Chapter 1

The Significance of PCR Primer Design in Genetic Diversity Studies: Exemplified by Recent Research into the Genetic Structure of Marine Species

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Abstract

Genetic markers are widely applied in the study of genetic diversity for many species. The approach incorporates a Polymerase Chain Reaction (PCR) amplification of targeted sequences in the genome. Crucial for the overall success of a PCR experiment is the careful design of synthetic oligonucleotide primers. Ideally designed primer pairs will ensure the efficiency and specificity of the amplification reaction, resulting in a high yield of the desired amplicon. Important criteria such as primer-sequence, –length, and -melting temperature (T_m) are fundamental for the selection of primers and amplification of targeted nucleotide sequences from a DNA template. There are many computational tools available to assist with critical bioinformatics issues related to primer design. These resources allow the user to define parameters and criteria that need to be taken into account when designing primers. Following the initial in silico selection, a primer pair should be further tested in vivo for their amplification efficiency and robustness.

Using examples taken from genetic diversity studies in a marine crustacean, this chapter provides outlines for the application of PCR technology and discusses details for the design of primers for the development and characterization of microsatellite and SNP-markers.

Key words Population genetics studies, Marine species, Molecular tools, Genetic markers, Microsatellite markers, SNP markers, Polymerase Chain Reaction, Oligonucleotide design

1 Introduction

Advances in the biotechnology and utilization of molecular markers facilitate the discovery of genetic variation among individuals, species, and higher order taxonomic groups. Generally, a marker of choice should have the capability of addressing the research issues while its genotyping procedure remains as simple and as low cost as possible. The most powerful and commonly used markers in the field of population genetics are microsatellite and single-nucleotide polymorphisms (SNPs) [1, 2].

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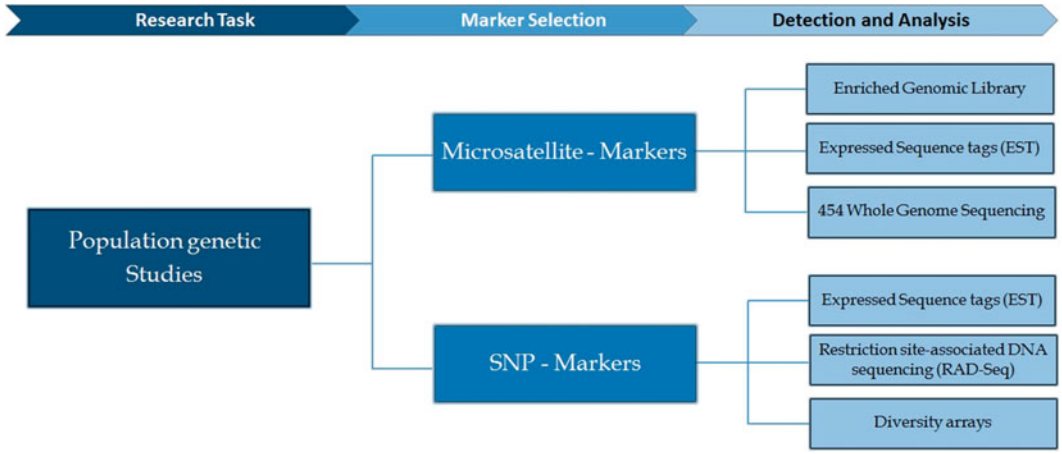

Fig. 1 A brief illustration of the classical approach for the development and characterization of microsatellite and SNP markers

Microsatellites are characterized by the high number of alleles present at a single locus resulting in high heterozygosity and ease of genotyping by PCR [3–5]. The multiallelic microsatellites exhibit more recent divergence due to their mutational rates of (1×10^{-5}) and are well suited to generate linkage maps in marine species [6], to prognosticate human prostate cancer [7], monogenic diseases [8], and neurological disorders [9, 10].

SNPs present biallelic markers, and are more reproducible and powerful for assessing gene flow, differentiation, admixture, migration, and connectivity in marine species [11–13]. They have also been utilized in a wide range of human disease prognostics such as lung [14] or pancreatic cancer [15] and vitamin D deficiency [16, 17].

For many species, the necessary genetic resources are lacking or have not been properly investigated. Hence, access to a wide range of functional marker types is fundamental for genomics-related studies (Fig. 1).

