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Genetic diversity, population structure, and demographic history of exploited sea urchin populations (*Tripneustes gratilla*) in the Philippines

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Abstract

The sea urchin *Tripneustes gratilla* is ecologically and economically important in the Indo-Pacific region. We use population genetic methods to investigate the population structure and historical demography of exploited populations in the Philippines. Sea urchins sampled in 6 localities in western Luzon and 4 outgroup sites were sequenced for mitochondrial cytochrome oxidase-1 gene (n = 282) and genotyped for seven microsatellite loci (n = 277). No significant genetic structure was found for either class of markers, indicating either extensive gene flow across the archipelago, or that populations have high genetic diversity and have not yet attained equilibrium between genetic drift and migration following large changes in demography. Interestingly, demographic inferences from the two types of markers were discordant. Mitochondrial lineages showed demographic expansion during the Pleistocene while microsatellite data indicated population decline. Estimates for the date of each event suggest that a Pleistocene expansion could have preceded a more recent population decline, but we also discuss other hypotheses for the discordant inferences.The high genetic diversity and broad distribution of haplotypes in populations that recently recovered from fishery collapse indicate that this species is very resilient over evolutionary timescales.

1. Introduction

The widely-distributed *Tripneustes gratilla* is a high-value sea urchin in the Indo-Pacific region. This ubiquitous herbivore occupies a key trophic position in nutrient cycling especially in tropical seagrass ecosystems (Koike, et al. 1987; Klumpp et al., 1993; Alcoverro and Mariani, 2002). *T. gratilla*, being an opportunistic grazer, also functions as a keystone species for coral reef and sea grass communities by controlling invasive macroalgae (Conklin and Smith, 2005; Stimson et al., 2007). Population outbreaks in some areas, however, have resulted in overgrazing of seagrasses (Eklöf et al., 2008) and foliose algae (Valentine and Edgar, 2010). This is also an economically-valuable sea urchin species and primarily collected for its high quality gonad – a specialty food item primarily in Japan (Lawrence and Agatsuma, 2001; Andrew et al., 2002). Many fisheries for this species have declined over time due to indiscriminate harvesting and lack of management measures which resulted to economic losses (i.e. Shimabukuro, 1991; Talaue-McManus and Kesner, 1995; Andrew et al., 2002). While these cases documented significant fisheries impacts on *T. gratilla* populations, they need to be placed into a broader spatial and temporal context. What does the regional population structure of the species look like? What is the demographic history of the species?

A population genetic approach can potentially provide these much needed insights for exploited invertebrate species (Thorpe et al., 2000). Molecular methods were initially used with marine species to better understand the effects of larval dispersal on the population structure (e.g. Waples 1987; Doherty et al., 1995) and determine the spatial scales of population connectivity (Palumbi, 2003; 2004; Hedgecock et al., 2007; Cowen and Sponaugle, 2009). Development of genetic markers and analysis have since extended the utility of genetic data to direct measurement of migration, estimation of effective population size, and examination of population demographic history (Emerson et al., 2001; Pearse and Crandall, 2004; Manel et al., 2005; Hellberg, 2009; Hare et al., 2011). To date, genetic assessments on *T. gratilla* populations have been limited to broad scale phylogeographic surveys of its genus (Lessios et al., 2003) and phylogenetic studies (Zigler and Lessios, 2003; Palumbi and Lessios, 2005). Mitochondrial sequence variation showed very weak regional divergence of *Tripneustes* populations across the Indo-Pacific region despite the significant local differentiation among the populations in this region (Lessios et al., 2003). This implies that *T. gratilla* in this region belongs to a large *Tripneustes* metapopulation (Lessios et al., 2003). The only finer-scale genetic survey on *T. gratilla* populations was carried out in western Luzon and eastern Philippines (Malay et al., 2002). This was conducted soon after the collapse of a local artisanal sea urchin fishery (i.e. Talaue-McManus and Kesner, 1995) to aid management of the heavily-exploited *T. gratilla* populations in northwestern Luzon (Malay et al., 2002). Based on six allozyme

markers, genetic differentiation of sea urchin populations within the western Luzon region was not significant, indicating extensive gene flow among populations (Malay et al., 2002).This lack of genetic structure of *T. gratilla* was initially explained by its potential for long-distance larval dispersal (Malay et al., 2002). This species has a relatively long planktonic larval duration of 20 to 52 days under culture conditions (Shimabukuro, 1991; Juinio-Meñez et al., 1998; Lawrence and Agatsuma, 2001). The planktonic stage or echinopluteus, though capable of movement using their ciliated bands (Emlet et al., 2006), is categorized as a weakly swimming larva and considered passive to the forces of oceanographic processes (Chia et al., 1984 cited in Weersing and Toonen, 2009). Thus, there is great potential for *T. gratilla* larvae to be dispersed over large distances before settlement. It is possible, though, that limited but significant genetic differentiation was not detected with the employed genetic marker, sampling design, or analysis (Ward, 2006). The availability of DNA markers for *T. gratilla* and recent developments in population genetics analysis presents an opportunity to re-examine the genetic variation in this exploited species.

Using multiple molecular markers, this study aims to obtain a more detailed characterization of *T. gratilla* populations in the Philippines to make inferences about population structure, effective size, and demographic history and gain insights on the vulnerability of the species to exploitation. Mitochondrial DNA has been noted for its relatively rapid development of population genetic structure due to its small effective population size. Based on this, it has become a preferred genetic marker over allozymes for initial examination of population differentiation (Bowen et al., in press). Given its non-recombining nature, mtDNA has been useful in gaining some insights on species evolutionary history and population demographic history based on maternal lineage (Avise et al., 1987; Liu and Cordes, 2004 but also see Ballard and Whitlock, 2004). On the other hand, microsatellites are inherited as codominant markers and thus provide insights on genetic differentiation of populations based on gene flow of both sexes (Liu and Cordes, 2004; Selkoe and Toonen, 2006). Highly polymorphic microsatellite loci also have the potential to reveal contemporary gene flow and effective population size (Ovenden et al., 2007; Saenz-Agudelo et al., 2011). The different characteristics of the DNA markers and new analytical approaches employed in this study should aid in elucidating historical and contemporary processes that influenced the current pattern of genetic variations in *T. gratilla*.

