# MATERIALS AND METHODS

**Selection of study sites**

Gujarat is the westernmost state of India and possesses the longest coastline among all the coastal states of the country, which stretches around 1600 km and comprises roughly 21% of the total coastline of India. The Saurashtra coast is a west-facing coast of Gujarat that is 850 km long. For the present study, the entire Saurashtra coast was surveyed, starting from Okha to Diu, for the purpose of site selection. During the survey, the maximum occurrence of *C. rhabdodactylus* individuals was recorded from the Veraval (20° 54’ 37" N, 70° 21’ 04" E), Sutrapada (20° 49’ 53" N, 70° 29’ 17" E), and Dhamlej (20° 46’ 29" N, 70° 36’ 19" E) coasts (personal observation), which were selected for the present study (Figure 8).

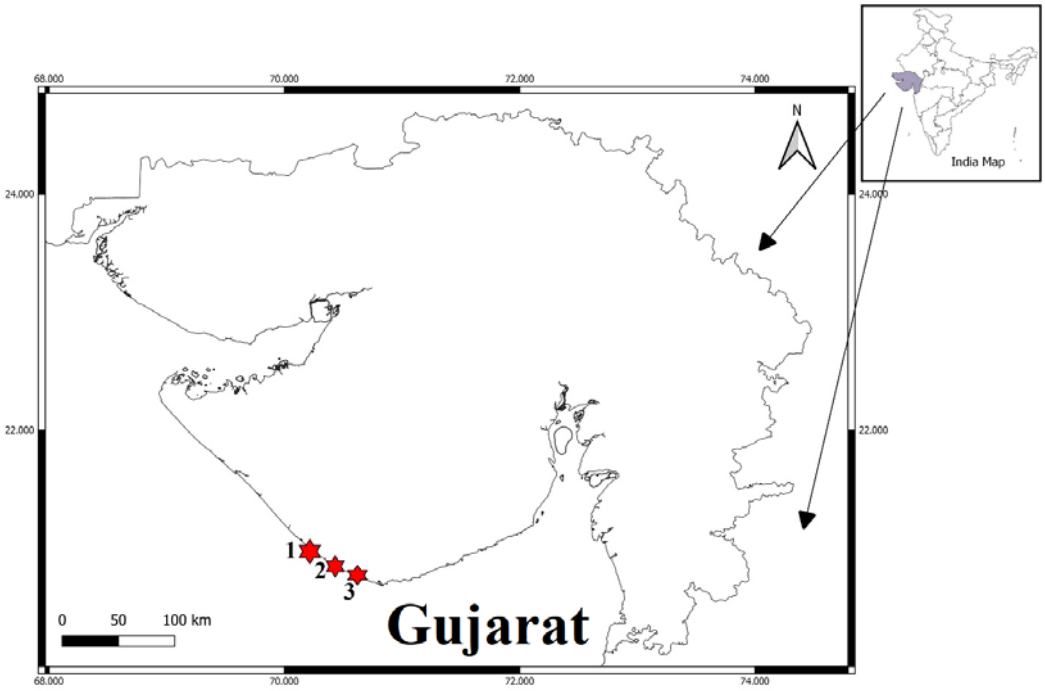


**Figure 8.** Location of study area 1. Veraval; 2. Sutrapada and 3. Dhamlej.

Veraval, Sutrapada, and Dhamlej are situated in the Gir Somnath district (formerly part of Junagadh district). All three sites have a rocky intertidal region, with the upper intertidal region composed of a sandy shore. The exposed intertidal length during low tide varied between different sites, with the maximum exposure observed at Dhamlej (150 metres) during low tide, followed by Sutrapada (120 metres), and the least exposure observed at Veraval (55

meters). The major microhabitat types in all three study sites were tidepools, crevices, and flat areas.

The abundance of *C. rhabdodactylus* was recorded from all three selected sites, and it was found that Veraval has a comparatively greater abundance of *C. rhabdodactylus* than Sutrapada and Dhamlej (Figure 9). Hence, Veraval was selected as a primary site for the present study.