Classically, microsatellite markers are developed de novo. The approach involves the construction of a genomic library enriched for repeated motifs, isolation, and sequencing of microsatellite-containing clones, primer design, optimization of PCR amplification for each primer pair, and a test of polymorphism on a few unrelated individuals [18–20].

Presently, SNP markers are possible to detect using high-throughput genotyping technologies, i.e., Next generation (e.g., Illumina HiSeq and MiSeq platforms) or third-generation sequencing (e.g., PacBio and Nanopore technology) [1, 3, 21, 22]. These approaches have been successfully used to detect SNPs for various marine species [11–13, 23].

The essential component for the approaches highlighted above is the PCR technique. Clearly, any successful PCR-based assay relies mainly on the careful design of synthetic oligonucleotide primers. To ensure the specificity and efficiency of the amplification, it is important to consider carefully key factors including primer-sequence, –length, and -melting temperature (T_m). Primers with poor specificity tend to cause a mismatch between primers and target genetic material, resulting in imbalanced PCR chemistry, impaired detection, and production of false-negative results.

Designing optimal primers is even more critical for the overall success of a multiplex PCR experiment, where a set of markers are used to amplify several segments of target DNA simultaneously. Multiplexing of PCR amplification requires that several primer pairs are included in the reaction mix. Incorrect design of primers will lead to mispriming of the target DNA or the formation of primer dimers and cause nonspecific amplification. Consequently, designing primers of adequate lengths and sequences will ensure an optimal annealing temperature and provide optimal conditions for a specific and efficient multiplex amplification.

This chapter describes the most frequent and widely used methods for the isolation and characterization of microsatellite- and SNP-markers. It also provides details for primer design, PCR amplification, and multiplexing of these markers and their utilization in comprehensive functional analysis.

2 Research Tasks

Marine environments are some of the most important and ecologically diverse biological systems on the planet. The current boom in intensive human intervention and exploitation of natural marine resources have a negative impact on marine biodiversity and ecosystems. To counter the impact of these interventions, an understanding of the broad scale ecological state and distribution of biodiversity is necessary. Coupling of established biodiversity assessment with advanced molecular genetic techniques can describe and monitor the marine biodiversity. Molecular markers that reveal variations in the DNA are the key tools in such studies. The selection and application of proper molecular markers depend on their power to address the task of study purposes. Microsatellite- and SNP-markers present powerful markers for large-scale biodiversity monitoring. While utilization of each marker alone might be limited in its effectiveness, the application of both markers combined, greatly increases the resolution in genetic diversity in marine species, remarkably.

Described below are details for primer design and application of microsatellite- and SNP- markers in research tasks related to marine population and biodiversity studies [6, 11, 24].

3 Sample Collection and DNA Extraction

Samples were obtained from a single walking leg of wild-caught scalloped spiny lobster *(Panulirus homarus)* adults and egg-carrying females or juveniles. They were preserved immediately in DMSO-salt preservative solution [25] until DNA extraction. Genomic DNA (gDNA) was extracted later using a modified CTAB (cetyltrimethylammonium bromide) protocol [26].

4 Microsatellite Markers

Details and procedures for the development of microsatellite markers using enriched microsatellite library techniques [27], available expressed sequence tags (EST) [28], or 454 whole-genome sequencing [20] (Fig. 1), are reported earlier. These techniques require no prior genomic knowledge and facilitate genotyping across large numbers (hundreds or more) of individuals for a range of markers (hundreds to hundreds of thousands). Further, all these approaches are based on the extraction of DNA from a particular organism, followed by the amplification of particular segments of DNA. Below are details for primer design for amplification and multiplexing of microsatellite markers presented. Further, outlines are highlighted for the successful application of these markers in the paternity testing and genetic diversity study of *P. homarus*.

4.1 Isolation, Primer Design, and Amplification

Genomic sequences from *P. ornatus* were obtained using a Roche 454 whole-genome sequence run and cross-species primer amplification strategy was used to isolate assayable microsatellite markers for closely related *P. homarus*. The sequence database was mined for perfect di-, tri-, and tetra-nucleotide microsatellite repeats using iQDD [29] and MSATcommander version 0.8.2 [30], which incorporate Primer3 software for PCR primer design (parameters: product length 150–400 bps; annealing temp 50–63 °C; GC content 20–80%). From this data mining, 370 independent sequence regions with microsatellite repeats with possible primers were identified. Potential loci with primers were subsequently filtered, based on the distance of primers from the beginning and end of a sequence (>10 bps), distance between primers and motif repeat (>10 bps), and PCR product length (from 75 to 400 bps). The quality of perfect di-, tri-, and tetra-nucleotide microsatellite repeats was validated by PCR amplification of 96 *P. homarus* gDNA samples [4]. Forty-six polymorphic microsatellites were reliably amplified across all DNA samples and were coamplified in 14 multiplexes. For fluorescent detection and multiplexing, the forward primers were dye labeled (NED, VIC or 6-FAM) (Life Science Technologies). A PIG-tail sequence 5′-GTTTCTT [31]