2. Materials and methods

2.1. Tissue collection and DNA extraction

Samples were collected from 6 sites along the western Luzon coast and 4 outgroup sites from other regions in the archipelago (Table 1). Only adult sea urchins with test diameter greater than 60 mm (Bangi, 2001) were collected on snorkel and tube feet and spines were sampled non-destructively. Individual tissue samples were kept in a vial and preserved in 90% ethanol. DNA was extracted from the tissue samples using a 10 % Chelex ^{TM} (Biorad) solution (Walsh et al., 1991).

2.2. DNA sequencing of the CO1 region

A region of the mitochondrial cytochrome oxidase subunit 1(CO1) gene was amplified using the universal forward primer: CO1f 5' CCTGCAGGAGGAGGAGAYCC and a *Tripneustes*-specific reverse primer CO1TR1 5'GGCATTCCAGCTAGTCCTARAAA (Lessios et al., 2003). PCR reactions were carried out in a final volume of 25-µl containing 1 µl genomic DNA extraction, 2.5 µl 10x PCR Buffer (PE-II), 2.5 µl of 8 mM dNTPs, 2.0 µl of 25 mM $MgCl_2$, 1.25 µl of each primer (10 mM), 0.125 µl of Amplitaq Gold polymerase (Applied Biosystems; 5 units/µl) and 14.5 µl of molecular grade water. The PCR temperature profile was as follows: initial denaturation for 7 min at 95^{o}C, 40 times of the main cycle: 30 s at 95^{o}C (denature), 30 s at 52^{o}C (anneal) and 1 min at 72^{o}C (extension), and 10 min of final elongation at 72^{o}C. After amplification, PCR products were run in 1% agarose gels stained with ethidium bromide to evaluate the quality and quantity of the amplified DNA. Excess dNTPs and primers were removed prior to cycle sequencing using an enzymatic method. Five (5) µl of the PCR product was incubated with 0.5 µl (0.5 U) of shrimp alkaline phosphatase and 0.5 µl (5 U) of exonuclease (GE Healthcare) for 30 min at 37^{o}C, 15 min at 80^{o}C, and 1 min at 25^{o}C. Purified DNA products were cycle sequenced in both directions using BigDye 3.0 terminator chemistry (Applied Biosystems, Foster City, CA). The cycle sequencing reaction was carried out in a 12-µl volume reaction: 2.5 µl of ABI 5x sequencing buffer, 0.5 µl of 10 mM primer, 0.5 µl BigDye, 0.5µl DMSO, 7.0 µl molecular grade water and 1 µl of cleaned PCR product. The sequencing program started with 25 cycles of 95^{o}C for 10 s, 50^{o}C for 5 s and 60^{o}C for 5 min and a final step of 5 min at 20^{o}C (Barber et al., 2006). Afterwards, the labelled DNA sequences were precipitated with ethanol, resuspended in formamide, and sent to Life Sciences Core Laboratories Center at Cornell University for sequencing on an Automated 3730 DNA Analyzer (Applied Biosystems, Inc.).

148

149 *2.3. Microsatellite PCR amplification and genotyping*

150 Seven out of 11 microsatellite loci developed for *T. gratilla* (Carlon and Lippé, 2007) were used
151 (Supplementary Table S1). The other four loci were excluded due to persistence of null alleles in all
152 subpopulations tested (Carlon and Lippé, 2007). PCR amplifications of three loci: Tgr-B11, Tgr-C117, and Tgr-
153 D134 were carried out in multiplex while the remaining four loci (i.e. Tgr-24, Tgr-A11, Tgr-C11, and Tgr-D5)
154 were individually amplified in separate PCR runs. The multiplex PCR consisted of 1 µl genomic DNA extracts,
155 1 µl 10x PCR buffer, 1 µl of 8 mM dNTPs, 1 µl of 1mg/ml Bovine Serum Albumin (BSA A7030 Sigma), 0.8 µl
156 of 25 mM MgCl2, 0.05 µl of Amplitaq polymerase (5 units/µl), 2.15 µl of molecular grade water, and the
157 forward and reverse primers (10 mM) of each locus, 0.25 µl Tgr-B11, 1.0 µl Tgr-C117, and 0.25 µl Tgr-D134
158 adding to a final volume of 10 µl.

159 For single-locus PCR, the 10-µl reactions contained 4.15 µl of molecular grade water, 0.5 µl of the
160 forward and reverse primers (10 mM) of a specific locus with same volume of the remaining reagents as
161 described above. The temperature profile of the PCR was the following: an initial denaturation at 94^{o}C for 7 min
162 followed by several cycles (38-40 times) of denaturing at 94^{o}C for 30 s, annealing at T_A for 30 s (Supplementary
163 Table S1), and elongation of 5 min at 72^{o}C. PCR products were then electrophorosed in 1% agarose gels,
164 stained with ethidium bromide, and examined under UV light.

165 For genotyping, microsatellite loci were divided into two pooling sets of PCR products. One pooling
166 set was a mixture of 0.5 µl PCR products of locus Tgr-24 and 1.0 µl of the multiplex PCR products. The other
167 set was comprised of 1.0 µl PCR products of locus Tgr-C11 and 0.5 µl PCR products of each locus, Tgr-D5 and
168 Tgr-A11. In each well of a 96-well plate, a set of pooled PCR products were combined with 9 µl Formamide-
169 GeneScan^{TM} 500 LIZ® (Applied Biosystems) mixture. The master mix solution was initially prepared in a
170 microcentrifuge tube by combining 985 µl of formamide and 15 µl of GeneScan^{TM} 500 LIZ®. The latter served
171 as internal molecular weight standard for allele calling. The PCR products were sent to Life Sciences Core
172 Laboratories Center at Cornell University and analyzed on Automated 3730xl DNA Analyzer (Applied
173 Biosystems, Inc.) with a fluorescent-based detection system.