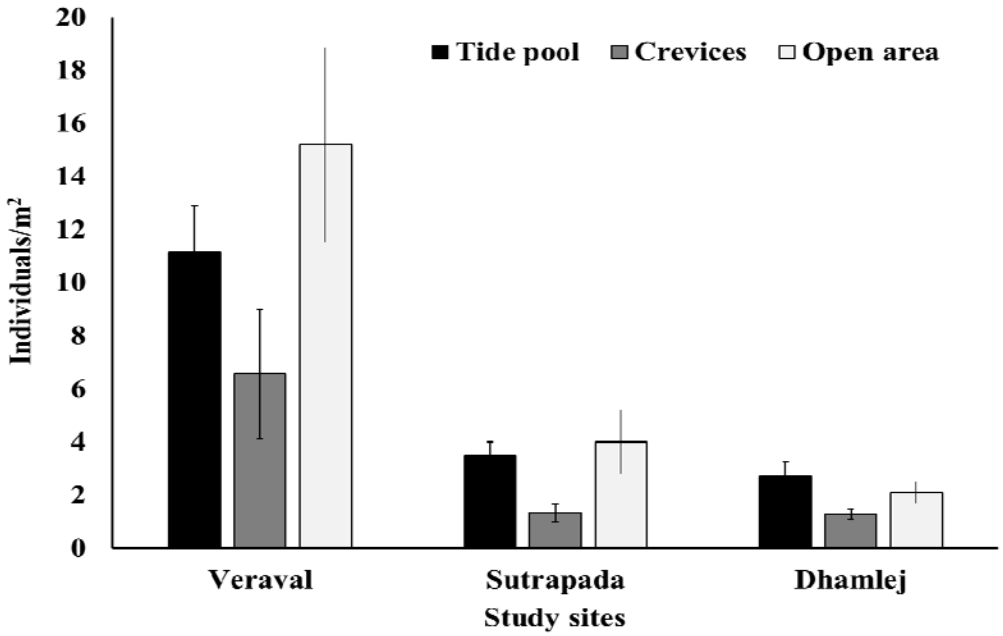


**Figure 9.** Abundance of *Clibanarius rhabdodactylus* in different microhabitats of study sites.

**Veraval:** Veraval is situated on the southwest coast of Saurashtra, and it is around 6 kilometres away from Somnath. Somnath Mahadev Temple is situated in Somnath, which has great importance in Hindu mythology as it is considered the first Jyotirlinga out of 16 Jyotirlingas. Geographically, Veraval is located at 20° 54’ 37" in the north and 70° 21’ 04" in the east, with the city having an average elevation of zero meters. The climate of Veraval is mostly warm, with temperatures ranging from the highest of 42°C in summer to the lowest of 7°C in winter. The climate in this region is semi-arid and hot, with an average humidity of 74%. Veraval is mostly famous for its fishing industry, as it is one of the largest fish landing centres in India. The local fisherman community known as "Kharwas" governs the fisheries industry in general. Along with this, Veraval is

also famous for building boats and trawlers. The sea food industry of Veraval is thriving and exporting their sea food products to several countries, including the USA, Europe, Persian Gulf countries, Japan, and Southeast Asia. The sampling site chosen for the current study in Veraval is located behind Veraval Fisheries College, close to the lighthouse at Chowpati. During low tide, around 30 to 50 metres of intertidal area get exposed. The intertidal region is roughly composed of around 41% of tidepools, 1.3% of crevices, and 57.7% of flat area, with the majority of shallow tidepools occurring in the upper and middle intertidal regions, while the lower intertidal region has very large tidepools and a steep, vertical slope towards the subtidal zone. The spray zone beyond the upper intertidal region is composed of sandy shores. The rocky shoreline is discontinuous and interrupted by large boulders or deep channels (Figure 10A).

