Heterozygosity fitness correlations and generation interval of the Norway lobster in the Aegean Sea, eastern Mediterranean

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Abstract
Background: Comprehensively detailed information on population dynamics for benthic species is crucial since potential admixture of individuals could shift the genetic subdivision and age structure during a full breeding period. The apparent genetic impact of the potential recruitment strategy of Norway lobster Nephrops norvegicus is still under research. For this reason the present study was focused on genetic variation of the species over a given continuous year period in a semi-enclosed gulf of the Aegean Sea.

Results: Analyses revealed that the relative smaller size class in females and the apparent faster growth of males may represent a key-role differential strategy for the two sexes, whereas females tend to mature slower. Heterozygosity fitness correlations (HFCs) showed substantially significant associations suggesting that inbreeding depression for females and outbreeding depression for males are the proximate fitness mechanisms, respectively.

Conclusions: Nephrops norvegicus uniformal genetic composition (background of high gene flow), could be attributed to potential population recolonization, due to a hypothesized passive larval movement from deeper waters, which may suggest that some offspring of local residents and potential male non-breeders from other regions admixture randomly.

Keywords: Microsatellites, Carapace length, Nephrops norvegicus, Generation interval, Heterozygosity fitness correlation, Inbreeding

Background
Lobsters are a quite expensive, nevertheless, valuable type of tasty seafood as they are considered as a “delicatessen” around the world. Global landings of lobsters for 2013 exceeded 230,000 mt, of which approximately 60,000 mt corresponded to Norway lobster, Nephrops norvegicus [1]. Pagasitikos gulf, eastern Mediterranean, is documented to be one N. norvegicus high population abundance site [2]. Fishing activity in Pagasitikos gulf is confined to small scale fisheries, since trawling is restricted, whilst there is a three-month period of creel ban during summer [3]. Indeed, this effective limitation of the fishing activity applied for over a decade, enhanced juvenile survival, protected stocks from overexploitation and increased yields in fishing grounds. Although overall landings of Norway lobster in Hellenic Seas, over the past 20 years were reduced by >69% (from 1600 mt at 1989 to 490 mt at 2009) [1], fishing pressure on this species remains heavy and the species appears to be withstanding overexploitation [4]. Nephrops norvegicus is a marine benthic decapod crustacean (Family Nephropidae) with a wide geographical and bathymetric distribution (captured even at 400 m in northern Aegean Sea fishing grounds). It is considered as highly commercial important species resulting in a recent interest as a new candidate species for aquaculture [3, 5, 6]. A high larval dispersal ability, although dependent *Correspondence: gkafas@uth.gr
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Knowledge of its population dynamic pattern (a background of quite high gene flow was previously recorded [9, 10]) will elucidate how genetic variation is partitioned among populations, thus, having important implications not only in Norway lobster’s evolutionary biology and ecology, but also in implementing conservation biology strategies. However, the current understanding on *N. norvegicus* ecology and evolution was focused so far on classical ecological [2, 11–13] and reproductive [3] approaches with, nevertheless, valuable information on the molecular level [9, 14–17]. Multiple evolutionary processes can affect the temporal and spatial variability of allelic frequencies in natural populations (e.g. [18]); such are migration, mutation, selection and genetic drift. Although marine species have the potential of migrating through long distances, genetic markers could reveal the presence of small, or large scale genetic structure (e.g. [19]). Since Norway lobster exhibits a burrowing lifestyle as an adult, the recorded lack of significant genetic differentiation could be mostly attributed to population mixing during larval pelagic phase. Indeed, *N. norvegicus* larval stage exceeds 50 days in plankton, before benthic settlement occurs. Juveniles appear to preferentially take up residence in vacated adult burrows, otherwise constructing their own burrows as an extension of the already existing ones [20]. Notwithstanding, larvae are able to migrate 100–300 km depending on local oceanographic characteristics and water mass transportsations [21]. Interestingly, low genetic variation was recorded through the species distribution, mainly due to genetic drift [22]. However, human activities could potentially modify the environment of larvae dispersal such as sound and light pollution, shipping and most notably pollution crisis (e.g. oil spills).

