (at standard length less than 25 cm); pectoral-fin rays 18 or 19 (often 19); total gill rakers 21 to 24 (modally 23); body width 1.4 to 1.8 times in body depth; lower jaw strongly projecting no dark
spots on distal part of pectoral fins; Dark spots on body elongate, oblique, and more numerous posteriorly; dark spots on head separated by more than 2 spot diameters; ventral edge of maxilla of subadults (33 to 40 cm standard length) a distinct step-like shape that develops into a bony knob in adults (Indian Ocean and Indonesia).......................... *E. longispinis*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); lower gill rakers 15 to 18; body depth 3.0 to 3.5 times in standard length; dorsal-fin rays 16 to 18; pelvic-fin length 1.9 to 2.3 times in head length (at standard length less than 25 cm); pectoral-fin length 1.2 to 1.6 times in head length (except fish from northwest Australia); serrate at corner of preopercle distinctly enlarged; oblique; two dark brown bands (or elongate dark blotches linked by narrow bands) on chest (Andaman Islands and western Pacific). ............................................. *E. quoyanus*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); lower gill rakers 15 to 18; body depth 3.0 to 3.5 times in standard length; pectoral-fin length 1.5 to 2.0 times in head length; dorsal-fin rays 15 to 18; serrae at corner of preopercle slightly enlarged; no oblique dark bands on chest; longest dorsal-fin spine 2.6 to 2.9 times in head length; lateral-scale series 92 to 120; Pectoral-fin rays 16 to 18; total gill rakers 23 to 27 (except *E. merra* with 21 to 25); dark spots on body larger than pupil; dark spots on fins smaller than those on body; maxilla usually reaching to or past a vertical at rear edge of orbit; some dark brown spots on body often joined to form short horizontal or oblique bands; black spots on pectoral fins very small and largely confined to rays (Indo-Pacific but not in Red Sea)......................... *E. merra*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); total gill rakers 29 to 31; body depth 2.6 to 2.9 times in standard length; Pectoral-fin length 1.5 to 2.0 times in head length; dorsal-fin rays 13 to 15; pectoral-fin rays 17 to 20; lateral-line scales 53 to 58; mid lateral-body scales rough; Second anal-fin spine 2.4 to 3.6 times in head length; longest dorsal-fin spine 2.8 to 3.8 times in head length; head and body pale yellowish brown, with irregular dark brown blotches and
numerous small close-set dark brown spots; black saddle spot on caudal peduncle; mid lateral-body scales of adults smooth (Indo-Pacific) .......... *E. fuscoguttatus*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); total gill rakers 22 to 29; body depth 2.7 to 3.7 times in standard length; pectoral-fin rays modally 19; pectoral-fin length 1.5 to 2.0 times in head length; dorsal-fin rays 13 to 16; lateral-line scales 54 to 65, the anterior scales of large adults with branched tubules; numerous small bony platelets on side of first gill arch; auxiliary scales present on body of adults; maxilla usually reaching past vertical at rear edge of eye; nostrils sub-equal or posterior nostrils enlarged, but not vertically elongate or more than twice diameter of anterior nostrils; pectoral-fin length 1.6 to 2.5 times in head length; lateral-scale series 95 to 130; body with 5 irregular dark bars which tend to bifurcate ventrally (bars may be faint or broken into series of 2 or 3 large blotches; head and body with numerous small well-separated black spots (largest spots about twice size of rear nostrils); irregular white or pale spots or blotches usually present on head and body; (Indo-Pacific) ............... *E. malabaricus*

Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); total gill rakers 22 to 29; body depth 2.7 to 3.7 times in standard length; pectoral-fin rays modally 20 pectoral-fin length 1.5 to 2.0 times in head length; dorsal-fin rays 13 to 16; lateral-line scales 54 to 65, the anterior scales of large adults with branched tubules; numerous small bony platelets on side of first gill arch; auxiliary scales present on body of adults; maxilla usually reaching past vertical at rear edge of eye; nostrils sub-equal or posterior nostrils enlarged, but not vertically elongate or more than twice diameter of anterior nostrils; pectoral-fin length 1.6 to 2.5 times in head length; lateral-scale series 95 to 130; head, body, and usually median fins with numerous orange, brownish orange, or reddish brown spots (diameter of largest spots about 4 or 5 times that of rear nostrils); no white or pale spots on head or body; orange spots become poorly defined and darker with growth; spots on head often coalesce and become elongate, arranged in irregular rows radiating from eye; fins brownish, with proximal parts brown spotted; orange spots turn brown after death and are poorly defined on preserved specimens (Indo-Pacific) .................... *E. coioides*
Caudal fin truncate to slightly rounded; inter-spinous membranes of dorsal fin not incised; Second dorsal-fin spine not elongate (third or fourth spines longest); total gill rakers 22 to 29; body depth 2.7 to 3.7 times in standard length; head length 2.1 to 2.4 times in standard length; pectoral-fin rays modally 20 pectoral-fin length 1.5 to 2.0 times in head length; dorsal-fin rays 13 to 16; numerous small bony platelets on side of first gill arch; auxiliary scales present on body of adults; maxilla usually reaching past vertical at rear edge of eye; nostrils sub-equal or posterior nostrils enlarged, but not vertically elongate or more than twice diameter of anterior nostrils; pectoral-fin length 1.6 to 2.5 times in head length; lateral-scale series 95 to 112; maximum length about 80 cm; Serrate at corner of preopercle slightly to moderately; 3 to 6 rows of teeth on midside of lower jaw of adults; head, body, and fins with orange-red to dark brown spots (those on body nearly as large as eye in young, about pupil size in large fish; spots usually absent in outer part of pectoral fins of adults); body often with faint oblique dark bars; blackish blotch often present at base of last 4 dorsal-fin spines and extending into lower part of fin (more evident in young) (Indo-Pacific) ................. **E. tauvina**

