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Applying ISSR markers for the genetic analysis of the invasive Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 (Portunidae) from Ghar El Melh Lagoon (Tunisia)

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Abstract:

The spread of invasive species is one of the most remarkable phenomena caused by human-induced global changes. Management actions that prevent their impacts on native species require knowledge of their ecological and genetic traits. The genetic characteristics of the Atlantic blue crab *Callinectes sapidus*, collected from the Ghar El Melh lagoon, were examined using inter-simple sequence repeat (ISSR) markers. A total of 30 individuals including 17 females and 13 males were studied. The analysis of data relies on polymerase chain reaction (PCR) amplification of DNA, followed by statistical processing of the resulting PCR products. We quantified the genetic diversity by the percentage of polymorphic loci, the expected heterozygosity and the Shannon information index and we compared them between males and females. Five ISSR primers yielded a total of 50 scored loci, 49 of which were polymorphic at the level of 99% displaying an average percentage of polymorphic loci of 98%. The mean expected heterozygosity and the Shannon information index in combined sex were relatively high and exceeded 0.30 and 0.46, respectively. Overall genetic diversity was found to be different for both genders, with significantly higher expected heterozygosity and Shannon information index values in females than in males. This work is an initial analysis of genetic aspects of the invasion of Atlantic blue crabs in Tunisia using ISSR. The technique has proven to be an excellent alternative for low-cost genetic monitoring focused on the identification and control of this invasive species and could be used in other regions of the Tunisian coasts.

Key words: Genetic diversity, Polymorphism, Tunisian water, *Callinectes sapidus*, ISSR markers, Atlantic blue crab.

Introduction

Invasive species are of ecological, evolutionary and conservation interests because they successfully adapt in areas where they have been introduced, sometimes despite low genetic diversity compared with their original habitat. One reason for this success may be multiple introductions with novel genotypes (Facon et al., 2008). Such introductions are, among other things, promoted by climate changes. The latter can directly or indirectly influence biological invasions by altering the likelihood of introduction, probability of establishment, geographic range size, environmental impacts, economic costs and/or ease of management (Hellmann et al., 2008; Hulme, 2017). Moreover, an introduced species can overcome the negative effects of founder events and genetic bottlenecks and create genetic variation in the introduced range when genotypes from different geographical areas are brought together. This has been termed the "genetic paradox" of biological invasion (Allendorf and Lundquist, 2003; Estoup et al., 2016). Since invasive species are known to endanger native biodiversity by unbalancing the stability of ecosystems and negatively impacting human health (Tsirintanis et al., 2022), research on their ecological and genetic traits is essential to develop appropriate conservation actions.

The Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 is a crustacean belonging to the family of Portunidae. It is an endemic species of the Eastern coasts of North America, commonly found between southern Canada and northern Argentina (Squires, 1990). *C. sapidus* can occupy the muddy and sandy

bottoms of estuaries, lagoons as well as coastal marine areas to approximately 90 m of deep (Nehring, 2011). The first detection of *C. sapidus* in the Mediterranean Sea dates back to the 1940s, precisely in the north Adriatic Sea (Suaria et al., 2017). The first record in Tunisia was reported on both the north and south coasts at the end of the 2010s (Katsanevakis et al., 2020; Ragkousis et al., 2020; Shaiek et al., 2021).

The Atlantic blue crab *C. sapidus* is a euryhaline and eurythermal species and can tolerate extreme variations in water conditions. The species is considered highly fertile given that females can produce more than 8 million eggs per spawn (Prager et al., 1990). The planktonic larval stage (Zoeae development) can last approximately 3 to 4 weeks (Epifanio, 2019). In addition, sexual maturity in both sexes occurs early between 1 to 2 years, depending on phenology and geographic location (Hines, 2007). The Atlantic blue crab is an important predator in benthic communities and can affect diversity and structure (Mancinelli et al., 2017). All these biological traits make *C. sapidus* one of the worst invasive species introduced into the Mediterranean Sea (Streftaris and Zenetos, 2006).

Inter-simple sequences repeats (ISSR) are type I dominant molecular markers (Zietkiewicz et al., 1994) used in studies on genetic diversity, species identification, phylogeny, gene tagging, genome mapping, evolutionary biology and conservation genetics. Their development does not require DNA sequence information, while detecting polymorphisms in inter-microsatellite DNA regions. Although criticized for its repeatability and reproducibility, the ISSR laboratory protocol is relatively quick with a low cost and potentially generates a large polymorphism.