It has been documented that during the protracted brooding periods and the periods of extended release of eggs, alteration of age and sex ratios in Norway lobster is possible [3]. Thus, such periods may influence population dynamics, in the sense of genetic differentiation, or even in subsequent recruitment of the species. On the other hand, this potential admixture of individuals could shift temporally the genetic subdivision and size structure during a full breeding period. However, the apparent genetic impact of the potential recruitment strategy is still under investigation. Consequently, by measuring the genetic diversity, it may be possible to assess the mechanism that generates a potential correlation between heterozygosity and life-history traits of body size of the species. The morphology of Norway lobster has already been thoroughly described [3] and it is well documented that growth play an important role to life-history success of the species in terms of reproduction. Published data have shown that heterozygosity is often correlated with indirect fitness measurements such as fluctuating asymmetry [23–26] and length measurements [27, 28]. According to the theory, low heterozygous individual have a relative reduced fitness, possibly due to inbreeding depression. Many earlier studies used microsatellite DNA markers, and due to the nature of these markers, they are not considered to represent genome-wide variability [29]. However, most important is not the panel of the markers used, but the level of identity disequilibrium in the studied populations [30]. On the other hand, more recent studies demonstrated the greater power availed by genome sampling (High-throughput sequencing, e.g. [31]), revealing new insights in genetic variability, which is however subjected to costs and high performance computing analyses.

To test this hypothesis, the present study was focused on assessing genetic variation in *N. norvegicus* over a continuous year period. Also, the generation interval of the given year was calculated in order to assess the age overlap of the species in Pagasitikos gulf. For this reason microsatellite markers were used as a molecular genetic tool and proved to be a comprehensively informative approach in the study area. Morphometric data were combined with the allele frequencies of *N. norvegicus* at a temporal scale in order to assess the generation interval separately for the two sexes. Moreover, heterozygosity-fitness correlations were tested regarding levels of genetic diversity and carapace length variability.

**Methods**

**Sampling**

A sampling scheme was designed in order to survey temporal variation of Norway lobster species during a full breeding period. Sampling of male and female individuals of *Nephrops norvegicus* was carried out during a given continuous year (2007) near the deepest area of Pagasitikos gulf (39°16′N; 23°02′E) (Fig. 1). It is worth mentioning that Pagasitikos gulf was selected because it is considered a sampling area of high *N. norvegicus* abundance throughout the Aegean Sea [13]. A total number of 764 specimens were collected through experimental trawling from a registered fishing ground approximately in the middle of a given month (subject to availability) (Additional file 1: Table S1). Following individual weighting and measuring, white muscle was dissected and stored at −20 °C. Carapace length was measured on each specimen; measurements were taken to the nearest 0.1 mm.
DNA extraction and amplification strategies

DNA was extracted from 50 mg of white muscle tissue from each individual using the standard phenol–chloroform protocol [32]. DNA pellet was finally diluted in 50 μL TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.00) and stored at −20 °C, for downstream PCR. Quantity and quality of template DNA were confirmed by measuring absorbance at 260 nm and 260/280 ratio, respectively, using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

Six microsatellite loci, specific to Nephropidae family [14, 33], were tested based on their recorded polymorphism in order to produce scorable amplification patterns (Table 1). Amplification reactions were carried out in a 20 μL volume using 10 ng of extracted genomic DNA, 2 μL reaction buffer (10×), 0.25 mM of each dNTP, 3 mM MgCl₂, 15 μM of each primer, 1 U Taq polymerase (KAPABIOSYSTEMS, Massachusetts, USA), and ddH₂O, up to the final volume. For all reactions, cycling conditions were 94 °C for 30 s, 57 °C for 30 s, followed by 72 °C for 30 s. Microsatellites were amplified with fluorescently labeled forward primers and PCR products were run with the internal ladder Genescan-500 LIZ Size Standard (Applied Biosystems, Foster City, CA, USA) in an ABI3700 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Genotypic data were analyzed using the STRAND 2.3.0.48 software package [34].