was included in the reverse primers. The PCR conditions for all multiplexes were optimized and carried out in a "Veriti Thermal Cycler" (Applied Biosystems Inc.; AB). The amplifications were applied in 12.5μl reaction volume containing 10–50 ng template, 6.25μl 1× Type-it Multiplex PCR Master Mix (Qiagen), and adjusted primer concentrations to yield consistent and relatively even fluorescence among loci [24]. Genotyping was performed using a 3130 Genetic Analyzer (AB). Data were collected automatically and sized with GeneMapper v4.0 software (AB) using the GeneScan-500-LIZ size standard (AB).

4.2 Paternity Testing

The successful application of microsatellite markers for successful parentage assignment in *P. homarus* has been reported earlier [24]. Here, two multiplex PCR protocols including seven microsatellites were developed (Fig. 2) for the study of maternal assignment of 24 larvae hatched from ten potential female spiny lobsters after a mass spawning in a common tank. Exclusion-based parentage analysis unambiguously assigned 83% of fry (20 of 24) to a single female parent. Of ten putative female parents, five have contributed to the 20 allocated offspring, with one being the true parent of 11.

This highlighted study demonstrates the usefulness of microsatellite markers for parentage analysis and their possible potential for application in a wide range of studies investigating population structure related to conservation and management planning, genetic diversity, and evolutionary relationships between *Panulirus* species.

4.3 Genetic Diversity Studies

Microsatellite markers have been applied successfully in a wide range of genomic studies of marine species [32–34]. An example of the utilization of microsatellite markers for the study of tropical spiny lobster population is given below. The study contains integrated microsatellite markers and a comprehensive sampling strategy for the assessment of the genetic structure of *P. homarus* along the coastline in Oman. To assess the level of genetic differentiation between individuals, Discriminant Analysis of Principal Components (DAPC) was used. DAPC was carried out using the optimum number of principal components (PCs) calculated with the α-score function in *adegenet* [35]. To assess both broad and fine-scale population structure, a network analysis with no prior population assumptions was performed using *NetView* R [36]. *NetView* was run through the R implementation of *NetView* P [37] at a mutual k-nearest neighbor k-NN range from 10 to 40 as determined by a k-NN selection plot. To visualize the extent of relatedness between individuals within each population and divergence among populations, a Neighbor-Joining (NJ) tree was constructed in MEGA6 [38]. The NJ tree was constructed using 1-proportion of shared alleles (1-psa) genetic distance matrix calculated in the R package *adegenet* using propShared function [39]. The findings