Caudal fin truncate to slightly rounded; inter-spinous dorsal-fin membranes moderately to deeply incised; Second dorsal-fin spine not elongate (third or fourth spines longest); total gill rakers 22 to 29; lower gill rakers 14 to 19; body depth less than head length, 2.5 to 3.6 times in standard length; head length 2.1 to 2.4 times in standard length; dorsal-fin rays 13 to 16 (rarely 16); pectoral-fin rays modally 20; pectoral-fin length 1.5 to 2.0 times in head length; 48 to 75 lateral-line scales; body usually with vertical or slightly oblique broad dark bars (faint or absent on some species). Body without dark vertical bars (except juvenile *E. bruneus* and *E. daemelii* which have dark oblique bars containing pale spots and streaks). No dark brown to black dots on body or head (some species with black spots, but these are larger than 2 mm); margin of inter-spinous dorsal-fin membranes black (red in fish from Western Australia and deep water); edge of orbit narrowly black, surrounded by a pale blue line; body usually with 5 faint dark bars often containing irregular pale spots (Indo-Pacific)......... **E. fasciatus**

Caudal fin truncate to slightly rounded; inter-spinous dorsal-fin membranes moderately to deeply incised; Second dorsal-fin spine not elongate (third or fourth
spines longest); total gill rakers 22 to 29; lower gill rakers 14 to 19; no dark bars on juveniles or adults; body depth 2.6 to 3.2 times in standard length; lateral-line scales 48 to 55; mid-lateral-body scales rough (except large adults of *E. marginatus* with smooth scales); body depth contained 2.6 to 3.2 times in standard length: body width 1.7 to 2.5 times in body depth; no bony platelets on first gill arch; third anal-fin spine less than 10% longer than second spine; lateral-scale series 95 to 110; pectoral-fin rays 15 to 17; obvious dark or light markings present on head and/or body; nostrils subequal; pectoral-fin length 1.3 to 1.7 times in head length; serrate at corner of pre-opercle slightly to moderately enlarged; mid-lateral part of lower jaw with 3 to 5 rows of teeth in Adults; body with auxiliary scales; colour pattern of white dots, spots, or blotches; shallow water species; snout length is 4.0 to 4.8 times in head length; head, body, and fins dark grey-brown with numerous small whitish spots and scattered large whitish blotches: maxillary streak black. Small whitish spots on adults tend to coalesce to form irregular longitudinal bands; paired fins of adults without whitish spots (Mozambique to western Pacific).……………….*E. ongus*

Caudal fin truncate to slightly rounded; inter-spinous dorsal-fin membranes moderately to deeply incised; second dorsal-fin spine not elongate (third or fourth spines longest); total gill rakers 23 to 28; lateral-scale series 102 to 125; body with prominent dark brown bands and/or spots; body with long curved dark brown bands or series of spots, the middle of each band or row of spots more ventral than the ends; Dark brown blotch on body at base of middle dorsal-fin spines joined to dark brown band passing to upper edge of gill opening; a second dark band from upper end of gill opening, bifurcating above pectoral fin, with branches to anterior and posterior dorsal-fin rays; 2 bands from eye, the upper branch to nape, the lower a broad curve or broken line to upper part of caudal peduncle (Red Sea to South Africa and central Pacific).………………………….*E. morrhua*

### 3.3.6 Key to the Species of *Plectropomus* genus

Body robust, elongate, the depth less than head length and contained 2.9 to 3.9 times in standard length; body width contained 1.6 to 2.1 times in its depth. Head length contained 2.8 to 3.2 times in standard length; snout distinctly longer than eye diameter, snout length 2.8 to 3.6 times in head length; pre-orbital depth contained 5.6
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to 10 times in head length; inter-orbital area concave or flat, the dorsal head profile convex; pre-opercle broadly rounded, with 3 large, ventrally directed spines (hidden by skin) along lower half; lower developed gill rakers 4 to 10; dorsal fin with VII or VIII slender spines and 10 to 12 rays, the fin membranes distinctly incised between the spines, the third or fourth spine usually longest, longest dorsal-fin ray 2.2 to 3.2 times in head length; pectoral-fin rays 16 to 18; pectoral-fin and pelvic-fin length 2.1 to 2.4 times in head length; caudal-fin length 1.5 to 1.8 times in head length; caudal fin emarginate, the caudal concavity 5 to 12 times in head length; no scales on inter-orbital area; blue spots round to oblong; head and body pale, with large saddle-like dark brown or black bars and a few small blue spots, the fins yellow; or head and body brownish with numerous small blue spots and with or without faint dark bars (Indo-Pacific).................. P. laevis