**Sutrapada:** Sutrapada is a small town that is located 20 km in the south direction from Veraval city. Despite its small size, it is well known locally for a large-scale industry, Gujarat Heavy Chemical Limited (GHCL), which produces approximately 60000 metric tonnes of refined $NaHCO_3$ per year. As a result of the industrial activity, the particulate-laden effluent released by GHCL is accumulating in the coastal region of Sutrapada. The sampling site selected for the present study is situated around 500 metres south of the local finishing port. During low tide, around 100 to 120 metres of intertidal area get exposed. The intertidal region is roughly composed of around 33% tidepools, 1.7% crevices, and 65.3% flat area. Overall, the intertidal region of Sutrapada has shallow tidepools, with the middle and lower intertidal zones comparatively flat in nature. The spray zone beyond the upper intertidal region is composed of sandy shores. The majority of the shallow and large tidepools occur in the middle intertidal zone, while the lower intertidal zone can be characterised by deep tidepools and a substantial abundance of barnacles and anthozoans (Figure 10B).

**Dhamlej:** Dhamlej is a small village that is located 40 km in the south direction from Veraval city and 20 km further south of Sutrapada. It is a small village with majority of the population is composed of fishermen community. The coastal region is only used by the local population of fishermen for fishing purposes. The sampling site selected for the present study is located around 500 metres north

of the local finishing port. The intertidal region is of rocky nature, while the spray zone is sandy in nature. During low tide, around 110 to 130 metres of intertidal area get exposed. The intertidal region is roughly composed of around 36.5% tidepools, 1.8% crevices, and 61.7% flat area. The middle and lower intertidal zones are mostly flat areas, while few shallow tidepools are observed in the middle intertidal zone (Figure 10C).

**Time Span:** The present study was carried out from December 2019 to November 2022. Initially, a pilot survey was carried out during October and November 2019 for site selection. On the selected sites, the abundance of *C. rhabdodactylus* was assessed in January and February 2020 using the transect method intercepted by quadrates. To study the population structure of *C. rhabdodactylus*, monthly visits were carried out from March 2021 to February 2022 on the selected study site, and the catch per unit effort method in the 500 $m^2$ area of the intertidal region was employed for sampling. To study the intertidal distribution of the *C. rhabdodactylus* population, monthly visits were carried out from September 2020 to August 2021 on the selected study site, and the transect method intercepted with a quadrate (0.25 $m^2$) was employed for sampling (Figure 10D). To study the shell utilisation pattern of *C. rhabdodactylus*, sampling was carried out from January to March 2021 on the selected study site, and the catch per unit effort method was employed in a 500 $m^2$ area of the intertidal region for sampling.