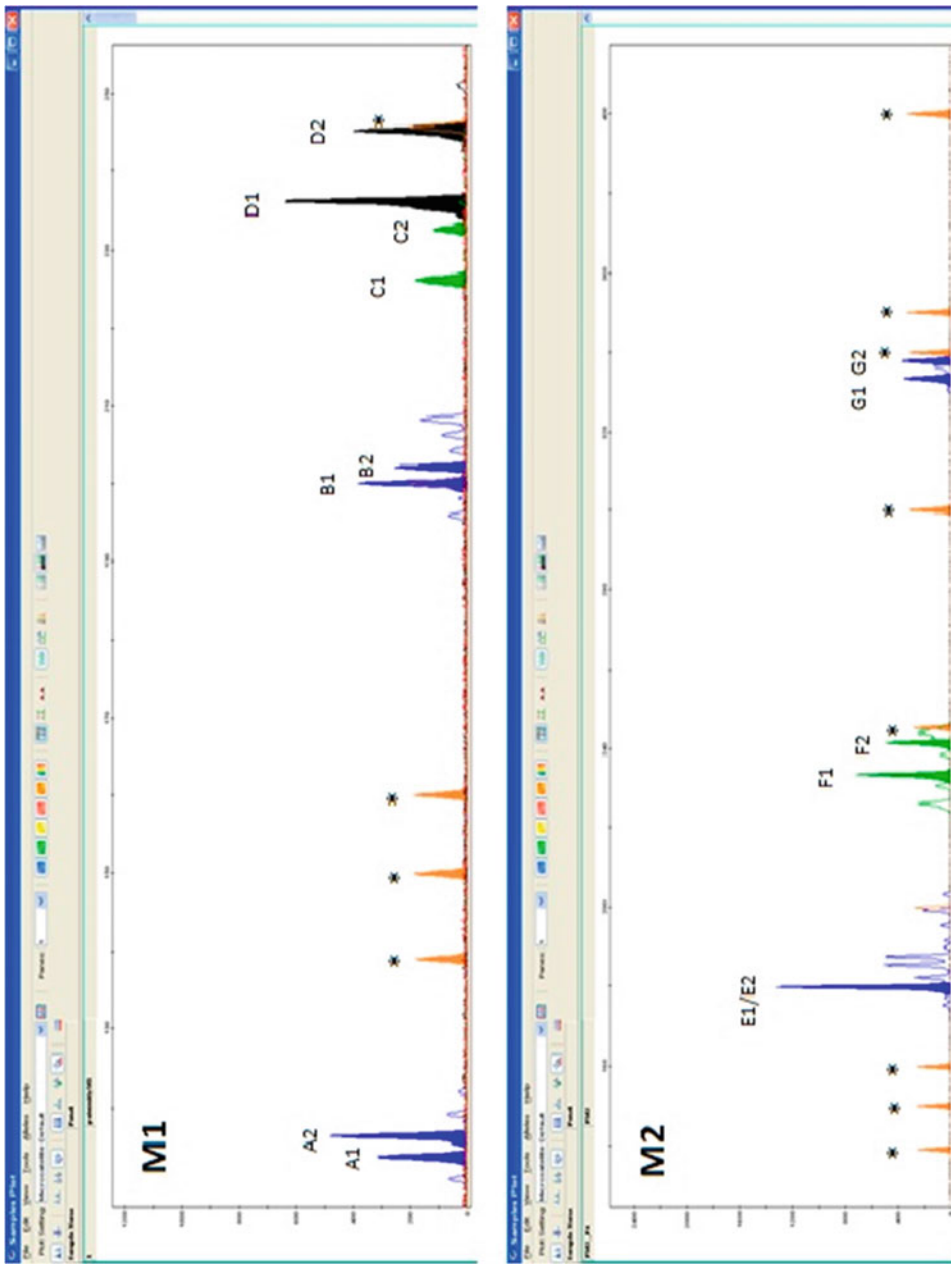

Fig. 2 Electropherogram of genotypes from seven microsatellite markers (A to G) coamplified in two multiplexes (M1 & M2) for parentage assignment in *P. homarus*. Alleles were typed simultaneously using DNA extract from one individual spiny lobster. The individual was heterozygous for all loci, except for the loci E. The allele sizes in basepairs were calculated automatically using commercial internal allelic ladder GeneScan-500 LIZ (AB) in a 3130*x*/Genetic analyzer. *Peaks from allelic ladder; A to G corresponds to seven microsatellites and digits to the two alleles of each markers