Body robust, elongate, the depth less than head length and contained 2.9 to 3.9 times in standard length; body width contained 1.6 to 2.1 times in its depth. Head length contained 2.8 to 3.2 times in standard length; snout distinctly longer than eye diameter, snout length 2.8 to 3.6 times in head length; no scales on inter-orbital area; pre-orbital depth contained 5.6 to 10 times in head length; inter-orbital area concave or flat, the dorsal head profile convex; pre-opercle broadly rounded, with 3 large, ventrally directed spines (hidden by skin) along lower half; lower developed gill rakers 4 to 10; dorsal fin with VII or VIII slender spines and 10 to 12 rays, the fin membranes distinctly incised between the spines, the third or fourth spine usually longest, longest dorsal-fin ray 2.2 to 3.2 times in head length; pectoral-fin rays 15 to 17; pectoral-fin and pelvic-fin length 1.7 to 2.3 times in head length; caudal fin emarginate, the caudal concavity 5 to 12 times in head length; caudal-fin length 1.3 to 1.5 times in head length; no broad dark saddle-like bars on body; blue spots round to oblong; head and body covered (except ventrally) with minute round blue spots, which are about the size of the nostrils, the distance between the spots more than twice their diameter; median fins also covered with blue spots (Western Australia and western)............. P. leopardus

Body robust, elongate, the depth less than head length and contained 2.9 to 3.9 times in standard length; body width contained 1.6 to 2.1 times in its depth. Head length contained 2.8 to 3.2 times in standard length; snout distinctly longer than eye
diameter, snout length 2.8 to 3.6 times in head length; no scales on inter-orbital area; pre-orbital depth contained 5.6 to 10 times in head length; inter-orbital area concave or flat, the dorsal head profile convex; pre-opercle broadly rounded, with 3 large, ventrally directed spines (hidden by skin) along lower half; lower developed gill rakers 4 to 10; dorsal fin with VII or VIII slender spines and 10 to 12 rays, the fin membranes distinctly incised between the spines, the third or fourth spine usually longest, longest dorsal-fin ray 2.2 to 3.2 times in head length; pectoral-fin rays 15 to 17; pectoral-fin and pelvic-fin length 1.7 to 2.3 times in head length; caudal fin emarginate, the caudal concavity 5 to 12 times in head length; caudal-fin length 1.3 to 1.5 times in head length; blue spots round to oblong; no broad dark saddle-like bars on body; Most blue spots on head and body more than twice the size of nostrils; some spots on head and body elongate (except juveniles); pelvic fins without blue spots; some spots on body of adults horizontally elongate; gill raker at angle of first gill arch longer than longest gill filament; pelvic-fin length 1.7 to 2.1 times in head length; nostrils subequal (Philippines to Australia).................. P. maculatus

3.3.7. Key to the Species of Variola genus

Body oblong, the depth less than head length and contained 2.8 to 3.2 times in standard length; body width contained 1.6 to 2.3 in the depth. Head length contained 2.5 to 2.8 in standard length; inter-orbital area of adults convex; dorsal head profile slightly convex; snout distinctly longer than eye diameter; pre-orbital depth contained 0.6 to 1.4 times eye diameter and 6 to 10 times in head length; pre-opercle rounded, finely serrate, the lower edge fleshy; opercle with 3 flat spines; upper edge of operculum almost straight; sub-opercle and inter-opercle smooth; posterior nostrils not much bigger than anterior nostrils; maxilla of adults with a distinct step on ventral edge; supra-maxilla well developed; both jaws with a pair of large canines at the front; 1 to 3 large canines at mid side of lower jaw; palatines with teeth.

Dorsal fin with IX spines and 13 to 15 rays, the dorsal-fin origin over rear end of operculum; dorsal-fin membranes not or slightly indented between the spines, the third to ninth spines sub-equal, the 11th or 12th ray elongated: anal fin with III distinct spines and 8 rays; rear margin of dorsal and anal fins falcate, the antepenultimate rays greatly elongated; pectoral fins rounded, with 16 to 19 rays, the middle rays longest,
their length contained 1.4 to 1.8 in head length; upper pectoral-fin rays joined to body by a scaly flap of skin; first two pelvic-fin rays elongated, usually much longer than pectoral fins and reaching to or beyond anal fin origin; caudal fin lunate (the lobes produced), with 8 branched rays and 10 pro-current rays in upper part and 7 branched rays and 10 pro-current rays in lower part. Mid-lateral-body scales cteneoid, without auxiliary scales. Second supra-neural bone about half length of first; epipleural ribs on first 9 or 10 vertebrae; dorsal fin with 4 to 6, anal fin with 4 trisegmental pterygiophores; rear edge of first dorsal-fin pterygiophore slightly excavated at tip of third neural spine; posterior part of parasphenoid deflected slightly ventrally; greatest width of cranium about 0.5 times its length; least interorbital width of frontals about 0.4 times postorbital width of frontals; parietal crest well developed, continued onto frontals where it joins the lateral crest to form a low ridge running anteromedially to meet its fellow of the opposite side; frontals rugose, not excavated anteriorly (no supra-ethmoid pit or depression); median supra-occipital crest low, not extending onto frontals; exoccipitals with a small notch in lateral edge of foramen magnum.

Rear margin of caudal fin with a black submarginal line and narrow white edge; dorsal, anal, and pectoral fins without a distinct yellow posterior border; lower gill rakers 13 to 16; pelvic fins usually not reaching anus; juveniles without a dark stripe on body dorsally and no dark spot at base of upper caudal-fin rays (western Indian Ocean to central Pacific Ocean).………………………….. V. albimarginata

3.3.8 Morphological identification of Grouper fishes from Andaman

Based on the above said key, the identified fish species were subjected to morphometric study to understand it relationship up to genus level to evaluate the molecular taxonomy. In this regard the following methodologies were adopted.