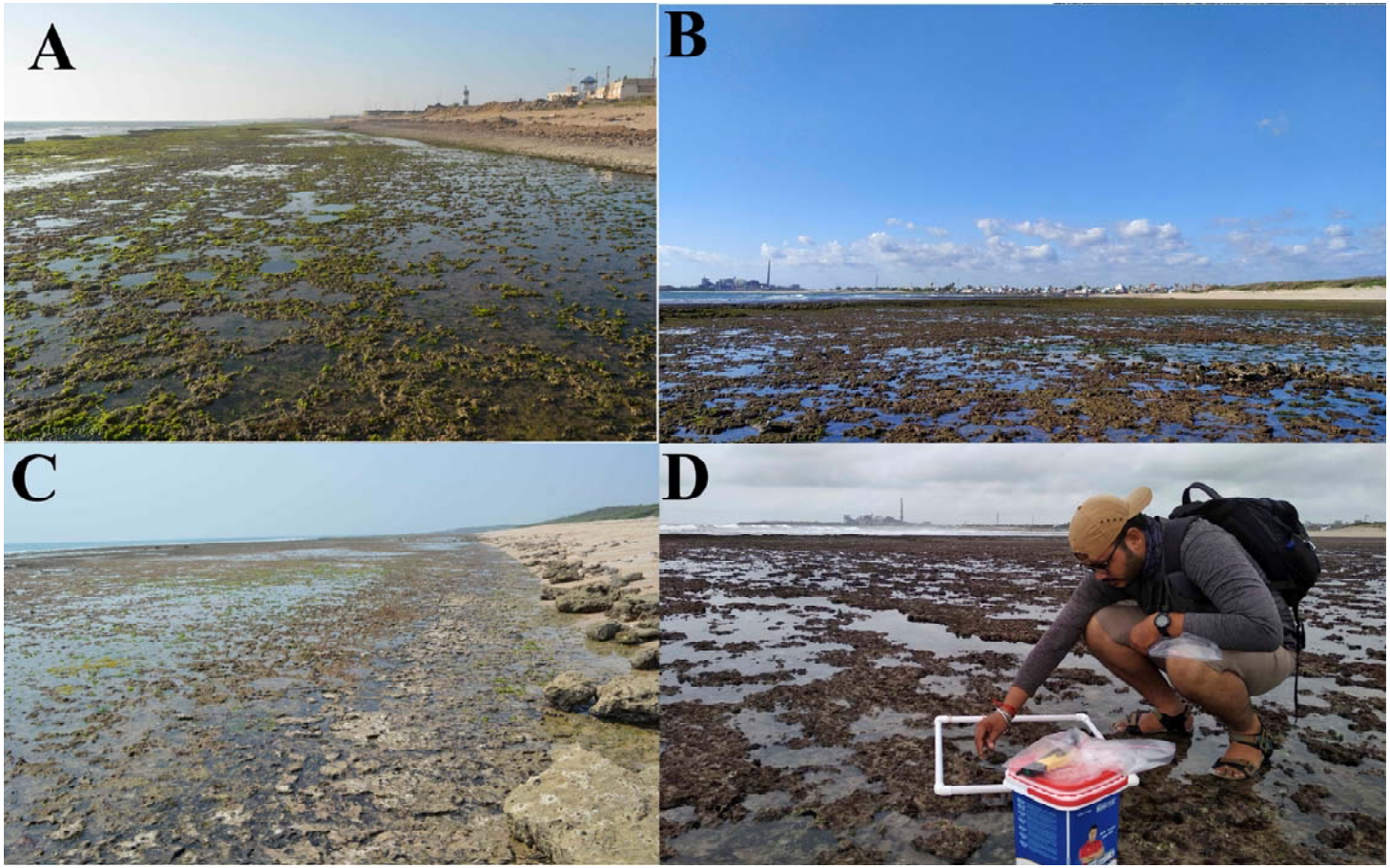


**Figure 10.** Habitat of study area (A) Veraval (B) Sutrapada (C) Dhamlej; (D) Quadrate sampling method for collection of *Clibanarius rhabdodactylus* individuals.

**Sampling Methodology**

1. **To study the population ecology of *Clibanarius rhabdodactylus*.**

**a) To study population structure of *Clibanarius rhabdodactylus*.**

- The sample collection was carried out for 12 consecutive months. The monthly data was compiled into different seasons following Rao and Rama-Sharma (1990), wherein November to February are defined as the winter season, March to June are defined as the summer season, and July to October are defined as the monsoon season period to observe the seasonal variation.

- Specimens were collected randomly from the intertidal region using catch per unit effort using handpicking method by one person during the period of 4 hours. In this method, first an intertidal area of 500 $m^2$ was marked during low tide, and it was thoroughly scanned for the presence of *C. rhabdodactylus* individuals. Whenever an individual was encountered, it was handpicked and collected in a zip-lock bag. All collected specimens were later kept in an icebox and transported to the laboratory, where they were transferred to 70% ethanol until further analysis. Abiotic factors like seawater and ambient temperature were also recorded using a digital thermometer.

- In the laboratory, the hermit crabs were removed from their shells by gently twisting them against the direction of the shell spiral. Only the individuals which remained intact after removal from their shell were used in the study.

- A stereomicroscope was used to examine the morphology of each hermit crab to confirm its species and gender. Individuals having gonopores on the ventral side of the first segment of their second pair of walking legs (third periopod) were considered females (Figure 11), while males do not have such a structure. If there were egg masses on the pleopods, the individuals were considered ovigerous females.