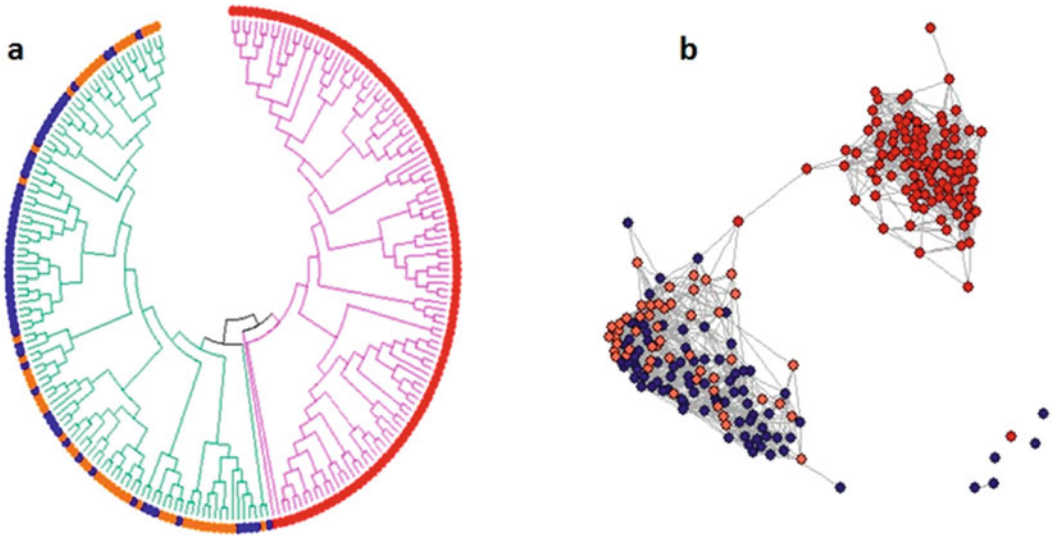

Fig. 3 Population structure of *P. homarus* across the sampled locations in Oman. Unrooted neighbor-joining tree (**a**) was drawn in MEGA6 using 1-psa genetic distances. Population network (**b**) was constructed in the R package *Netview* using 46 microsatellite markers

have a potential impact on fisheries management and aquaculture of the species. Genomic DNA from 220 *P. homarus* individuals were genotyped using 46 microsatellite loci (14 multiplexes) as described above. PCR amplification and multiplexing conditions were the same as outlined in Subheading 4.2. The results from this study indicated the presence of two major stocks of scalloped spiny lobster in Oman (Fig. 3). Further, the findings deliver support for regional fishery management measures and contribute to sustainable fishery management and protection of spiny lobster stock in Oman.

5 SNP Markers

High-throughput DNA sequencing technologies are widely applied for the discovery and analysis of SNP markers (Fig. 1). Details and procedures for the identification of SNP makers from EST sequences [23] and Diversity array technology [11] are reported earlier. These approaches include the preparation of genomic libraries of pooled genomic DNA extracts from a species of interest. The genomic library construction is a multistep process including specific primer design for sequencing and PCR amplification.

The following section provides an example of SNP discovery utilizing the existing EST library for the Atlantic cod *(Gadus morhua)* and highlights the successful application of Diversity array technology for genetic diversity study of *P. homarus*.

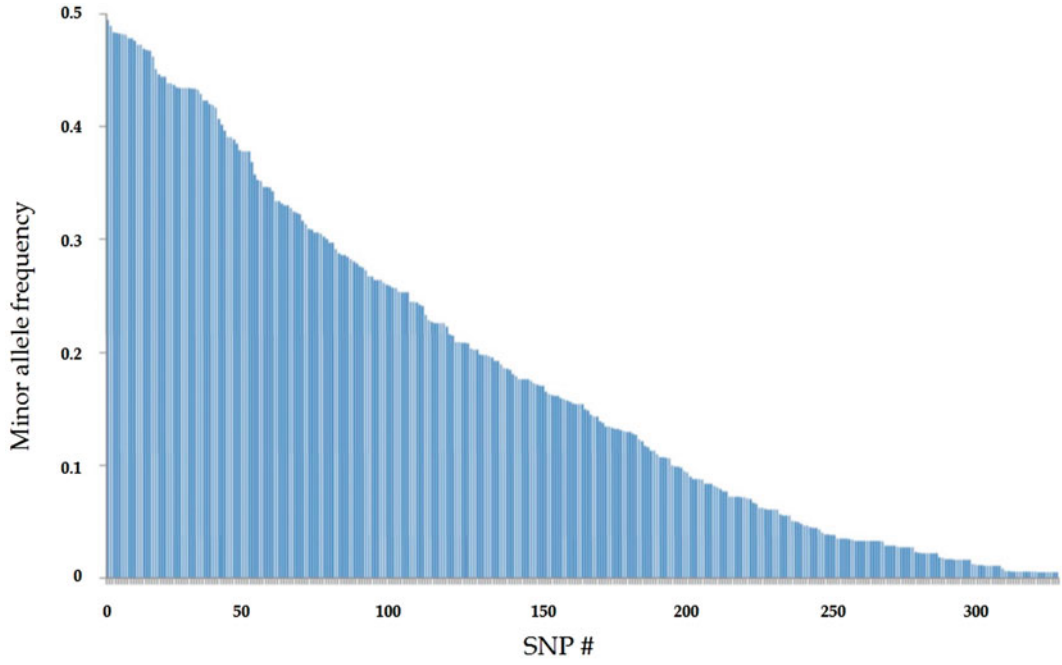

Fig. 4 Overall allele frequency distribution of 318 isolated SNPs for Atlantic cod, ranked in descending order [23]