174

175 *2.4 Sequence data analysis*

176 Electropherograms were visualized, proofread and compiled in Sequencher v4.8 (GeneCode, Ann
177 Arbor, MI) and the resulting sequences were aligned in MUSCLE (Edgar, 2004). Sequence data was collapsed

178 into unique haplotypes using the web tool, FaBOX (Villesen, 2007). A haplotype network was created in TCS
179 v1.21 (Clement et al., 2000) using a statistical parsimony procedure. A minimum spanning tree based on
180 pairwise differences was also generated in Arlequin v3.5 (Excoffier and Lischer, 2010) and visualized in
181 FigTree (Rambaut, 2009). To illustrate the relationships between unique haplotypes, the minimum spanning
182 haplotype tree was edited to reflect key results from TCS v1.21 (Clement et al., 2000). The dominant haplotypes
183 were also compared with sequences deposited in Genbank with BLASTN v2.2.24+ (Zhang et al., 2000) at the
184 nucleotide collection (nr/nt) database. A number of sequences from different localities were downloaded and a
185 neighbor-joining tree was generated using MEGA4 (Tamura et al., 2007) under nucleotide models of Log-Det
186 and Kimura 2-parameter (following Lessios et al., 2003) with 1000 bootstrap replicates.
187 Genetic diversity indexes were calculated for each population with Arlequin v3.5 (Excoffier and
188 Lischer, 2010): number of haplotypes (Nh), number of polymorphic sites (Np), haplotype diversity (h), and
189 nucleotide diversity (π). The same program was used to examine genetic differentiation among populations
190 using an analysis of molecular variance (AMOVA) based on haplotype frequency and sequence divergence (F_{ST}
191 and Φ_{ST}, respectively). Pairwise genetic distances (F_{ST} and Φ_{ST}) between populations were also calculated and
192 significance values were determined by performing 10000 permutations among the individuals between
193 population.
194 The relationship between genetic distance and geographical distance among populations was examined
195 using IBDWS v3.16 (Jensen et al., 2005). Mantel tests were performed with 10,000 permutations on genetic
196 distance obtained from Arlequin v3.5 and the measured geographical distances between localities determined
197 from Google Earth® as the shortest distance over the sea.
198 The sequence data were further analyzed for information on changes in the long-term effective
199 population size of *T. gratilla*. Fu's F_S (1997) generated in Arlequin v3.5 was examined to test for significant
200 departures from the neutral model. These analyses were performed for each population as well as for the pooled
201 Philippines sequences. In addition, a Bayesian skyline plot was generated in BEAST v1.5.3 (Drummond et al.,
202 2005) to estimate past population dynamics over time back to the most recent common ancestor of the gene
203 sequences. The program utilizes a standard Markov chain Monte Carlo (MCMC) sampling procedure in
204 estimating a posterior distribution of effective population size through time from sequence data given any
205 specified nucleotide-substitution model (Drummond et al., 2005). Due to computational constraints for a large
206 dataset, a subsample of 100 sequences randomly chosen from unstructured populations was analyzed on
207 processors provided by the Cornell Computational Biology Service Unit web-computing facility

208 (http://cbsuapps.tc.cornell.edu/beast.aspx). The subsampled dataset was run 6 times for 40 million steps to
209 ensure convergence under an HKY+G model of nucleotide substitution, strict clock model (fixed clock rate of
210 1.0), and linear skyline model (skyline groups = 5) with the default priors for model parameters and statistics as
211 specified in BEAUti (κ: Gamma prior [0.05, 40], initial = 1.0; α: Uniform [0, 1 000], initial = 0.5;
212 skyline.popSize: Uniform [0, ∞], initial = 0.0030). The mutation model for this sequence data was determined
213 through jModelTest v.0.1.1 (Posada, 2008) implementing Bayesian Information Criterion selection strategies.
214 Logfiles and treefiles from the replicate runs were examined for convergence in Tracer v1.5 and were combined
215 together using LogCombiner. The skyline plot was created in Tracer v1.5 from the combined treefiles and
216 logfiles (Rambaut and Drummond, 2009). The estimates from BEAST were converted into units of time and
217 effective population size using a lineage mutation rate of 1.4%, 1.75%, and 2.67% per million years (MY) as a
218 heuristic values based from studies on *Tripneustes* (Lessios et al. 2003), *Echinometra* (McCartney et al., 2000)
219 and *Protoreaster nodosus* (Crandall et al., 2012), respectively.
220
221 *2.5. Microsatellite analysis*
222 Chromatograms were examined in STRand v2.3 (Toonen and Hughes, 2001) to determine the fragment
223 sizes (alleles) of each locus per sample. All samples were checked manually and samples with ambiguous peaks
224 were not scored. Descriptive statistics including number of alleles, allelic richness (standardized to the smallest
225 sample size, n=29), observed (H_o) and expected (H_e) heterozygosity (Nei, 1978) were determined for each
226 population at each locus using FSTAT v. 2.9.3 (Goudet, 1995). For each locus and each sampled population, the
227 inbreeding coefficient (F_{IS}) was calculated and significance was estimated with 840 randomizations as
228 implemented in the same program. Genotypic linkage disequilibrium (LD) of all locus pairs and deviations from
229 Hardy-Weinberg equilibrium (HWE) of each locus in every population were tested using the web version of
230 Genepop v4.0.10 (Rousset, 2008). For the evaluation of HWE, data for each locus were initially analyzed by
231 testing for the general probability of departure from HWE. When a significant departure was found, the more
232 explicit hypotheses of heterozygote excess ($H_e<H_o$) and heterozygote deficit ($H_e>H_o$) were tested. In all tests,
233 parameters were set at 1000 dememorizations, 1000 batches, and 10000 iterations per batch. The classical one-
234 stage corrections for the false discovery rate (FDR) method (Verhoeven et al., 2005; Pike, 2010) were applied to
235 the p-values obtained in tests with multiple comparisons (i.e. HWE, LD, heterozygote deficits, and inbreeding
236 coefficient) to generate q-values. Micro-Checker v2.2.3 (Van Oosterhout and Hutchinson, 2004) was also used
237 to identify possible genotyping errors due to null alleles, large allele dropout or mis-scoring of stutter peaks.

238 Results of analysis in Micro-checker indicated the possible presence of null alleles in all populations at locus
239 Tgr-24, Tgr-A11, Tgr-B11; 4 populations at locus Tgr-D5; and 2 populations at locus Tgr-C11 and Tgr-C117.
240 Among these loci, Tgr-24 was the most likely to have null alleles based on the highest frequency of PCR failure,
241 significant departure from HWE in all populations, and highest level of inbreeding coefficient.Tgr-24 is thus
242 considered a deviant locus in this study and subsequent analyses were carried out without this locus.