- As a morphological characteristic of the hermit crabs, the shield length (from the midpoint of the rostrum to the midpoint of the posterior margin of the shield) of each individual was measured using vernier callipers (±0.01 mm accuracy), and wet weight was measured using a weighing balance (0.001 g

accuracy). Size at first maturity (SFM) was considered as the smallest ovigerous female measured from the collected samples (Pinheiro and Fransozo, 2002), and any male or female individuals smaller than the smallest ovigerous female were classified as juveniles (<2.98 mm SL) (Baeza et al., 2013).

- For the fecundity test, five ovigerous females were randomly selected from the collected specimens. The egg mass was carefully removed from the pleopods of these ovigerous females and kept in 20 ml of sea water, which was gently mixed so that the eggs got distributed in the water evenly.
- Now, from the above 20-ml solution, 3 samples of 2 ml each were taken in a petri dish and observed under a stereomicroscope to count the total number of eggs present in the petri dish. To calculate the total number of eggs, an average was calculated from the three samples, and the value was multiplied by the dilution factor (10) (Llodra, 2002; Litulo, 2004).
- In order to record the weight of egg mass in ovigerous females, first the wet weight of the ovigerous female with egg mass was recorded. After that, the entire egg mass was removed, and again, the ovigerous female was weighed, this time without egg mass, and the weight was recorded. The difference between the weight of the ovigerous female with egg mass and without egg mass was considered the weight of the total egg mass. The size range of eggs (n = 10) from each crab was measured by means of an ocular micrometre under a microscope (Figure 12), and the diameter of the eggs was considered the measurement for egg size.
- The population size structure was analysed as a function of the size frequency distribution of the individuals. Specimens were grouped in 1 mm size-class intervals, from 1 to 8 mm SL. For month-wise variation in the size and gender composition, the monthly data of hermit crab size (shield length) and sex were plotted.
- For month-wise variation in the size and sex composition (modal distribution), the monthly data of hermit crab size (shield length) and sex were plotted. The occurrence of more ovigerous females in the monthly

samples indicates the breeding season of the species, while the occurrence of smaller specimens in the monthly samples indicates the juvenile settlement season.

- Normality test of the collected data was tested using the Shapiro Wilk test, suggesting that the data was not normally distributed ($p<0.001$), hence non-parametric methods were used.

- Variation in mean values of shield length of different sexes (males, non-ovigerous females and ovigerous females) of *C. rhabdodactylus* was evaluated using the Kruskal–Wallis (KW) test to assess sexual dimorphism. On finding a significant difference ($p<0.001$) in the KW test, Dunn's post hoc test was carried out for multiple comparison tests.

- The chi-square test ($\chi^2$) was calculated to evaluate the sex ratio. Monthly estimates of the proportions of juveniles were tested for correlation with temperature using Pearson's correlation. Moreover, Pearson correlation coefficients were calculated to determine the relationship between crab densities and environmental parameters.

- To determine the size at first maturity of females, the percentage of ovigerous females in different size classes was calculated for all 12 months (March 2021 to February 2022) since ovigerous females occurred throughout the year. The size at which the frequency of ovigerous females was estimated to be over 50% (SM50) was the maturity size (Wada et al., 2000; Mishima and Henmi, 2008).

- The monthly data on juvenile and ovigerous female occurrences was plotted against the ambient and water temperatures to understand the effect of temperature on the breeding and juvenile settlement of *C. rhabdodactylus*.

- Pearson's correlation analysis was carried out between the relative frequency of juveniles and the mean ambient temperature. Monthly data on the relative frequency of juveniles was plotted against ambient temperature to understand the relation between temperature and juvenile settlement.

- Regression analysis was performed to find out the relationship between the morphological parameters of eggs and hermit crabs' morphology (shield length and weight).
- All the statistical analysis was performed using PAST software (4.03 version) and Microsoft Excel 2019.