5.1 SNP Discovery from EST Sequences

The methodological details for SNP marker discovery from EST-sequences of existing cDNA libraries are described earlier. The work describes the successful discovery of 318 SNPs from 17,056 EST sequences originated from cDNA libraries of *G. morhua* (Fig. 4). Genotyping of SNPs was performed using the MassARRAY system from Sequenom (San Diego, USA). PCR-primers and extension-primers were designed using the software SpectroDESIGNER v3.0 (Sequenom). All SNP genotyping was performed according to the iPLEX protocol from Sequenom [40]. For allele separation, the Sequenom MassARRAY™ Analyzer (Autoflex mass spectrometer) was used. Genotypes were assigned in real-time [41] by the MassARRAY SpectroTYPER RT v3.4 software (Sequenom) based on the mass peaks present. All results were manually inspected, using the MassARRAY TyperAnalyzer v3.3 software (Sequenom). SNPs were classified as "failed assays" (meaning that the majority of genotypes could not be scored and/or the samples did not cluster well according to genotype), "SNPs w/all animals heterozygous", "SNPs w/all animals homozygous", or "polymorphic SNPs", based on this manual inspection. SNPs that were out of HWE in one or several populations were double-checked. The SNP markers reported in this work proven to be powerful tools for genetics work and studies on the population structure of Atlantic cod [6].

5.2 SNP Discovery and Genotyping by Diversity Array Technology

Genomic DNA extracts were standardized to 50 ng/μl and sent for sequencing and genotyping using DArTseq™ technology, with Diversity Arrays Technology, Canberra, Australia [42, 43]. Library preparation was completed as described [43, 44] with all *P. homarus* DNA samples being digested by a combination of *PtsI* and *HpaII* restriction enzymes. Multiplexed reduced representation libraries were then sequenced on the Illumina HiSeq2500 platform for 77 cycles. To determine SNPs and genotype for each individual, raw Illumina HiSeq2500 data were first demultiplexed into individual samples based on sample-specific barcode sequences. Demultiplexed samples were then assessed for overall sequence quality, with any fragments with an average Q-score of <25 being removed from the dataset. Sequences were also compared to public databases for identification of contaminant sequences and any non-target sequences (including bacterial and viral fragments) were removed. SNP calling was conducted using the *DArTsoft14* algorithm within the KDCompute framework developed by Diversity Arrays Technology (http://www.kddart.org/kdcompute.html) with initial calling parameters and filtering methods [22, 45].

To eliminate potentially aberrant SNPs, stringent quality controls were applied using custom python scripts within the DArTQC pipeline (https://github.com/esteinig/dartQC) [37]. Initially, all duplicated sequences with >95% similarity were identified using CD-HIT and collapsed into a single cluster or removed [46]. Further, SNPs with a call rate <70% and those where technical replicates did not return a repeatability value of >95% were also removed. Additionally, individuals and SNPs with >20% missing data and SNPs with a Minor Allele Frequency (MAF) < 0.02 were excluded using Plink v1.07 [47]. To investigate the effect of sequencing depth, F_{is} and H_o were calculated for each population at different reads depth (Average SNP Counts) thresholds (3, 5, 7, and 10) to discover the degree of potential bias caused by lower call depths. Accordingly, four subsets of SNPs were generated at these sequencing depths. To detect potential genotyping artifacts, SNPs were tested for significant deviation from Hardy-Weinberg equilibrium (HWE) using Arlequin v.3.5.2.2 [48]. Any SNP loci which significantly deviated from HWE were excluded following Bonferroni correction ($P < 0.000004$). To assess the impact of deviation from HWE, F_{is} and H_o were calculated before and after the removal of significantly deviated SNPs.

5.3 Genetic Diversity Studies

Spiny lobsters are among the world's most valuable and highly priced seafood, captured and marketed in over 90 countries. Recent assessment studies show that the local spiny lobster stock is heavily exploited. For the successful fishery management of this species, it is vital to understand the population genetic structure and to delineate the boundaries of unique genetic stocks. The results of a study utilizing genome-wide single-nucleotide polymorphisms to

study the genetic structure of scalloped spiny lobsters from Oman are summarized below. The findings demonstrate the successful application of genome-wide single-nucleotide polymorphism markers to shed light on the genetic structure of *P. homarus* populations along the Omani coast.