3.3.9 Fish Morphometry

Thirty three species of five genus’s belonging to Epinephelinae subfamily were collected from landing centres, situated around Port Blair, to a tune of 15-24 individuals. These specimens subjected for the morphological and morphometric analysis. The morphological characters and morphometric count data were taken and recorded as per the standard procedure. Morphometric measurements were taken on
22 characters (Table 3.1.1) to the nearest 0.1 mm using Vernier Callipers following Osuka et al. (2011), and total length, standards length and head length measured in fish scale instrument (BIOTECH - FISH SCALE READER). The 11 morphometric characters were counts (Table 3.1.2).

![Fig. 3.1.1 Grouper](image1)

![Fig. 3.1.2 Morphometric Measurements](image2)
Table 3.1.1 Morphometric measurements details for the studied species of subfamily Epinephelinae

<table>
<thead>
<tr>
<th>Sl. Nos.</th>
<th>Measurement</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total length</td>
<td>TL</td>
<td>The straight-line distance from the tip of the anterior most jaw to a vertical line passing through the posterior tip of the longest caudal-fin ray</td>
</tr>
<tr>
<td>2</td>
<td>Standard length</td>
<td>SL</td>
<td>A measurement from the rostral tip of the upper jaw to the origin of the caudal fin, caudal measuring point is the middle of the borderline between the caudal peduncle and caudal fin.</td>
</tr>
<tr>
<td>3</td>
<td>Body depth</td>
<td>BD</td>
<td>The greatest vertical depth of the body mostly between points near rostral origins of dorsal fin and of the pelvic fin.</td>
</tr>
<tr>
<td>4</td>
<td>Head length</td>
<td>HL</td>
<td>A measurement from the rostral tip of the pre-maxillae to the caudal end of the gill cover.</td>
</tr>
<tr>
<td>5</td>
<td>Pre-dorsal fin length</td>
<td>PRDFL</td>
<td>A measurement between the most anterior and the most posterior point of the pectoral fin base.</td>
</tr>
<tr>
<td>6</td>
<td>Head Depth</td>
<td>HD</td>
<td>The greatest vertical depth of the body mostly between points near rostral origins of dorsal fin and of the pelvic fin.</td>
</tr>
<tr>
<td>7</td>
<td>Pre-ventral fin length</td>
<td>PRVFL</td>
<td>Measured from the rostral tip of the upper jaw to the most anterior point of the pelvic fin base.</td>
</tr>
<tr>
<td>8</td>
<td>Ventral &amp; dorsal fins origin</td>
<td>VDOL</td>
<td>Distances between Ventral and dorsal fins origin</td>
</tr>
<tr>
<td>9</td>
<td>anal and dorsal fin ends</td>
<td>ADFEL</td>
<td>Distances between anal and dorsal fin ends</td>
</tr>
<tr>
<td>10</td>
<td>Dorsal fin base length</td>
<td>DFBL</td>
<td>Dorsal fin base length</td>
</tr>
<tr>
<td>11</td>
<td>Caudal peduncle depth</td>
<td>CPD</td>
<td>The distance between the vertical line through the caudal point of the anal fin and that of through the caudal border.</td>
</tr>
<tr>
<td>12</td>
<td>ventral fin origin and end of anal fin</td>
<td>VOAE FL</td>
<td>Distances between the ventral fin origin and the end of anal fin.</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Code</td>
<td>Description</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>13</td>
<td>Spine of the Dorsal fin &amp; end of Anal fin</td>
<td>SDAEF L</td>
<td>Distances between first spine of the dorsal fin and the end of anal fin</td>
</tr>
<tr>
<td>14</td>
<td>Dorsal fin end and ventral fin</td>
<td>DEVO FL</td>
<td>Distances between dorsal fin end and ventral fin origin</td>
</tr>
<tr>
<td>15</td>
<td>ventral fin end and anal fin origin</td>
<td>VEOAO FL</td>
<td>Distances between ventral fin end and anal fin origin</td>
</tr>
<tr>
<td>16</td>
<td>Dorsal and ventral caudal fin origin</td>
<td>DVCF L</td>
<td>Distances between dorsal and ventral caudal fin origin</td>
</tr>
<tr>
<td>17</td>
<td>Dorsal fin end and Dorsal caudal fin origin</td>
<td>DEDC FL</td>
<td>Distances between dorsal fin end and dorsal caudal fin origin</td>
</tr>
<tr>
<td>18</td>
<td>Anal fin end and ventral caudal fin origin</td>
<td>AEVC FL</td>
<td>Distances between anal fin end and ventral caudal fin origin</td>
</tr>
<tr>
<td>19</td>
<td>Caudal fin dorsal end to ventral fin origin</td>
<td>DEVC L</td>
<td>Distances between dorsal fin end and ventral caudal fin origin</td>
</tr>
<tr>
<td>20</td>
<td>Caudal fin dorsal origin and anal end</td>
<td>AEDC FL</td>
<td>Distances between anal fin end and dorsal caudal fin origin</td>
</tr>
<tr>
<td>21</td>
<td>Eye diameter</td>
<td>ED</td>
<td>Maximum eye length from the most anterior point to the most posterior point of the orbit.</td>
</tr>
<tr>
<td>22</td>
<td>Snout length</td>
<td>SNL</td>
<td>A measurement from the rostral tip of the pre-maxillae to the rostral point of the bony orbit.</td>
</tr>
<tr>
<td>23</td>
<td>Lateral line</td>
<td>LL</td>
<td>The series of pored or tubed scales that run from the upper end of the gill cavity to the caudal fin</td>
</tr>
<tr>
<td>24</td>
<td>Lateral-scale series</td>
<td>LSS</td>
<td>The oblique series of scales that run above the lateral line from the upper end of the gill opening to the base of the caudal fin</td>
</tr>
</tbody>
</table>
These measurements are described in Table (3.1.3) determined as follows: Dorsal fin spine and ray counts, anal fin spines and rays, pectoral fin rays, scale rows between lateral line and dorsal fin, scale rows between lateral line and ventral fins, number of caudal fin rays, number of the gill rakers on the ceratohypobrachial portion of the first left gill arch ascending order, number of the gill rakers on epibranchial portion of the first leaf gill arch in order to descending and total gill rakers on the first left gill arch and lateral line scales from operculum to caudal fin base and pre dorsal scales similarly later line scale serious also counted.