**Figure 11**. Ventral view of female *Clibanarius rhabdodactylus* showing gonopores on the first segment of the second pair of walking legs (third periopod).

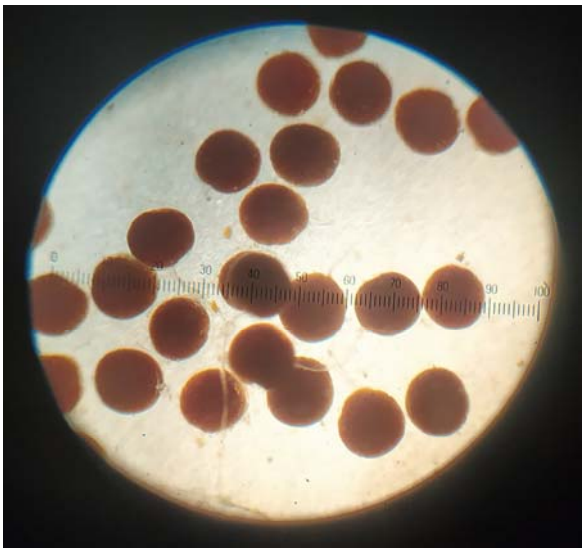

**Figure 12.** Eggs of *Clibanarius rhabdodactylus* measured under ocular micrometre.

**b) To study the seasonal variation and intertidal distribution pattern of *Clibanarius rhabdodactylus.***

- The sample collection was carried out for 12 consecutive months. The monthly data was compiled into different seasons following Rao and Rama-Sharma (1990) to observe the seasonal variation.

- The sample collection was carried out for 12 consecutive months. The monthly data was compiled into different seasons following Rao and Rama-Sharma (1990) to observe the seasonal variation.

- For sampling, the rocky intertidal region of the study site was firstly divided into three different zones: the upper intertidal (0 to 20 m), the middle intertidal (20 to 40 m), and the lower intertidal zones (40 to 60 m).

- The quantification of *C. rhabdodactylus* individuals was carried out using the line transect method, intercepted with a quadrate. A total of three transects were laid, which were 100 m apart from each other. The transects were laid perpendicular to the shoreline, from the upper intertidal zone to the lower intertidal zone. On each of the three transects, a quadrate of 0.25 $m^2$ was laid at an interval of every 5 m, starting from the upper intertidal mark till the accessible lower intertidal region. Moreover, for each and every quadrate on each of the transects, a separate zip-lock bag was tagged; for example, T1Q1 indicates quadrate 1 of transect 1, T2Q1 indicates quadrate 1 of transect 2, T3Q1 indicates quadrate 1 of transect 3, and so on.

- Each quadrate was thoroughly scanned for the presence of *C. rhabdodactylus* individuals. All the *C. rhabdodactylus* individuals occurring in each quadrate were collected using the hand-picking method and kept in separately tagged zip-lock bags. Later, all the collected specimens were kept in an icebox and brought to the laboratory for further analysis.

- For abiotic factors, the temperature of tide pool water from the upper, middle, and lower intertidal zones was measured during each hour of sampling. Moreover, the ambient temperature of the study site was also recorded every hour during the sampling period. The tide pool water

temperature and ambient temperature were recorded using a digital thermometer.

- For the purpose of microhabitat analysis, three different types of microhabitats in the rocky intertidal region of the study area were identified, which were as follows: tide pools, crevices, and open areas. The percentage area coverage of each type of microhabitat in the study area was further analysed and measured using a transect intercepted with the quadrate method.

- A total of 10 transects were laid, which were 50 m apart from each other. The transects were laid perpendicular to the shoreline, starting from the upper intertidal mark till the lower intertidal mark in the rocky intertidal region of the study area. On each of the transects, a quadrate of 1 $m^2$ was laid at an interval of every 5 m, starting from the upper intertidal mark till the accessible lower intertidal region. Each quadrate was further divided into 100 sub-quadrates of 10 × 10 cm. A photograph was captured from the top view of each quadrate placed on the transect for later microhabitat analysis.