### Table 1 Gene diversity (G), number of alleles (N_o), allelic richness (R), inbreeding coefficient index (F_IS) per locus and sex for N. norvegicus

| Loci               | Females | | | | Males | | | |
|--------------------|---------|---|---|---|-------|---|---|---|
|                    | G        | N_o | R   | F_IS | G   | N_o | R   | F_IS |
| Nnmic2-E4          | 0.898    | 15  | 14.158 | 0.157 | 0.898 | 10  | 9.976 | −0.054 |
| Nnmic1-F2          | 0.955    | 23  | 22.168 | 0.122 | 0.941 | 21  | 20.232 | 0.024 |
| Nnmic1811          | 0.830    | 8   | 7.834 | 0.153 | 0.825 | 10  | 9.644 | 0.247 |
| Nnmic1-C12         | 0.716    | 9   | 8.129 | −0.084 | 0.676 | 7   | 6.765 | −0.438 |
| NnmicT-G2          | 0.958    | 24  | 23.068 | 0.125 | 0.941 | 23  | 21.845 | −0.033 |
| Lobp3              | 0.797    | 11  | 10.620 | 0.186 | 0.783 | 11  | 10.485 | 0.034 |
| Total              | 0.859    | 15  | 12.559 | 0.109 | 0.844 | 13.66 | 13.157 | −0.041 |

| Females | | | | | Males | | | | |
|---------|---|---|---|---|-------|---|---|---|
| MLH     | 0.528 ± 0.23 | 0.471 ± 0.23 | 0.067 ± 0.23 | 0.533 ± 0.15 | 0.456 ± 0.156 | 0.046 ± 0.16 |
| IR      | | | | 0.0057 (−0.0638; 0.0752) | 0.16 | 88 | 0.872 |
| HL      | | | | −0.0057 (−0.0754; 0.0640) | −0.16 | 88 | 0.872 |

### Data analysis

All loci were tested for the presence of null alleles, or allelic dropout using the software MICROCHECKER v.2.2.3 [35]. The software Bayescan v.1.0 [36] was used to identify candidate loci under natural selection. Exact tests for Hardy–Weinberg equilibrium and Linkage Disequilibrium (using Fisher’s exact tests) were carried out using the software Genepop v.1.2 [37]. F_IS index [38], number of alleles, allelic richness and gene diversity
Table 2 Estimates of generation effective size ($N_e$) and generation interval (GI) for *N. norvegicus* sampled individuals

|          | $N_e$ | GI paternal | GI maternal | Overall GI |
|----------|-------|-------------|-------------|------------|
| Estimate | 1902  | 5.89        | 6.28        | 5.41       |
| CI 95% low | 1749  | 5.89        | 6.08        | 5.25       |
| CI 95% upper | 2444  | 5.97        | 6.69        | 5.71       |

per locus and per sex were calculated using the FSTAT v.2.9.3.2 software [39].

Species generation interval was assessed with the Age-Structure software [40] based on genotype parentage assignment. Length Frequency Distributions (LFD) were calculated separately for male and female individuals per month using carapace length. Class interval was calculated as 3.72 mm when using the formula described by Sokal and Rohlf [41]. All LFD analyses were carried out in SPSS 14.0 (SPSS Inc., Chicago, USA).

Mean multilocus heterozygosity (MLH) and inbreeding measures (Internal Relatedness-IR and Homozygosity by Locus-HL) were performed using the software IRmacroN v.4.0, an EXCEL macro written in Visual Basic [42]. Linear regressions were used to investigate possible relationships between measures of genetic diversity and carapace length for both sexes using Minitab v.17.0 (Minitab Ltd., Coventry, UK).

Results

Factorial Correspondence Analysis (FCA) reveal a single population (Additional file 1: Fig. S1), thus individuals were treated as such. Significant departures from Hardy–Weinberg equilibrium occurred at random loci and Linkage Disequilibrium was found at multiple loci pairs. On the other hand, no evidence of selection was detected across all six loci. Female individuals favored significant evidence of high inbreeding index (overall $F_{IS}=0.109$, $p<0.05$), while in male individuals this was not the case (overall $F_{IS}=-0.041$; $p>0.05$). The number of alleles ranged from 9 (*Nnmic1-C12*) to 24 (*Nnmic1T-G2*) with a mean value of MLH at 0.528 and 0.533 for female and male individuals, respectively. Summary statistics on genetic variability are presented in Table 1.

Mean Generation interval was calculated at 5.41 years (CI 95% 5.25–5.71), which is considered rather informative (Table 2), since previous ecological data remain unclear on age clustering of the species [6]. Generation interval for female and male individuals was calculated at 6.28 and 5.89, respectively. Each individual was assigned accordingly to each carapace length class (see review in [43]) as shown in Fig. 2. Female lobsters attain sexual maturity at approximately 2.5–3 years of age at a carapace length of 21–22 mm. Males become mature after 3 years at a carapace length of 25 mm [44]. The present dataset recorded a female size at the onset of maturity at 28.1 mm [3], a fact which is consistent with the proposed generation interval calculation. Monthly pairwise differentiation of mean carapace length per sex was significant for all comparisons (Chi square analysis), except for January and October (Fig. 3).