3.3.10 Data analyses:

The raw data were entered in MS Excel spread sheets then exported to Plymouth Routines in Multivariate Ecological Research Software, Version 6 (PRIMER-6) for the cluster analysis. The data were pre-treated and then standardised for similarity. The standardised data were subjected to cluster analysis based on group analysis and the similarity tree was generated, the results were discussed for further interpretation.

3.4 Molecular Taxonomy Methods

3.4.1 DNA Isolation

About 30 - 50mg of preserved tissues of each fish was taken and the chromosomal DNA was extracted based on the standard protocol of Phenol – Chloroform – Iso-amyl alcohol and proteinase K method (Sambrook et al., 1989). The collected tissue samples of each fish was thoroughly washed using ultrapure MilliQ water, then placed in to a centrifuge tubes containing 1ml of TE buffer (Tris Cl + EDTA buffer 10mM) and centrifuged at 12,000 rpm for 10 mints at 15°C. After the centrifugation, the TE buffer was decanted. Then 100 µl of lysis buffer was added into the microfuge tube and incubated at 50°C using water bath for 30 - 45 minutes. Subsequently, tissue was homogenized using sterile micro-pestle by adding 100 µl of lysis buffer, 100µl of 10% Sodium dodecyl sulphate and 20 µl of proteinase K solution.
The homogenized tissue was incubated at 65°C for 30 minutes in thermo regulated water bath. After the incubation, 100 µl of 5M Sodium chloride was added to the homogenized tissue lysate in the microfuge tubes. After the short spin, add 100 µl of CTAB (N-acetyl N N tri methyl ammonium bromide + Nacl) and mix slowly for 2 minutes. Then add slowly 10 µl RNase solution (10mg/ml) and vortex briefly. The mixture was incubated at 65 °C for 30 min in water bath thermo-regulator and cool to room temperature for 10 minutes.

The lysate in the microfuge was mixed well after addition of 300 µl of Phenol: Chloroform: Iso-amyl alcohol (25: 24: 1) and then the tube was vortex and centrifuged at 12,000 rpm for 15 minutes at 4°C. Following centrifugation, upper most aqueous layer was collected in new microfuge tubes. Along with the supernatant 200 µl of chilled Absolute alcohol was added and kept in -70°C for 1 hour. Later the microfuge tube was kept in room temperature for few minutes and centrifuge at 12,000 rpm for 30 minutes at 4°C. After centrifugation, the supernatant was discarded carefully without disturbing the DNA pellet and it was allowed to air dried for 30 minutes. Finally the DNA pellet dissolved in 20 µl of TE buffer and store at -20°C and till further use. The extracted DNA was subjected to following quantification and quality analysis to confirm its activity as suggested by Persis et al., (2009).

3.4.2 DNA Quantification and Quality Analysis:

The isolated DNA was subjected for quantification using spectrophotometer. The protocol is as follows: initially 1ml of TE buffer was taken in a cuvette as a blank and calibrated the spectrophotometer at 260 nm as well as 280 nm. Subsequently, DNA sample was diluted i.e. 10 µl of DNA sample in to new microfuge tube with 990 µl of TE (Tris-EDTA) buffer and mixed thoroughly. Then the quantification of DNA analysis was carried out with TE buffer as a blank solution in the spectrophotometer. After standardization of spectrophotometer using the blank, DNA sample was analysed in two different wave lengths such as OD_{260} and OD_{280}. The OD_{260}/OD_{280} ratio was calculated for confirmation of DNA.
3.4.3 Standard Inferences and Comments

A ratio range 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids.

A ratio below 1.8 indicates the presence of proteins and/or other UV absorbers.

A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (<1.8 or >2.0) it is advisable to re-precipitate the DNA.

Table 3.1.2 Standard References of OD 260/280 ratio

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>% Protein</th>
<th>% Nucleic acid</th>
<th>260:280 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0.57</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>5</td>
<td>1.06</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>10</td>
<td>1.32</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>30</td>
<td>1.73</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>70</td>
<td>1.94</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>90</td>
<td>1.98</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>95</td>
<td>1.99</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>100</td>
<td>2.00</td>
</tr>
</tbody>
</table>

3.4.4 Calculation:

The amount of DNA can be quantified using the following formula:

\[
\text{DNA concentration (\mu g/ml) = } \frac{\text{OD}_{260} \times 100 \times \text{(dilution factor)}}{1000} \times 50 \mu g/ml
\]
3.4.5 Spectrophotometric Conversions for Nucleic Acids

1 A 260 of ds DNA = 50 µg/ml
1 A 260 of ss oligonucleotides = 33 µg/ml
1 A 260 of ss RNA = 40 µg/ml

The concentration of DNA was estimated using a 260/280nm UV spectrophotometer method. Subsequently, the DNA was diluted to a final concentration of 100ng/µl for further use.