243 Genetic differentiation among the sampled sea urchin populations was initially examined by testing the
244 significance of genotypic differentiation for all populations and all pairs of populations in Genepop v4.0.10 on
245 the web (1000 dememorizations, 1000 batches, and 10000 iterations per batch). In Arlequin v3.5, genetic
246 differentiation of populations was evaluated under the assumptions of unstructured AMOVA (or single group of
247 populations) and infinite allele model. Because high levels of heterozygosity can lower the maximum value of
248 F_{ST}, we also measured G'_{ST} and D_{EST}, which correct for this problem using SMOGD (Crawford, 2010). Another
249 test of population differentiation was carried out in BAPS v5 (Corander et al., 2008) which was based on a
250 Bayesian clustering method. This population mixture analysis requires input of the maximum number of
251 genetically diverged groups (K). In this analysis, clustering of groups of individuals was carried out using
252 several K values (1, 2, 3, 4, 5 and 6) and analyses were run three times for each K value. In addition we used the
253 Bayesian coalescent sampler Migrate v3.5.1 (Beerli and Palzewski, 2010) to test a model of genetic structure
254 (k=2, Guimaras vs. Northern Luzon sites) against no structure (k=1). We used a Brownian motion model of
255 mutation, and windowed, exponential priors on Θ (1×10^{-4} to 1×10^{3}) and m/μ (1×10^{-3} to 1×10^{4}). Each model
256 was run for 1 million steps, with 10,000 steps removed as burnin, and 10 heated chains ranging in temperature
257 from 1 to 1×10^{5}. Model selection was based on Bayes Factors calculated from the marginal likelihood of each
258 model, as estimated from a Bezier approximation to thermodynamic integration over the heated chains (see
259 Beerli and Palzewski 2010, Crandall et al., 2012). Based on unbiased genetic distance (Nei, 1978), a UPGMA
260 (unweighted pair-group method using arithmetic averages) tree of sampled populations was generated (1000
261 bootstraps) in TFPGA v1.3 (Miller, 1997). A Mantel test was carried out in IBDWS v3.16 (Jensen et al., 2005)
262 to test for a positive relationship between geographical distance (measured as shortest distance by sea) and
263 pairwise genetic differentiation obtained from Arlequin v3.5 based on 10000 random permutations.

264 To estimate contemporary effective population size of the *T. gratilla* population in Northwest Luzon,
265 we employed a method that utilizes the mean squared correlation in allele frequencies (i.e. linkage
266 disequilibrium) implemented in the LDNe software (Waples, 2006; Waples and Do, 2008). We combined data
267 from all localities except for Guimaras and Lucero (which showed a small amount of non-significant structure

with other localities), and only included alleles with a frequency greater than 0.02, which Waples and Do (2010) have shown to balance the precision provided by many alleles with the bias created by rare alleles. Confidence intervals were determined with a one-delete jackknife over loci.

To infer past demographic events from the microsatellite data, Bottleneck v1.2.02 (Piry et al., 1999) was used to determine whether the sampled populations have experienced a reduction in their effective population size. In principle, a severe reduction of effective population size results in a progressive reduction of number of alleles and heterozygosity at polymorphic loci. However, the number of alleles is reduced faster than the heterozygosity (H_e); hence, a transient excess in H_e is expected to characterize a bottlenecked population. To detect heterozygosity excess ($H_e > H_{eq}$), this program compares the expected heterozygosity (H_e) calculated from allele frequency and expected equilibrium heterozygosity (H_{eq}) (i.e. no bottleneck) derived from the number of alleles found in the samples. The comparison of these heterozygosity parameters (H_e and H_{eq}) is in the context of Nei's gene diversities (1978). Thus, this heterozygosity excess ($H_e>H_{eq}$) should not be confused with excess of heterozygotes ($H_e<H_o$) which compares the proportion of heterozygotes with expectations of Hardy-Weinberg equilibrium. The calculations were performed using the infinite allele model. Since there were fewer than 20 loci, Wilcoxon's test was used to determine the significance of the observed heterozygosity excess of the population (Piry et al., 1999). Bottleneck analyses excluding one or two and all of the putative loci with null alleles (i.e. Tgr-24, Tgr-A11, Tgr-B11) using the original and Brookfield corrected data were also carried out.

3. Results

3.1. Genetic diversity

Mitochondrial CO1 sequences were obtained from 282 individuals of *T. gratilla* sampled from 10 sites. The 605-bp sequences were aligned without indels and collapsed into 79 unique haplotypes characterized by 69 polymorphic sites (Supplementary Table S2; GenBank accession numbers: **JX661089-JX661167**). Most of these haplotypes occurred only once (57 singleton or 72% of the total unique haplotypes). Based on overall frequency, the haplotypes found to be dominant were sequence 1 (34.8%), 10 (11.7%), and 3 (9.3%) while the rest of the haplotypes occurred less often (<2.5%). The minimum spanning tree revealed three star-like polytomies with the dominant haplotypes separated by only one nucleotide difference (Fig. 1). All haplotypes were closely related as they differed by only 1 to 10 unique mutations. The most frequent and broadly distributed haplotype 1 was also identified by TCS v1.21 (Clement et al., 2000) as the most probable ancestral haplotype. A neighbor-joining tree generated from a number of sequences from Lessios et al. (2003) showed

298 that the dominant haplotypes in this present study were also shared with other populations from different parts
299 of the world (Fig. 2). Interestingly, the most dominant haplotype 1 was shared by *T. gratilla* and *Tripneustes*
300 *depressus* populations from several localities across the Indian and Pacific Oceans (Fig. 2). Haplotype 1 was
301 also the most common haplotype in the global phylogeographic survey comprising about 18% of the pooled
302 sample of *T. gratilla* and *T. depressus* (Lessios et al., 2003).