MLH and inbreeding measures (IR, HL) did not differ significantly between male and female individuals (Table 1). On the contrary, HFC analysis showed significant associations for all three different measures of heterozygosity against the carapace length for both male and female individuals (Fig. 4). Interestingly, male individuals showed significant negative correlation with respect to MLH, IR and HL, suggesting outbreeding depression. The correlations in male individuals were strongly negative regarding the carapace length measure (Table 3) and remained highly significant even after Bonferroni correction ($r_{MLH}^2=0.352$, $p<0.001$; $r_{IR}^2=0.352$, $p<0.05$; $r_{HL}^2=0.306$, $p<0.001$). On the other hand, female individuals showed relatively low, but significantly positive association ($r_{MLH}^2=0.047$, $p=0.03$; $r_{IR}^2=0.047$, $p=0.03$; $r_{HL}^2=0.052$, $p=0.023$) with carapace length (Table 3), implying that inbreeding depression, this time, is the main fitness mechanism.

Discussion

This study represents one of the first attempts dealing with the ecological aspects of mixed gene pools in the marine environment, regarding different reproductive strategies with respect to sex. Male and female Norway lobsters, surprisingly, favored different evolutionary mechanisms suggesting that even relatively high migration movements (i.e. outbreeding), or high inbreeding could adapt robustly in terms of fitness. Previous genetic studies in *Nephrops norvegicus* demonstrated a low but significant genetic heterogeneity [9, 16] through the species distribution. However, an unclear geographical pattern among lobster populations has been recorded; an IBD model of geographical and genetic distances was not valid [9]. These findings have been discussed under a background of high gene flow, thus temporal sampling over differential generations was of great interest among *N. norvegicus* populations, in order not only to test if the observed genetic pattern remains stable over time, but also in order to clarify the model of genetic and size classes structuring.

In both sexes we observed significant but different HFC regression slopes according to carapace length. In males, the associations of different levels of heterozygosity with the fitness related trait were negative, suggesting that outbreeding depression is likely to be the
Fig. 2  Frequencies of Carapace Length (CL) distribution in each size class per month, separately for female (white bars) and male (black bars) individuals.
substantial conservation scenario due to an apparent movement of lobsters. The latter suggests that, highly heterozygous male individuals grew less and suffered much greater loss of fitness. The mechanism that underlies this observed pattern may be local adaptation; offspring of genetically distant mates may be less adapted to the environment than their parents [45]. In relatively small sized populations of several other species, local adaptation is often attributed to impose a strong impact on HFC and therefore reflects outbreeding depression [26, 46–48].

Despite this observed alteration in allele frequencies, little variation in levels of multilocus heterozygosity was recorded, suggesting high levels of inbreeding in female individuals. Interestingly, female individuals showed a rather low but also significant relationship between carapace length and overall heterozygosity measures, implying that inbreeding depression this time, is the primary mechanism for such an association. Indeed, high levels of $F_{IS}$ may suggest non-random mating, thus indicating high inbreeding within females. Given this pattern, the observed loss of allelic frequencies from the apparent local population, might imply some degree of adaptiveness to the local environment. The specific analyzed microsatellite loci were somewhat variable in all females and most of the individuals were homozygous at all these six loci. Such a documented absence of a fine-scale structure of the species to date [9, 16], may lead to smaller local effective population sizes, and the possibility of a greater impact of inbreeding on fitness [49].

Moreover, significant levels of HFC differentiation among the two sexes could be explained by the actual migration of individuals favoring rather low relatedness within the population, suggesting a putative replacement by immigrants. To this extent, this study illustrates that the near-panmictic $N. norvegicus$ populations may profound a temporal genetic variation in a local-scale level, demonstrating the presence of a non-inbreeding enhancement as a process of rare alleles transition. Although high inbreeding levels within temporal samples could affect the effective population size promoting low genetic differentiation among samples, the presence of non-breeders seems to have a greater local genetic impact than the dispersal of in-breeders. Nonetheless, the level of gene diversity was moderately high, implying either a degree of structuring, or an exchange of genes occurred in the past. Thus, taking into account the presently calculated mean generation interval of approximately 5.41 years for $N. norvegicus$, one could assume that at least one effective migrant each year might explain the recorded levels of pairwise genetic differentiation. It has been suggested [50] that a minimum of one and a maximum of ten migrants per generation would be the appropriate empirical rule for genetic conservation purposes.