3.4.6 Polymerase Chain Reaction (PCR) for COI gene Amplifications

PCR amplification was done in 25-μL reaction mix, containing 5µl DNA template with 1µM of each forward and reverse primers were used. The 650 - 655bp section of the mitochondrial (mt) DNA genome from the COI gene was amplified using already published universal primer (Ward et al., 2005) synthesised by Sigma Aldrich Chemicals India Pvt. Ltd.

**Table 3.1.3 Master Mix preparation**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>5.0µl</td>
</tr>
<tr>
<td>DNTP (5mM)</td>
<td>1.50µl</td>
</tr>
<tr>
<td>COI gene forward Primer F1 or F2 (0.5µM)</td>
<td>1.0µl</td>
</tr>
<tr>
<td>5’-TCA ACC AAC CAC AAA GAC ATT GGC AC-3’</td>
<td></td>
</tr>
<tr>
<td>COI gene reverse primer R1 or R2 (0.5µM)</td>
<td>1.0µl</td>
</tr>
<tr>
<td>5’-TAG ACT TCT GGG TGG CCA AAG AAT CA-3’</td>
<td></td>
</tr>
<tr>
<td>Taq polymerase 3U</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Mgcl₂</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>RNase free water</td>
<td>10.25µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

20 µl reaction mix either 5 µl DNA were mixed
Table 3.1.4 Degenerated Primers used this study list

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of the Primer</th>
<th>Name of the gene and Length of sequence</th>
<th>Publisher</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forwarded Primer F1\n5’TCAACCAACCACAAAGACATTGGGCAC3’\nReverse Primer R1\n5’TAGACTTCTGGTGAGGCAAAGAATCA3’</td>
<td>mtDNA COI (645 - 650bp)</td>
<td>Ward et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Forwarded Primer F2\n5’TCGACTAATCTATAAGATATCGGCAC3’\nReverse Primer R2\n5’ACTTCAGGGTGACCAAGAATCAGAA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Forwarded Primer\n5GAGGGCCATCTCCTCTCTCCA-3’\nReverse Primer R5\nGTCTGTAGAGTGCTCACAGGAGAGCA3’</td>
<td>nDNA RAG2 (847 nt)</td>
<td>Westneat and Alfaro, 2005</td>
</tr>
</tbody>
</table>
Fig. 3.1.3 Schematic representation of Thermal cycle conditions
3.4.7 Thermal Cycle Condition

PCR was carried out in Applied Bio systems AB-2720 Thermal cycler. The initial denaturation was performed at 95°C for 5min, followed by denaturation at 94°C at 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 60 sec for 40 cycles followed by final extension at 72°C for 10 min. PCR products were resolved in 1% agarose containing 0.5 µg/ml of ethidium bromide and viewed under UV Transilluminator and documented.

3.4.8 PCR Condition for Nuclear DNA Recombination-Activating Protein 2 (RAG2) gene

PCR amplification was done in 25 µl reaction mix, containing 5µl DNA template with 1µM of each forward and reverse primers were used. The 750 – 850 bp section of the Nuclear gene (nDNA) genome from the RAG2 gene was amplified using already published primer (Westneat and Alfaro, 2005) synthesised by Sigma Aldrich Chemicals India Pvt. Ltd.

3.4.9 Thermal Cycle Condition

PCR was carried out in Applied Bio systems AB-2720 Thermal cycler. The initial denaturation was performed at 95°C for 3min, followed by denaturation at 95°C at 30 sec, annealing at 55°C for 60 sec and extension at 72°C for 90 sec for 40 cycles followed by final extension at 72°C for 10 min. PCR products were resolved in 1.2 - 2% agarose containing 0.5 µg/ml of ethidium bromide and viewed under UV Transilluminator and documented.
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Fig. 3.1.4.1 - DNA Conformation

Fig. 3.1.4.2 - COI gene PCR products gel Check

**Fig. 3.1.4 Gel Pictures**
Fig. 3.1.5 Gel Pictures
Table 3.1.5 Master Mix preparation for Nuclear DNA RAAG2 gene

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>5.0µl</td>
</tr>
<tr>
<td>DNTP (5mM)</td>
<td>1.50µl</td>
</tr>
<tr>
<td>nDNA RAG2-F-5-GAG GGC CAT CTC CTT CTC CAA-3’</td>
<td>0.5µl</td>
</tr>
<tr>
<td>nDNA RAG2-R-5 GTC TGT AGA GTC TCA CAG GAG AGCA-3’</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Taq polymerase 3U</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Mgcl2</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>RNase free water</td>
<td>11.25µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20 µl</strong></td>
</tr>
</tbody>
</table>

20 µl reaction mix either 5 µl DNA were mixed

Fig. 3.1.6 Schematic representation of Thermal cycle conditions

**Denaturation**

**START**  Step – 1  2  3  4  5
95°C for 3mins  95°C for 30 Sec  472°C for 9 Sec  72°C for 10mins  4°C Hold

**Elongation**

**PCR**  Applied Biosystems
40cycles  Multiplication
3.5 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a technique used for the separation of DNA fragments according to size as they migrate through a gel matrix. Agarose is a highly purified polysaccharide isolated from seaweed. Its major constituent is a linear polymer of alternating D-galactose and 3, 6-anhydro-L-galactose units. Bromo phenol blue (BPB), a ‘tracking dye’ in a loading buffer is mixed with DNA to make DNA loading easier and DNA migration visible. The phosphate groups in the DNA backbone carry negatively charged oxygen which gives a DNA molecule overall negative charge. In an electric current the negatively charged DNA moves towards the positive pole of the electrophoresis chamber. The DNA fragments are separated by size and visualized by staining with the fluorescent dye (Ethidium bromide, EtBr) that intercalate between bases of DNA.