303 Geographical distribution and relative frequency of unique haplotypes are shown in Fig. 3. The three
304 dominant haplotypes comprised the major proportion of the samples in all sites except Burgos where haplotype
305 3 was not sampled. Minor haplotypes were also shared by 2 to 6 populations. Private or site-specific haplotypes
306 were present in each population but comprised a small proportion of the samples (range 9.5 to 23.3%). In each
307 sampled population, the number of haplotypes ranged from 12 to 20 and number of polymorphic sites ranged
308 from 12 to 24 (Table 2) indicating high degree of genetic diversity. Overall, the haplotype diversity was high
309 (mean $h = 0.8554 \pm 0.0592$) while nucleotide diversity was low (mean $\pi = 0.0031 \pm 0.0005$) (Table 2).

310 A total of 277 sea urchins from 6 localities were genotyped at seven microsatellite loci (Supplementary
311 Table S3) but a number of individuals (no more than 12 per locus) had missing data at some loci due to
312 technical causes (i.e. PCR failure, ambiguous peaks). There was no significant linkage disequilibrium after
313 adjustment for multiple comparisons indicating that none of the loci were physically linked. Overall, there were
314 153 alleles found in all loci ranging from 9 to 41 alleles per locus. When averaged across populations, the
315 number of alleles per locus ranged from 7 to 28 alleles (or 6 to 24 alleles in terms of allelic richness). There
316 were 27 private alleles occurring at low frequency (0.010 to 0.021) across the sampled localities. High genetic
317 diversity is indicated by the mean and total expected heterozygosities of each locus ranging from 0.658 to 0.952
318 and 0.663 to 0.952, respectively.

319 There were significant discrepancies in the observed and expected heterozygotes per locus averaged
320 across the populations, indicating a high level of deviation from Hardy-Weinberg equilibrium (HWE). Out of 42
321 single locus tests per population, 27 (64.3%) did not conform to the expectations of HWE (Probability test,
322 $p<0.05$, Supplementary Table S3). Further evaluation of HWE showed that majority of the populations that were
323 not in equilibrium had deficits in the number of heterozygotes (34 out of 42 tests were significant) and none had
324 heterozygote excesses (data not shown). In all sampled populations, more than half of the microsatellite loci
325 used in the study had heterozygote deficiency (Supplementary Table S3). Locus Tgr-24 was the most notable for
326 its significant HWE departure and heterozygote deficiency in all populations. Likewise, Tgr-A11 and Tgr-B11
327 also exhibited high degree of HWE deviation. The remaining four loci had fewer departures from HWE and

were at equilibrium in at least three sites. Null alleles have been identified as the most probable technical cause of observed heterozygote deficit for these microsatellite loci as also reported in the primer note (Carlon and Lippé, 2007). As we found no evidence for nulls at 4 loci and estimates of F_{IS} were positive at all loci, the presence of null alleles can only partly explain the HWE departures observed in *T. gratilla* populations and other processes (e.g. selection, demographic effects) may still have significant role in reducing heterozygosity.

3.2. Population genetic structure

Analysis of molecular variance (AMOVA) based on mitochondrial sequence data found no significant population differentiation among population within western Luzon (F_{ST}=-0.004, p=0.696 and Φ_{ST} =-0.004, p=0.727). Genetic differentiation among sea urchin populations sampled across the archipelago were also not significant (F_{ST}=-0.003, p=0.736 or Φ_{ST}=-0.003, p=0.767). Pairwise F_{ST} values were less than 0.02 and none were significant (data not shown). The genetic and geographic distance were not positively correlated (Z=-69.6316, r=-0.1081, Mantel test, p= 0.7202).

Similar results were found in the analysis of microsatellite data. The fixation index values were also very low (F_{ST}<0.01) and genetic differentiation among populations were not significant (AMOVA, global F_{ST}=0.001, p=0.719). Per-locus estimates for G'_{ST} and D_{EST} were all below 0.02 (Supplementary Tables S4 and S5).The UPGMA dendrogram also showed that the clustering of *T. gratilla* populations did not conform to the expected grouping based on geographical distance between locations (Supplementary Fig. S1). This is consistent with a lack of correlation between genetic and geographic distance (Z=1.4041, r=-0.3958, Mantel test, p=0.8591). For example, Lucero and Victory populations were not clustered together despite their proximity of less than 10 km apart. Further analyses also showed that genotypic differentiation for all populations and pair-wise population comparisons were not significant (Fisher's method: Chi^2=6.072, p=0.965). The Bayesian approach employed in BAPS v5 was also not able to partition the sampling localities. A model with a single population (k=1) had a higher marginal likelihood value (logML=-8067.51) than the rest of population models tested with probability values ranging from -8224.19 to -8916.66. Similarly, the k=1 model in Migrate had a much higher marginal likelihood (-132,118) than a k=2 model (-775,726), although both models showed good convergence as evaluated by effective sample sizes greater than 400.

358 *3.3. Demographic history inferences*

359 Fu's F_S values were significantly negative (Table 2, p=0.0001) in all sites, indicating an excess of rare
360 haplotypes and the rejection of the null model of neutral evolution. Similarly, the Bayesian skyline plot revealed
361 population growth in local sea urchin populations (Fig. 4). The abrupt increase of effective population size was
362 within the range of 30000-55000 years ago (median time) assuming a lineage mutation rate maximum at 2.67%
363 and minimum at 1.4%, respectively. Long-term coalescent N_e was estimated to range between 0.6 and 13.5
364 million effective females depending on which mutation rate was used. LDNe estimated contemporary effective
365 size for Northwest Luzon to be 6535.5 from six microsatellite loci, with a lower bound of 448.4 and an infinite
366 upper bound. This infinite upper bound is consistent with the absence of significant linkage disequilibrium
367 found by Genepop (Waples and Do, 2010). Bottleneck analysis of the microsatellite data revealed an excess in
368 expected heterozygosity, which indicates a decline in the effective population size of *T. gratilla*. Expected
369 heterozygosity (H_e) was generally found to be higher than the expected equilibrium heterozygosity (H_{eq}) in 33
370 out of 42 comparisons (Table 3) and the multi-locus statistical test showed that the observed heterozygosity
371 excess was significant in all sampled populations (Wilcoxon test, p=0.008-0.016, Table 3). Analyses that
372 excluded Tgr-A11 or Tgr-B11, which were also suspected with null alleles, still showed significant
373 heterozygosity excess in all populations (data not shown). In addition, this result stayed highly significant when
374 genotype data were corrected for null-alleles (Brookfield, 1996) and with Tgr-24 re-added to the dataset
375 (Supplementary Table S6).