The objective of this study is to determine genetic diversity in the *C. sapidus* sample collected from Ghar El Melh Lagoon using ISSR markers and to compare it between males and females. The information obtained can serve as a starting point for a subsequent study of population genetics in Tunisian waters.

Materials and Methods

Sample collection

Samples of *C. sapidus* were obtained on 15 August 2021 from the local fishermen of the Ghar El Melh Lagoon situated in the extreme north of the Gulf of Tunis (Fig. 1). The specimens were accidentally caught with nets. Sampled crabs were checked for species levels following Williams (1974) and a total of 30 individuals were correctly identified and retained for this study. Sex was determined according to the shape of the abdomen, triangular in males, whereas wider and circular in females (Fig. 2). Individuals were transported frozen in dry ice to the laboratory and they were stored at -20°C until further analysis. In the laboratory, the maximum carapace length (CL) and maximum carapace width (CW) of each individual were determined to the nearest 0.01 mm using a digital caliper (RUPAC µ). Total wet body weight (W_b) was measured using a digital balance with a precision of 0.01 g. biological material consisted of a piece of muscle tissue dissected from the chela and preserved in the alcohol 95° for molecular analysis. The sample measurement details by sex are mentioned in Table 1.

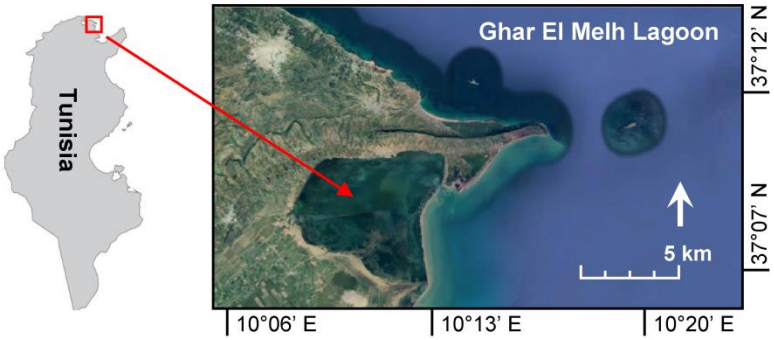

Figure 1. Map of Tunisia showing the collected region of *Callinectes sapidus* sample from Ghar El Melh lagoon (Tunisia).

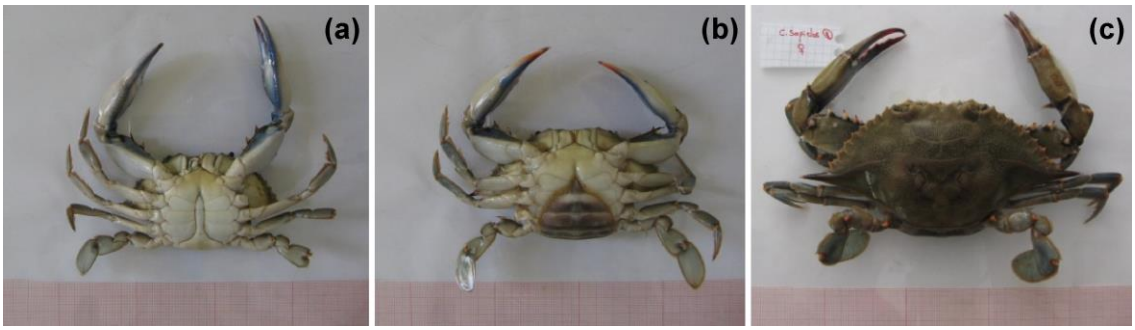

Figure 2. Photographs of male (a) and female (b) specimens of *Callinectes sapidus*, the Atlantic blue crab, collected from Ghar El Melh Lagoon in Tunisia, showing the distinguishing features of their abdomens. (c) Dorsal view of the carapace.

Table 1. Sample measurement details for *Callinectes sapidus* from Ghar El Melh lagoon (Tunisia).

	N	**CL ± SE (mm)**	**CW ± SE (mm)**	**W_b ± SE (g)**
Females	17	60.33 ± 1.90	118.87 ± 4.86	107.51 ± 8.80
Males	13	62.12 ± 1.74	113.24 ± 3.26	104.82 ± 5.43
Total	30	61.11 ± 1.30	116.43 ± 3.09	106.34 ± 5.44

Abbreviations: N, sample size; CL, maximum carapace length; CW, maximum carapace width; W_b, total wet body weight and SE, standard error.