The PCR products obtained after amplification of COI genes were resolved and analysed by electrophoresis using 2% agarose gel (Sigma) prepared in 1X TAE buffer (Tris Acetate EDTA) containing EtBr. A 100 bp DNA ladder (1ug/L, Sigma) was used as DNA marker for estimation of size of the PCR products were visualized under UV-Transilluminator (548nm) and photograph of the gels were recorded using Gel documentation system (Bio-Rad).

3.5.1 Gel extractions and purification of PCR products

All PCR products were excised from the gel and purified using QIAquick gel extraction kit (Qiagen, Germany). The QIAquick system uses spin column technology with the selective DNA binding properties of silica membrane. DNA is absorbed to the silica membrane in the presence of high concentration of salt while contaminants like unused primers; EtBr, Molten agarose, etc., pass through the column. After removal of impurities, the pure DNA is eluted in the elution buffer or water.

3.5.2 Procedure

Three volumes of (300 μl) of QG buffer were added to 1 volume (100 mg of gel slice) of gel containing excised DNA fragment. The incubation was carried out at 56°C for 15-20 min. or until the gel was completely dissolved in QG buffer. Isopropanol (100 μl) was added in a proportion equal to gel volume (100 mg) and
mixed properly. The mixture was applied to the QIAquick column placed on a collection tube (provided in the kit) and centrifuged at 10,000 rpm for 1 min at room temperature. After discarding the flow through, 500 µl QG buffer was added to the column and centrifuged at 10,000 rpm for 1 min at room temperature. The flow through was discarded and 750 µl of PE buffer were added to the column and incubated at room temperature for 5 min. The columns were centrifuged at 10,000 rpm for 1 min and the flow-through was discarded. In order to remove PE buffer completely, an additional spin at 13,000 rpm for 1 min was given. The QIAquick columns were placed on the new collection tube and DNA was eluting in 20 µl of EB (Elution Buffer) by spinning the column at room temperature. The purified DNA was stored at -20°C until utilized.

3.5.3 Nucleotide Sequencing

Both strands forwarded and Reverse DNA of purified PCR products were sequenced using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit, V3.1. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes of different wavelengths of fluorescence and emission. The Terminator Ready reaction mix (TRRM) contains A-Dye Terminator labelled with dichloro [R6G], G-Dye Terminator labelled with dichloro [R110], C-Dye Terminator labelled with dichloro [ROX], T-Dye Terminator labelled with dichloro [TAMARA], deoxy-nucleotide triphosphates (dATP, dCTP, dITP, dGTP, dUTP), AmpliTaq DNA polymerase, MgCl2 and Tris-HCL buffer, pH 9.0 (Appendix II).

Thermal cycling of the sequencing reactions creates and amplifies extension products that are terminated by one of the four dideoxynucleotides. In normal condition, the chain elongation takes place when the 5’ carbon of an incoming dNTP is joined to the 3’carbon at the end of the chain by phosphor-diester linkage. A dideoxy NTP lacks 3’ hydroxyl group necessary to form the linkage with an incoming nucleotide. Hence, the incorporation of a dideoxy NTP halts the chain elongation and incompletes the products of various lengths are created. The ratio of deoxynucleotides to dideoxy nucleotide is optimized to produce a balanced population of long and short extension products.
3.5.4 Preparation of cycle sequencing reaction mix

Two cycle sequencing reactions (one with forward and the other with reverse primer) were carried out of each PCR product. The composition of the reaction mix was as follows:

3.5.5 Post cycle sequencing purification of PCR products

After cycle sequencing reaction, the PCR products were purified using Ethanol/EDTA/sodium acetate method. The purpose of purification is to completely remove unincorporated dye terminators prior to capillary electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with base calling. Ethanol/EDTA/sodium acetate precipitation is recommended to obtain signal from base 1. Sodium acetate precipitates the DNA and EDTA helps to stabilize extension products during precipitation, and wash out unincorporated dyes from the completed reaction.

3.5.6 Procedure

Master mix 1 -2 µl 125 mM EDTA + 10 µl nuclease free water Master mix 2 – 3 µl of 3M sodium acetate Absolute + 50 µl Absolute Ethanol to 10 µl of cycle sequenced products, 12 µl of mix was added and mixed properly. This was followed by addition and proper mixing of 52 µl of mix 2. The mixture was incubated for 15 minutes and centrifuged at 10,000 rpm for 30 minutes at room temperature. The supernatant was discarded and the tubes were centrifuged again at 10,000 rpm for 5 minutes at room temperature. Ethanol was removed completely by air drying the pellet at room temperature for 30 min. the pellet was reconstituted in 20 µl of Hi-Di™ formamide (Applied Bio-system, U.S.A). The denaturation of DNA was carried out at 95°C for 2 min. followed by snap chilling on ice for a minute. The contents were mixed by using vortex mixture (Remi Equipments, India), spun and loaded in automated sequencer, ABI 3010 Genetic analyser (Applied Bio-system, U.S.A). PCR was carried out in the MJ Research thermal cycler, under following cycling conditions:
Table 3.1.6 Composition of cycle sequencing reaction mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPRM</td>
<td>4 µl</td>
</tr>
<tr>
<td>Forward/Reverse primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Template*</td>
<td>1-5 µl</td>
</tr>
<tr>
<td>Nuclease free distilled water</td>
<td>10 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20 µl</strong></td>
</tr>
</tbody>
</table>