376

377 **4. Discussion**

378 *4.1. Non-equilibrium populations and absence of genetic structure in the Philippines*

379 Western Luzon populations of *T. gratilla* were found to be genetically homogeneous and not
380 significantly distinct from populations in other regions of the Philippines. These results from seven
381 microsatellite loci and a mitochondrial locus are consistent with the findings of the previous genetic survey that
382 used allozyme markers (Malay et al., 2002). The observed genetic homogeneity of *T. gratilla* populations along
383 the western Luzon coasts is consistent with its life history features that predict long-distance larval dispersal.
384 The low and non-significant F_{ST} values ($F_{ST} < 0.02$) obtained for this species indicate the persistence of enough
385 gene flow among the sampled populations of about 10 or more migrants per generation (Lowe and Allendorf,
386 2010). Based on a passive particle dispersal model in the Western Luzon region (Bernardo, 2011), the estimated
387 range of dispersal distance for *T. gratilla* larvae was about 116 to 1060 km after 52 days depending on source

388 location and season (L. Bernardo, unpubl data). Considering that *T. gratilla* is a year-round spawner (Tuason,
389 1980), larvae can be effectively supplied over a wide range of oceanographic conditions (e.g. Addison et al.,
390 2008), exposing them to current vectors that may move in opposite directions depending on the monsoon
391 seasons (e.g. Juinio-Meñez and Villanoy, 1994), thereby further enhancing the extent of larval exchange within
392 the region.

393 The lack of genetic structure across the archipelago, however, could not be entirely explained by
394 contemporary oceanographic processes. This is in contrast with the findings of other local genetic studies in the
395 Philippines on marine species with biphasic life-histories that revealed broad to fine-scale genetic structure
396 within the archipelago that conformed with current patterns or biogeographic divisions (e.g. *Linckia laevigata* -
397 Juinio-Meñez et al., 2003; *Siganus fuscescens* - Magsino and Juinio-Meñez, 2008; Ravago-Gotanco and Juinio-
398 Meñez, 2010). Perhaps a better explanation for the absence of detectable genetic structure in our data is that the
399 underlying structure has been obscured by non-equilibrium processes such as the demographic changes
400 described below (Excoffier et al., 2009; Marko and Hart, 2011). Based on the large effective population size
401 estimated from microsatellites for the Northwest Luzon population (N_e = 6535.5), and assuming an ecologically
402 high rate of gene flow (m = 0.1), we estimate that it would take ~4700 generations for F_{ST} values to move
403 halfway to their equilibrium value following any of these demographic changes (Crow and Aoki 1984).The
404 inferred absence of equilibrium between gene flow and genetic drift is initially evidenced by the lack of
405 significant correlation between geographic and genetic distance for both markers (Slatkin, 1993). This is further
406 supported by the observed sharing of the dominant mitochondrial haplotypes with samples from Reunion Island
407 in Western Indian Ocean and even with its congeneric species, *T. depressus* in the Galapagos Islands in the
408 eastern Pacific (Lessios et al., 2003). This lack of divergence across the Eastern Pacific Barrier and the
409 incomplete sorting of the mitochondrial lineage for the genus *Tripneustes* indicate the persistence of genetic
410 patterns shaped by evolutionary events in the past (Benzie, 1999; Lessios et al., 2003).

411 For the microsatellite data, significant departures from Hardy-Weinberg equilibrium due to
412 heterozygote deficiency also substantiate the non-equilibrium state of *T. gratilla* populations. Heterozygote
413 deficiency has been associated primarily with ecological processes such as inbreeding, recent admixture,
414 selection, or accumulation of genetically distinct cohorts (e.g. Watts et al., 1990; Addison and Hart, 2004; van
415 Oppen et al., 2008). Considering that populations in this study were genetically homogeneous and have high
416 inbreeding coefficients without linkage disequilibrium, the most probable explanation for the heterozygote
417 deficiency would be the significant genetic differentiation among cohorts or the temporal Wahlund effect (i.e.

418 Watts et al., 1990). This has been suggested to be a consequence of large variance in reproductive success or
419 differential survival during the planktonic larval stage or immediately after settlement at early benthic stage
420 (Watts et al., 1990; Hedgecock, 1994; Hedgecock et al., 2007; Hedgecock et al., 2011).This hypothesis, also
421 known as sweepstakes reproductive success, suggests that this large reproductive variance is due to asynchrony
422 between oceanographic and biological conditions that may influence larval development, dispersal, and
423 recruitment (Hedgecock, 1994; Hedgecock et al., 2007, Hedgecock et al., 2011). *T. gratilla* is predisposed to
424 large variance in reproductive or recruitment success based on its life history features (i.e. high fecundity,
425 broadcast spawning, long planktonic larval duration), patchy distribution, and dynamic habitat (Shimabukuro,
426 1991; Lawrence and Agatsuma, 2001). In support of this idea, preliminary genetic analysis of *T. gratilla* recruits
427 and adults sampled from local populations in Santiago Island, Bolinao indicate genetic variability among
428 cohorts (Casilagan, 2011). Overall, these non-equilibrium results at the archipelagic scale are in agreement with
429 the chaotic population structure and absence of equilibrium that has been found across the entire species range
430 (Lessios et al., 2003).
431
432 *4.2. Discordance in demographic trends inferred by mtDNA and microsatellites*
433 Estimates of long-term coalescent effective population size from the mtDNA data were orders of
434 magnitude larger than estimates of contemporary effective size based on linkage disequilibrium in the
435 microsatellite data. This is unsurprising, because the two markers and methods are estimating over very different
436 temporal and spatial scales. The mtDNA data integrates over the coalescent history of the genetic sample, and
437 therefore reflects the population size of the global *T. gratilla* population, which may include the entire Indo-
438 Pacific, as indicated by the vast ranges of shared haplotypes in Fig. 2 (Lessios et al., 2003). On the other hand,
439 linkage disequilibrium is a transient phenomenon that occurs among loci, and N_e estimates from the
440 microsatellite data reflect only the effective number of parents from which the current sample was drawn. Thus,
441 this estimate represents the effective size of the breeding population in Northwest Luzon in 2007-2008. The
442 linkage disequilibrium method has been shown to be robust to migration from outside the sampled region unless
443 the percentage of migrants from outside is greater than 5%-10% (Waples and England, 2011).
444 Similarly, demographic analyses of mitochondria and microsatellite data suggested contrasting
445 demographic events in the history of *T. gratilla* populations in the Philippines. The mitochondrial lineages
446 indicated population expansion while microsatellite data exhibited genetic signatures of a population decline. In
447 particular, the highly negative Fu's F_S, star-like haplotype network, high haplotype diversity and low nucleotide