DNA Extraction

The total genomic DNA of each crab was extracted using a modified salting-out protocol as described by Sunnucks and Hales (1996). Small muscle fragments (≈ 30 mg) were cleaned by distiller water to remove alcohol traces and immediately transferred into a 1.5 ml microfuge tube containing 400 µl DNA extraction buffer (50 mM Tris-HCl pH 7.6, 400 mM NaCl, 20 mM EDTA, 0.5% SDS, and freshly added 0.2 mg/ml proteinase K). The lysates were incubated at 55 °C overnight under slight agitation. DNA was extracted with sodium acetate (3 M pH 5.2) by adding one-tenth volume to the lysates. Proteins were pelleted in a microfuge at 12,000 rpm for 20 min. DNA was precipitated by adding two volumes of ice-cold isopropanol. DNA was collected by brief centrifugation and washed twice with 70% ethanol, air-dried, and resuspended in Tris-EDTA 10/1 buffer. The quality of the isolated DNA was analyzed by gel electrophoresis on a 1% agarose gel using 1×Tris-Acetate EDTA (1×TAE) buffer, stained with Ethidium Bromide (0.35 µg/ml) and visualized under UV light. The DNA was quantified spectrophotometrically at 260 nm and 280 nm and diluted to 50-100 ng/µl for use as a template in Polymerase Chain Reactions (PCR).

PCR for Inter-Simple Sequences Repeats

The DNA was amplified with PCR using ISSR primers developed by researchers from the University of British Columbia (UBC, Vancouver, Canada). Ten ISSR primers were screened for their repeatable amplification. From these primer sets, 5 ISSR primers produced clear and reproducible bands and were retained for the present study (Table 2). Each primer set was amplified in a total volume of 25 µl, consisting of 2.5 µl of genomic DNA, 5 µl 10×PCR buffer, 2 µl MgCl2 (25 mmol/l), 2 µl dNTPs (1.25 mmol/l), 0.4 µmol/l primer, 1 U of Taq polymerase enzyme (Promega) and sterile distilled water. PCR amplification was carried out in a DNA thermal cycler (Life technology Applied biosystems 2720 Thermal cycler) with initial denaturation for 5 min at 94 °C followed by 45 cycles, denaturation for 40 sec at 94 °C, annealing for 40 sec at respective annealing temperature (Tm, Table 2), and extension at 72 °C for 1 min 30 sec, with a final extension at 72 °C for 8 min. The amplified products (6 µl of PCR products mixed with 2 µl of bromophenol blue) were resolved on a 2.5% agarose gel in 1×TAE buffer containing 0.35 µg/ml Ethidium Bromide. A DNA ladder was applied as a size marker (Invitrogen, 100 bp DNA ladder). Electrophoreses were run for 60 min at 100 V. DNA fragments were identified by visual analysis after being photographed with a gel documentation system under a UV transilluminator.

Table 2. Characteristics of ISSR markers retained with the best resolution for *Callinectes sapidus* from Ghar El Melh lagoon (Tunisia) and their polymorphism indices.

Marker	**Sequence**	**Tm (°C)**	***TB***	***PB***	***%P***	***PIC***	***H'***
UBC 811	5'-GAGAGAGAGAGAGAGAC-3'	52	9	8	88.89	0.325	2.201
UBC 814	5'-CTCTCTCTCTCTCTCTA-3'	54	9	9	100	0.258	2.218
UBC 834	5'-AGAGAGAGAGAGAGAGYT-3'	54	12	12	100	0.367	3.665
UBC 844	5'-CTCTCTCTCTCTCTCTRC-3'	52	11	11	100	0.239	2.758
UBC 898	5'-CACACACACACARY-3'	56	9	9	100	0.375	2.839
Average			10	9.8	98	0.313	2.736

R = (A, G); Y = (C, T)

Abbreviations: Tm, annealing temperature; *TB*, total number of bands; *PB*, number of polymorphic bands; %*P*, percentage of polymorphism; *PIC*, polymorphic information content; and *H*', Shannon's index.