- Percentage microhabitat cover was assessed by counting the number of sub-quadrates occupied by each microhabitat type and giving 1% cover to each sub-quadrate. All sampling was carried out by a single person to avoid potential observer influence.

- In the laboratory, for each quadrate sample, the hermit crabs were removed from the gastropod shell by gently twisting the crabs against the direction of the shell spiral. Only those individuals who remained intact after being removed from their shells were used in the study. The hermit crabs were classified as male, non-ovigerous female, or ovigerous female.

- Monthly data for each season was compiled, and quadrate-wise abundance was calculated for each season. The abundance data was used to plot kite diagrams in order to obtain information about the variation in the distribution pattern of *C. rhabdodactylus* in the different zones of the intertidal region during different seasons.

- Variation in the mean abundance of *C. rhabdodactylus* during different seasons was analysed using a one-way ANOVA. Seasonal variation in the tide pool water and ambient temperature between different intertidal regions was calculated using a two-way ANOVA.
- All the statistical analysis was performed using PAST software (4.03 version) and Microsoft Excel 2019.

**2. Study of behavioural ecology of *Clibanarius rhabdodactylus.***

**c) To study the shell utilisation pattern of *Clibanarius rhabdodactylus.***

- There were two dominant species occurring in the rocky intertidal region of the study area, viz., *C. rhabdodactylus* and *C. ransoni*, which may affect the shell use patterns of each other. Hence, in the present study, the difference in gastropod shell utilisation pattern as well as the relationship between the morphology of the hermit crab species and the different morphological parameters of gastropod shells utilised by these two hermit crab species were studied.
- The specimens of *C. rhabdodactylus* and *C. ransoni* were collected randomly from January to March 2021 during low tide, and the collected specimens were kept in an ice box and brought to the laboratory for further analysis.
- In the laboratory, hermit crabs were removed from their shells by gently twisting them against the direction of the shell spiral, and only intact individuals were used for the study.
- The gender of each individual was identified on the basis of the presence or absence of gonopores and egg masses using a stereomicroscope (Metlab PST 901) and categorised into male, non-ovigerous female, and ovigerous female.
- Two morphological characteristics of the hermit crab: hermit crab weight (HW) (0.01 g) and shield length (0.01 mm) were measured for each individual using a digital weighing scale (0.01 gm accuracy) and vernier callipers (±0.01 mm accuracy), respectively. Ovigerous females were weighed with the egg mass.

- Further, the hermit crabs were sorted according to different size classes on the basis of their shield length (SL).
- The occupied gastropod shells were identified up to species level using a monograph on gastropod shells by Apte (2014).
- Five morphological parameters of gastropods, like shell total length (SHL), shell aperture length (SHAL), shell aperture width (SHAW), shell dry weight (SHW) and shell volume (SHV), were analysed (Figure 13).
- SHL is the greatest distance between the tip of the apex and the base of the aperture, parallel to the shell axis. SHAL is the greatest opening of the gastropod shell, parallel to the shell length. SHAW is the greatest opening of the gastropod shell perpendicular to the shell length (Argüelles-Ticó et al., 2010).
- For SHW, the shells were dried at 60 °C in a laboratory oven for 24 h and weighed (Argüelles-Ticó et al., 2010). For SHV, the empty shells were filled with water using a syringe and needle, drop by drop to avoid the formation of bubbles (0.1 ml) till the edge of the aperture, and the total volume of water filled is considered the shell volume ($mm^3$).
- Individuals smaller than the smallest ovigerous female (2.98 mm CW) were considered juvenile (Baeza et al., 2013).
- The abundance of five highly occupied gastropod shells was quantified using line transects intercepted with a 0.25 $m^2$ quadrate every 5 m.
- A total of ten line transects were laid randomly, perpendicular to the shoreline, from the high tide to the low tide mark to quantify the abundance of live and empty shells.
- The morphological parameters of gastropod shells and hermit crabs were correlated using regression analysis to find out the relationship between them (Sant'Anna et al., 2006).