448 diversity provided initial evidence of demographic expansion in the mtDNA. As shown by the Bayesian skyline
449 plot, the initiation of the abrupt population growth occurred during Pleistocene. Demographic expansion during
450 the Pleistocene also been reported for other marine taxa in the Indo-Pacific region such as gastropods (Crandall
451 et al., 2008a), echinoderms (Crandall et al.,2008b; Kochzius et al., 2009), crustaceans (Benzie et al., 2002), and
452 fish (Rohfritsch and Borsa, 2005; Liu et al., 2007; Ravago-Gotanco and Juinio-Meñez, 2010). The Pleistocene
453 era was characterized by changing sea levels and temperatures due to glaciations and deglaciations (Roy et al.,
454 1996; Rohling et al., 1998; Siddall et al., 2003). Particularly in Southeast Asia, sea levels were reduced to more
455 than 120 m below the present levels (Voris, 2000) which would have exposed reef flats causing local extinction
456 especially of species in shallow habitats. Following the Last Glacial Maximum (~20000 years BP), flooding of
457 the shelves provided newly-available habitat for re-colonization and subsequent population expansion (e.g.
458 Crandall et al., 2008a, 2008b; Ravago-Gotanco and Juinio-Meñez, 2010). The populations of *T. gratilla*, being a
459 shallow-water echinoid, were likely been influenced by these historical events.

460 On the other hand, microsatellite data indicate demographic decline in *T. gratilla* populations.
461 Significant heterozygosity excess was observed in most loci in all populations, and indicates a deficiency in
462 alleles that characterizes bottlenecked populations. It might at first be thought that, because microsatellites
463 evolve relatively faster than the mitochondrial markers, the bottleneck might be of anthropogenic origin,
464 associated with the recent collapse in the *T. gratilla* fishery (Talaue-McManus and Kesner, 1995). However, the
465 method used here detects bottlenecks that happened around 0.2-4.0 N_e generations ago (Cornuet and Luikart,
466 1996). Based on our estimates of contemporary effective size, this means that the bottleneck could have
467 occurred between 90 to more than 26,000 generations ago or similar values in years ago, assuming 1 year
468 generation time (Bangi, 2001; Lawrence and Agatsuma, 2001). This estimate suggests that the bottleneck
469 detected by the microsatellites cannot be attributed to the most recent collapse of the *T. gratilla* fishery, which
470 occurred in the 1990's, but might be attributed to earlier undocumented anthropogenic pressure, or else to
471 environmental changes during the late Pleistocene.

472 The seemingly discordant inferences of a population expansion in mtDNA and a bottleneck in
473 microsatellites can be reconciled in a number of ways. First, microsatellites are better suited for detecting
474 population declines than they are for detecting expansions (Cornuet and Luikart, 1996), so both inferences may
475 be correct. Given the rough range of dates that we inferred for each type of marker (expansion at 30-55 kya in
476 mtDNA, contraction at 0.009 – 26 kya in microsatellites), it is probable that a population decline detected by the
477 microsatellites occurred after the expansion detected in the mtDNA. Second, due to lack of recombination, it is

478 also possible that the mitochondrial genome had undergone a selective sweep or introgression (Ballard and
479 Whitlock, 2004). The sharing of dominant haplotypes among Indo-Pacific *T. gratilla* and eastern Pacific *T.*
480 *depressus* suggests recent introgression among these species, especially since a recent global survey of
481 *Tripneustes* using microsatellite markers revealed that *T. gratilla* can be genetically distinguished from the *T.*
482 *depressus*at nuclear loci (Carlon, pers comm). Hence, the observed genetic signature of population growth could
483 also be a consequence of selective sweep following introgression. Finally, it is possible that departures from the
484 neutral model detected in both mtDNA sequence and microsatellites could be the result of purifying selection.
485 While the mtDNA and microsatellite variation that we measured is putatively neutral, selection could have
486 occurred on nearby linked genes (i.e. background selection). Background selection has been shown to produce
487 patterns similar to population growth in sequence data (Fu, 1997) as well as to cause a loss of rare alleles that
488 might indicate a bottleneck in the microsatellite data (Charlesworth et al., 1993). Discordant inferences between
489 mtDNA and microsatellites have been noticed in a number of other marine taxa as well, and likely have to do
490 with the large variance in genealogies that is possible in species with large effective population sizes (DiBattista
491 et al., 2012). To disentangle the signals of historical processes related to demographic fluctuation from those of
492 selective processes, it might be necessary to obtain sequence data from multiple nuclear regions (e.g. Calderón
493 et al., 2008)
494
495 *4.3. Conservation implications*
496 Conservation of *T. gratilla* as a species is a relevant concern because it is the top target echinoid
497 species for commercial harvesting in the tropical and subtropical region. In this context, identification of
498 evolutionary significant units (ESUs, Moritz, 1994) and evaluating species vulnerability would be important.
499 The genetic analysis did not reveal distinct ESUs or indications of cryptic speciation based on the absence of
500 deep phylogenetic divergence. Based on the species' wide distribution range and the estimates of population
501 genetic diversity and variability (i.e. high genetic diversity, broad distribution of major haplotypes, and
502 extensive genetic exchange), the vulnerability of the species to extinction is low. Our data, however, also
503 suggest that populations in the Philippines have undergone large demographic fluctuations in the past, perhaps
504 similar to the anthropogenic changes that we are seeing now. Although recent recovery of overharvested
505 populations was observed (Juinio-Meñez et al., 2008), the persistent harvesting and habitat degradation still pose
506 a serious threat of local depletion. With the absence of genetic structuring particularly in western Luzon region,
507 a precautionary approach to management (e.g. McCook et al., 2009) should be practiced with a degree of