Data analysis

Each amplified ISSR locus was transformed into a binary character matrix (1 = presence, 0 = absence). The capacity of each primer was evaluated by the following parameters: the total number of bands (*TB*), the number of polymorphic bands (*PB*), the percentage of polymorphism (%*P*) at the level of 99%, the polymorphic information content (*PIC*) (Roldàn-Ruiz et al., 2000) and Shannon's information index (*H'*) (Shannon and Weaver, 1949). *PIC* of dominant bi-allelic data was estimated by the formula:

$PIC = (\Sigma 2p_i q_i)/PB$

Where p_i is the frequency of the visual allele (band present) and q_i is the frequency of the null allele in each locus of the considered ISSR primer. Allele frequencies were estimated assuming the population is at Hardy-Weinberg equilibrium for this locus, so q_i is the squared-root of the frequency of individuals in which the band is absent. Shannon's information index was calculated by the formula:

$H' = -\Sigma p_i \text{ Ln } p_i$

The genetic diversity by sex and for the total sample was estimated by the observed number of alleles per locus (n_a) and by the effective number of alleles per locus (n_e) estimated as follows (Kimura and Crow, 1964):

$n_e = 1/(p_i^2 + q_i^2)$

We also estimated the number of polymorphic loci (*PL*), the percentage of polymorphic loci (%*P*), the expected heterozygosity (H_{exp}), and the Shannon-Wiener diversity index (*H'*). Differences in H_{exp} and *H'* between both sexes were tested for significance using paired *t*-tests of arcsine-square-root-transformed values of a single locus (Archie, 1985). All these parameters were processed using POPGENE software version 1.32 (Yeh et al., 1999) and R environment version 4.1.2 (R Core Team, 2021). The statistical significance for all tests was set to $P < 0.05$ after the application of the False Discovery Rate of Benjamini and Hochberg (1995).

Results

The five ISSR primers employed revealed a total of 50 loci, of which 49 are polymorphic. The scored fragment sizes ranged from 250 to 2000 base pairs in length, while the number of amplification products ranged from eight to twelve products per marker (Table 2). Only, the UBC 811 marker gave a single monomorphic locus, showing accordingly an average percentage of polymorphic loci of 98%. An example of ISSR banding patterns for each primer for *C. sapidus* is given in Fig. 3. These banding patterns could be used as a template for the gynogenetic identification of the species. The maximum *PIC* of 0.375 was obtained from UBC 898, while the minimum of 0.239 was observed in UBC 844. The Shannon's index (*H*') average was 2.736 ± 0.487 for all ISSR markers, the lesser value was for UBC 811 (2.201), and the major was for UBC 834 (3.665).

Data from the polymorphic bands revealed that n_a and n_e values ranged from 1.620 in males to 1.960 in females and 1.352 in males to 1.577 in females, while total sample description statistics gave the values of 1.980 and 1.519 alleles, respectively (Table 3). The higher value of n_a than n_e indicates that this sample has a greater number of individuals with two different alleles at a given locus than the theoretical value of an equilibrium population. The expected heterozygosity (H_{exp}) at the sample level gives an average value of 0.307 ± 0.158 and the Shannon-Wiener diversity index (*H'*) of 0.468 ± 0.196.

High genetic variation was observed in females than in males of *C. sapidus* with a percentage of polymorphic loci of 96% and 62%, respectively (Table 3). The pairwise *t*-tests between males and females of arcsine-square-root-transformed values of H_{exp} and *H*' yielded highly significant *P*-values (*P* = 0.00006 and 0.00004, respectively) even after applying the Benjamini and Hochberg (1995) correction.