To assess population structuring and genetic differentiation of *P. homarus* populations along the Omani coastline, 180 pleopod samples were collected from nine sites. A reduced-representation sequencing GBS approach was used for SNP discovery and 3095 highly informative markers were selected for analysis following stringent filtering (Subheading 5.2). The markers were then used to assess population differentiation and genetic structure among collected samples. The extent of pairwise population differentiation was evaluated using Weir and Cockerham's unbiased F-statistics [49] through Genetix v.4.05.2 [50]. To assess hierarchical levels of population structuring, an analysis of molecular variance (AMOVA) using Arlequin v.3.5.2.2 [48] was calculated. In addition, the function *find.clusters* in the R package *adegenet* [39] was used to determine the optimal number of clusters with the Bayesian Information Criterion (BIC) method. To assess levels of differentiation between the obtained genetic clusters, DAPC was performed using the optimum number of PCs calculated using the α-score function in *adegenet* [35].

Finally, a network analysis with no prior population assumptions was performed to assess both broad and fine-scale population structure using the R package *NetView* [36], an R implementation of *NetView* P [37]. *NetView* was run at a k-NN range from 25 to 65 as determined by a k-NN selection plot.

The findings revealed five clearly distinguished genetic clusters of *P. homarus* (Fig. 5) along the coastline of Oman, suggesting spatially customized management strategies for the species along the coastline of Oman.

6 Notes

1. An extensive literature search should be performed to ensure the choice of the most suitable genetic markers for a specific research task.
2. Prior to the selection of suitable genetic markers, it is beneficial to look for an existing genomic library for the species of interest, as it will save time and resources.
3. For the development of microsatellites, a cross-species primer amplification strategy should be considered.
4. High-throughput sequencing is the method of choice for large-scale genetic marker development.

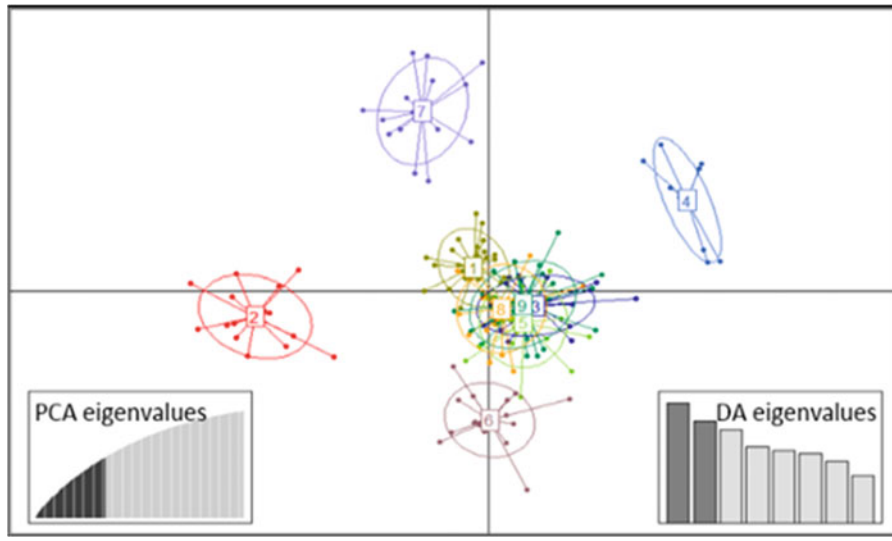

Fig. 5 Population structure of *P. homarus* along the sampled locations in Oman.Discriminant Analysis of Principal Components (DAPC) scatter plot using 3095 highly informative SNP markers in 180 *P. homarus* individuals, in the R package adegenet. Dots represent individuals

5. General recommendations and guidelines should be carefully applied for the design of an optimal primer.
6. Primer3 software is a convenient and user-friendly tool for primer design.
7. A reference sequence of proximally 500 bp will facilitate the design of more specific and efficient primers.
8. Ideally designed primers are located >10 bps from the beginning and end of the target sequence and produce a PCR product of 75 to 400 bps.
9. A primer size of 21–30 bp, annealing temperature 50–63 °C, and GC content 20–80% are favorable features for any primer.
10. Primer-length, primer-sequence similarity, primer-annealing temperature, and the PCR product size should be taken into account before combining primers for multiplexing purposes.
11. Inclusion of a PIG-tail sequence at the reverse primer end (5′-GTTTCTT) will significantly reduce undesirable PCR products.

Acknowledgments

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