- Variation in the mean values of shield length of different sexes of hermit crabs was analysed using a one-way ANOVA. The shell species occupation rate was estimated as a percentage.

- Mean values of different morphological parameters of five highly occupied gastropod shells by different sexes as well as reproductive stages of *C. rhabdodactylus* and *C. ransoni* were calculated to understand the sexes or reproductive stage-wise shell occupation pattern.

- Canonical Correspondence Analysis (CCA) was carried out to analyse the relationships between hermit crab morphometry (SL and HW) and shell parameters (SHL, SHAL, SHAW, SHW, and SHV) and visualise the main features of crab (species, sex, and size) distribution according to the gastropod shell species and characteristics.

- The data set of the hermit crab species, comprised of sex and size class, was correlated, while the environmental data set consisted of the shell parameters (SHL, SHAL, SHAW, SHW, and SHV). The data set for hermit crab species for different sexes and sizes was given specific codes as: CRH = *C. rhabdodactylus*, CRS = *C. ransoni*, M = male, F = female, whereas the size classes are represented by numerals.

- All the statistical analysis was performed using PAST software (4.03 version) and Microsoft Excel 2019.

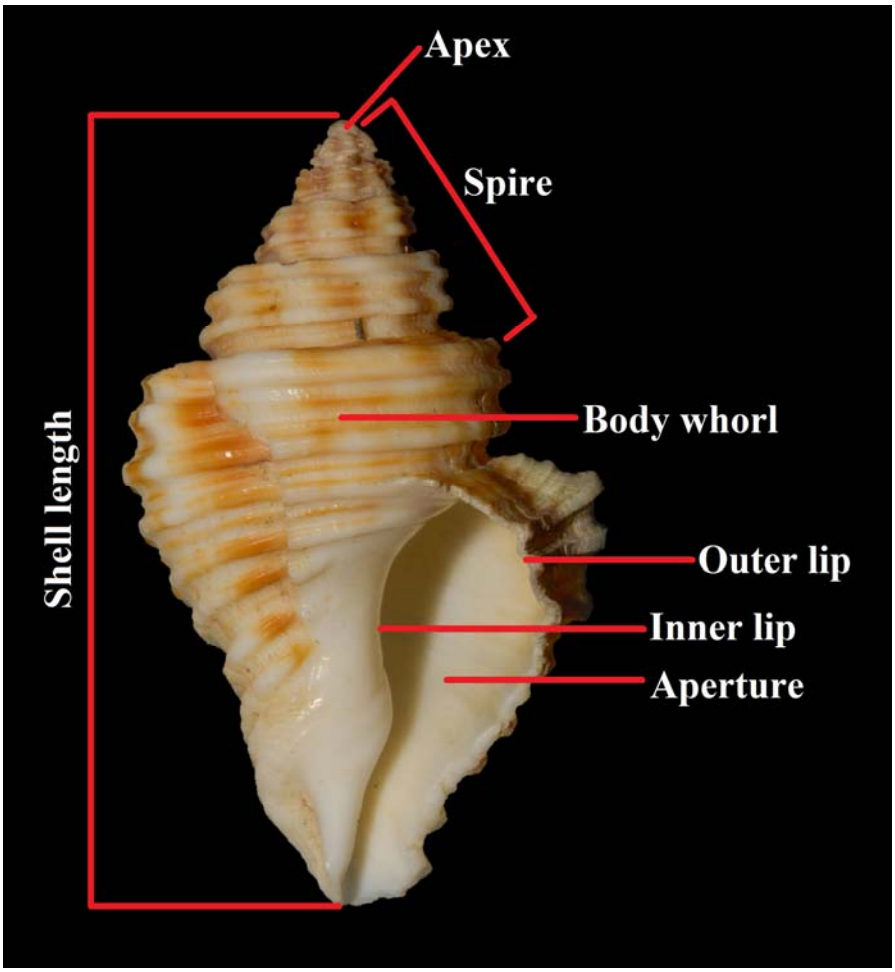


**Figure 13.** External morphology of a gastropod shell.