* Depending on the intensity of the PCR products

Fig. 3.1.7 Schematic Representation of Thermal cycle Conditions

Start

Step 1
94°C for 5 mins

Step 2
94°C for 10 seconds

25 cycles

Step 3
55°C for 5 Seconds

Step 4
72°C for 2 mins

4°C Hold
3.5.7 Nucleotide sequences

The nucleotide sequences were collected using an automated sequencer, ABI 3010 Genetic analyser (Applied Bio-system, U.S.A). The cycle sequenced products are subjected to capillary electrophoresis. During capillary Electrophoresis, the extension products of the cycle sequencing reaction enter in to the capillary as a result of electro kinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments in to the capillaries. The extension products are separated by size. Shortly before reaching the positive electrode, the fluorescent labelled DNA fragments separated by size, move across the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. An optical detection device detects the fluorescence. The data collection software converts the fluorescence signal to digital data and records the data, because each dye emits light at a different wavelength when excited by the laser. All four bases labelled with four different colours can be detected and distinguished in one capillary injection.

3.5.8 Sequences assembling

The forward and reverse sequences collected from sequencer were checked manually in the electropherograms using the SeqMan II version 5.03 (DNASTAR). The checked and corrected nucleotide sequences of Serranidae fishes COI gene sequences were adjusted in FASTA format. Then nucleotide sequences of COI gene sequences F and R genomic region of Serranidae fishes from NCBI database (www.ncbi.nlm.nih.gov/nuccore) were retrieved for sequence assembling.

3.5.9 Phylogenetic trees for COI gene sequence

Phylogenetic tree is a graphical representation of the evolutionary relationship between taxonomic groups. It is a specific type of cladogram (a branched diagram similar to family trees) in which the branch lengths are proportional to the predicted or hypothetical evolutionary time between organisms or their sequences. The phylogenetic trees depicted the evolutionary relationship between taxonomic groups were generated for all structural as well as non-structural genes sequences using molecular evolutionary genomic analyser software MEGA 4.0 (Tamura et al., 2007). Genetic distances were calculated by using the Kimura2 parameter model at the nucleotide level and phylogenetic trees were constructed by using neighbour-joining method.
3.5.10 Bootstrap analysis

The reliability of phylogenetic trees was tested by applying the bootstrap test with 1000 bootstrap replications. Bootstrapping is a way of testing the reliability of the dataset. In phylogenetic analyses nonparametric bootstrapping is the most commonly used method. The pseudoreplicate dataset are generated by randomly sampling the original character matrix to create new matrices of the same size as the original. The frequency with which a given branch is found is recorded as the bootstrap proportion. These proportions can be used as a measure of the reliability (within limitations) of individual branches in the optimal tree.

3.5.11 Pairwise distances and homology calculation

Pairwise (P) distances between Andaman fishes as well as reference sequences were identified through BLAST analysis. In the present study it was calculated by using Kimura 2-Parameter (K2P) distance and pairwise method in MEGA 4.0 software. The corresponding percentages of homologies were calculated using K2P distance converter. The K2P distances were also calculated for serranidae COI sequences from different part of the world.

3.5.12 Data analysis of nDNA RAG2 gene sequences and DLST

The FASTA file for 11 species of groupers, forty sequences of COI gene (630 – 655bp) and forty sequences of RAG2 (670 – 750bp) were analysed for data processing. The forward and reverse DNA sequence were analysed for every individual fish sequences using the SeqMan II version 5.03 (DNASTAR). The aligned sequences were also subjected to a Nucleotide BLAST search to know the identity and further strengthen our results. The sequence analysis was done along with reference sequences of various species belonging to the family Serranidae, retrieved from NCBI GenBank for COI gene sequence.

However, the nDNA RAG2 gene sequences belonging to studied species not available in NCBI database. Two references same species sequences only are available in NCBI database which are also considered for further reference comparisons studied (AY279869 Epinephelus polyphekadion; DQ874793 Epinephelus gestates) the reference sequences deposited by Westneat and Alfaro, (2005). The other reference sequences of JQ769471 Scorpis lineolata from other
family had very distance genetic identity observed in BLSATN analysis, all the reference sequences deposited by Davis et al., (2012).

The sequence diversity analysed the both COI and RAG2 gene sequences used MEGA software and their results were presented. Nucleic acid sequences were also aligned using CLUSTALW and MEGA4 software (Tamura et al. 2007).

The COI gene sequences of studied species and the related reference sequences were aligned to form the phylogenetic tree analysis. The Pair-wise evolutionary distances calculated and then the reference sequences were compared to Andaman species sequence clusters based on Kimura two-Parameter (K2P) method (Tamura, Dudley, Nei, and Kumar 2007) obtained with K2P genetic distance.
Fig. 3.1.8 Raw Data Electrogram shown

Sample Name: **Epinephelus fasciatus**

File: *1st_set-Sp3-R1.ab1*  
Run Ended: 2011/9/17 2:20:8  
Signal G: 829

A: 810  C: 705  T: 848

Sample: *1st_set-Sp3_R1*  
Lane: 13  Base spacing: 14.549236  
681 bases in 16300 scans