The differential status with regards to sex was also profound in size classes. Pairwise comparisons of the mean length-at-size class showed that female individuals were smaller compared to males. This may be due to the different reproductive behavior of the species in question, resulting to decreased catchability of female individuals [51]. On the other hand, due to
the continuous breeding period [3] females’ adaptation possibly rests to their reproduction strategy rather than growth, thus resulting in lower growth rates [12, 41, 51]. The documented relative smaller size classes in females and the faster growth of males maybe represent a key-role differential strategy for the two sexes, whereas females tend to mature slower. Indeed, paternal generation interval is smaller compared to the maternal one, indicating a cryptic and complex social behavior. Nevertheless, the apparent movement of male individuals as stated in the genetic analyses might explain at some extent the differences in size classes between the two sexes. The differences in monthly mean carapace length between the two sexes

Table 3 Regression analysis of variance of carapace length against MLH, IR and HL for female and male N. norvegicus sampled individuals; significances are in italics

|          | MLH        | IR         | HL         |
|----------|------------|------------|------------|
| **Females** |            |            |            |
| Equation  | \( y = 33.30 + 4.053x \) | \( y = 37.34 − 4.042x \) | \( y = 37.39 − 4.188x \) |
| \( r^2 \) | 0.047      | 0.047      | 0.052      |
| \( F \)  | 4.870      | 4.870      | 5.36       |
| \( p \) value | 0.030     | 0.030      | 0.023      |
| **Males** |            |            |            |
| Equation  | \( y = 54.01 − 24.89x \) | \( y = 29.18 + 24.83x \) | \( y = 30.42 + 22.22x \) |
| \( r^2 \) | 0.352      | 0.352      | 0.306      |
| \( F \)  | 17.95      | 17.95      | 14.55      |
| \( p \) value | 0.001     | 0.001      | 0.001      |
were found to be statistically significant in most of the cases. Non-significance in January and October might be explained by the fact that the brooding period presented the highest peaks just before these 2 months [3].

Sea water circulation pattern of the Aegean Sea depicts a far eastern movement of surface currents along Chalkidiki Peninsula (see [52]), following the eastern Greek mainland coastline entering Pagasitikos gulf through the Trikeri Strait with several, throughout, inflow and outflow patterns (see [53]). Evoikos gulf communicates with the Aegean Sea through Oreoi Channel and is mainly associated with frequent and intense tidal water movements (Fig. 1). The overall oceanographic pattern facilitating larval movements through these areas for several marine species amplifies the precautions that should be taken into account, when recruitment and gene pool conservation strategies are implemented for *N. norvegicus*, besides specificities on fishing, spawning and feeding grounds/banks [54].

**Conclusions**

Conclusively, the northern/central Aegean Sea is subjected to a strong influence of more eutrophic waters compared to the southern Aegean, featuring higher zooplankton abundance. Richness of a number of bentthic species was negatively correlated with depth, partly reflecting the intense research activities in shallower waters and the poor scientific knowledge of the deeper ones [55]. In that sense, *N. norvegicus* uniformal genetic composition (background of high gene flow), could be attributed to potential population recolonization, due to a hypothesized passive larval movement from deeper waters, which may suggest that some offspring of local residents and potential male non-breeders from other regions admixture random. Norway lobster has relatively high FIS values in the study area, suggesting that potential populations of the central Aegean Sea need to be identified and to apply conservation measures. Considering the above along with the apparent absence of physical barriers, individuals of the species in question within the study area may be favored to recruit from an apparent nearby large population encountered in deeper waters as local fishermen claim.

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s40709-019-0103-0.

**Additional file 1:** Table S1. Number of individuals per gender and month. Figure S1. Factorial Correspondence Analyses for all individuals. Result suggest a single population.

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**Authors’ contributions**

GAG, DV and AE conceived the study, ITK, EMI provide with samples. EEM, GAG processes the labwork, GAG, AE, CST and MH ran the analyses. GAG, AE, DV an MH wrote the manuscript. AE and DV supervised the study. All authors read and approved the final manuscript.

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**Availability of data and materials**

The data have been presented with the article.

**Ethics approval and consent to participate**

Not applicable.

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Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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