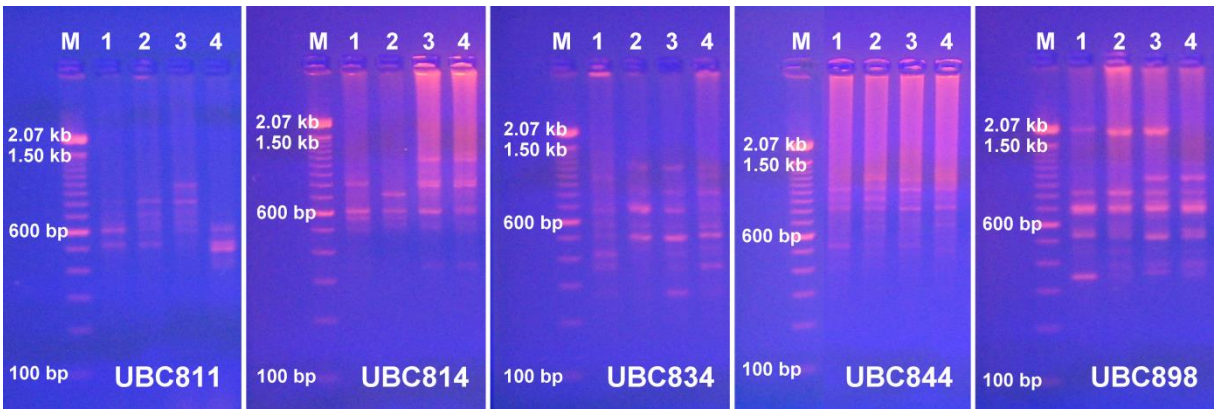

Figure 3. Examples of ISSR marker profiles retained for the Atlantic blue crab *Callinectes sapidus* from Ghar El Melh Lagoon (Tunisia) and generated in 2.5% agarose gel 1×TAE. For each ISSR marker, we used the same four individuals preceded by a 100 bp DNA ladder noted M (Invitrogen).

Table 3. Genetic diversity parameters by sex and total sample for *Callinectes sapidus* from Ghar El Melh lagoon (Tunisia) using ISSR markers.

	n_a ± SD	n_e ± SD	*PL*	%*P* (99% criterion)	H_{exp} ± SD	*H*' ± SD
Females	1.960 ± 0.198	1.577 ± 0.335	48	96%	0.335 ± 0.151	0.501 ± 0.190
Males	1.620 ± 0.490	1.353 ± 0.397	31	62%	0.207 ± 0.200	0.312 ± 0.282
Total	1.980 ± 0.141	1.519 ± 0.343	49	98%	0.307 ± 0.158	0.468 ± 0.196

SD, standard deviation.

Abbreviations: n_a, Number of observed alleles; n_e, number of effective alleles; *PL*, number of polymorphic loci; %*P*, percentage of polymorphic loci; H_{exp}, expected heterozygosity; and *H*', Shannon-Wiener diversity index.

Discussion

The genetic diversity in *C. sapidus* is considered high when we compare our results with those obtained with its homologous invasive species in southern Tunisia *Portunus segnis* using the same genetic markers (mean H_{exp} = 0.265 ± 0.15; mean *H'* = 0.417 ± 0.19) (Hatira et al., 2020). In the same context, high genetic diversity in *C. sapidus* was also reported in its native range or the Mediterranean and Black Seas based on mitochondrial (McMillen-Jackson and Bert, 2004; Feng et al., 2017; Williams et al., 2017; Öztürk et al., 2020; Schubart et al., 2023) and nuclear markers (Yednock and Neigel, 2014; Macedo et al., 2019).

The level of genetic diversity is strongly related to effective population size, which depends mainly on the mating system and gene flow. It determines the ability of populations to evolve to cope with environmental change on an evolutionary time scale (Frankham, 2005). Usually, invasive species,

recently established in a new region, are expected to suffer from reductions in genetic diversity because they are founded by a limited number of individuals (Dlugosch and Parker, 2008). Accordingly, natural selection and genetic drift will act to result in lower genetic variation than in native populations. The high genetic diversity observed in *C. sapidus* proves that the species has long been established in the Mediterranean Sea. This may be due to gene flow and/or multiple introductions, thus creating the conditions for high adaptable capacity and expanding potential. Additionally, long pelagic larval duration (Epifanio, 2019) facilitates a high level of gene flow and leads to the maintenance of genetic diversity.

It seems that genetic diversity in *C. sapidus* is maintained primarily by the high variance of reproductive success of females. The production of multiple offspring by a female in a single spawning might play an important role in determining population effective size and, therefore, in maintaining the genetic diversity of the population. Further molecular analyses using codominant neutral markers are needed to confirm the current results.

Conclusion

This study utilized a molecular approach based on ISSR Markers to characterize *C. sapidus* in Tunisia for the first time. Moreover, genetic diversity between the two sexes was assessed. A high genetic variability was observed in the total sample and the females seem to be the ones maintaining this diversity and could therefore be partly responsible for the spread and the invasion character. Our study showed that ISSR analysis is quick, reliable, and produces sufficient polymorphisms and information which could help for the genetic identification as well as for the implementation of further conservation programs to control or enhance this species.

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Author Contributions

Chaima Jebali designed the study, carried out the sampling and laboratory work;

Chiheb Fassatoui wrote the manuscript, treated the statistical data, interpreted the results, edited and contributed to supervise the work;

Mohamed Salah Romdhane supervised the work, contributed to the writing of the manuscript, revised, and contributed to the interpretation of results.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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