508 "spatial bet-hedging" (sensu Larson and Julian, 1999). This would entail region-wide management interventions
509 to protect adequate spawning stocks and ensure reliable recruitment in the localities. This can be carried out, for
510 example, by establishing network of marine protected areas and sea urchin grow-out cage culture sites along the
511 western Luzon coasts which serves as reproductive reserves and recruitment sites (Malay et al., 2002; Juinio-
512 Meñez et al., 2008; 2009). Consideration of genetic impacts is also still vital especially in implementing the
513 culture-based management interventions such as the release or grow-out culture of hatchery-produced juveniles
514 (Ward, 2006). As with all fisheries species, hatchery-based supportive breeding efforts should aim to maximize
515 genetic diversity in the captive population released into the wild (Ryman and Laikre 1991). Integration of these
516 insights with other information (i.e. recruitment patterns, demographic data) would facilitate the development of
517 an effective management scheme that would ensure sustainability of the *T. gratilla* fishery.
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829 **Fig. 1** Minimum spanning tree for the CO1 haplotypes of *T. gratilla*. Filled circle represents a unique haplotype
830 which was sized proportionally to its absolute frequency, ranging from 1– 98. The dominant haplotypes were
831 identified by their sequence number. The asterisk denotes the most probable root haplotype while the square
832 represents unsampled haplotypes as revealed by TCS v1.21 (Clement et al. 2000).

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834 **Fig. 2** Neighbor-joining tree illustrating the relationship of the major CO1 haplotypes in this study and the CO1
835 sequences from Lessios et al. (2003) based on LogDet substitution model. Numbers next to nodes indicate
836 bootstrap support from 1000 iterations and nodes with less than 50% support have been collapsed.

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838 **Fig. 3** Relative frequency of mitochondrial haplotypes per population. Represented in the pie graph are the
839 major haplotypes (sequence 1, 10, and 3), minor haploytpes (pooled haplotypes with frequency of 2 to 7), and
840 private haplotypes (haplotypes found in only one site).

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842 **Fig. 4** Bayesian skyline plots of effective population size (N_e) scaled by generation time for CO1 mitochondrial
843 DNA. The plots run from the present to their median time to most recent common ancestor (T_{MRCA}). Grey dotted
844 lines represent the 95% CI for Net.

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Table 1
Sampling location, population code, collection dates, and the number of individuals (n) screened at mitochondrial (CO1) and microsatellite markers.

				Mitochondrial		Microsatellite	
Sampling sites	Lat (N)	Long (E)	Population Code	Collection date	n	Collection date	n
Study Region: Western Luzon							
Bubon, Burgos, Ilocos Norte	18°30'31"	120°34'45"	Burgos (BUR)	Nov-08	29	Nov-08	34
Dadalaquiten, Sinait, Ilocos Sur	17°53'34"	120°26'28"	Sinait (SIN)	Dec-08	28	*n.d.*	*n.d.*
Lucero, Bolinao, Pangasinan	16°24'09"	119°54'30"	Lucero (LUC)	Nov-08	21	Nov-09	50
Victory, Bolinao, Pangasinan	16°23'35"	119°57'59"	Victory (VIC)	Nov-08	24	Nov-09	49
Panglit Is., Masinloc, Zambales	15°29'42"	119°55'09"	Masinloc (MAS)	Apr-09	30	Apr-09	48
Matuod, Lian, Batangas	13°59'11"	120°37'40"	Lian (LIA)	Feb-09	30	Feb-09	47
Outgroup Sites							
Bacon, Sorsogon	13°02'24"	124°02'56"	Sorsogon (SOR)	Jul-08	21	*n.d.*	*n.d.*
Lawi, Jordan, Guimaras	10°32'45"	122°31'16"	Guimaras (GUI)	Jan-09	47	Dec-09	49
Cantaan, Guinsiliban, Camiguin	09°06'25"	124°48'14"	Camiguin (CAM)	Mar-09	32	*n.d.*	*n.d.*
Simunul, Tawi-Tawi	04°54'03"	119°50'56"	Tawi-tawi (TAW)	May-09	20	*n.d.*	*n.d.*

Table 2
Summary statistics of *Tripneustes gratilla* populations based on CO1 sequences. Population, sampling size (n), measures of genetic diversity (Nh = no. of haplotypes; Np = no. of polymorphic sites; h = haplotype diversity; π = nucleotide diversity; s.d. = standard deviation), and Fu's neutrality test (Fu 1997). All F_S values were found to be significant (p=0.0001) indicating deviation from neutral equilibrium model.

Population	**n**	**Nh**	**Np**	**h (s.d.)**		**π (s.d.)**		**Neutrality test (F_S)**
Burgos	29	13	15	0.7660	(0.0816)	0.0030	(0.0020)	**-7.851**
Sinait	28	15	16	0.9101	(0.0370)	0.0033	(0.0022)	**-10.583**
Bolinao	45	20	21	0.8737	(0.0415)	0.0030	(0.0020)	**-17.172**
Lucero	21	11	11	0.9143	(0.0380)	0.0029	(0.0020)	
Victory	24	14	16	0.8333	(0.0767)	0.0031	(0.0020)	
Masinloc	30	13	12	0.7678	(0.0749)	0.0022	(0.0015)	**-10.235**
Lian	30	15	21	0.8736	(0.0498)	0.0035	(0.0022)	**-9.610**
Sorsogon	21	12	13	0.9095	(0.0479)	0.0036	(0.0023)	**-6.915**
Guimaras	47	18	23	0.8372	(0.0403)	0.0026	(0.0018)	**-14.628**
Camiguin	32	17	24	0.8347	(0.0646)	0.0036	(0.0023)	**-12.468**
Tawi-tawi	20	13	13	0.9263	(0.0431)	0.0033	(0.0021)	**-9.810**

Table 3
Bottleneck analysis on *Tripneustes gratilla* microsatellite data from 6 loci (without Tgr-24) under the assumption of infinite allele model. Asterisk (*) indicate heterozygosity excess (H_e>H_{eq}) based on the comparison of expected heterozygosity (H_e) and expected equilibrium heterozygosity (H_{eq}) of each locus per population. Wilcoxon's test determined the significance of heterozygosity excess across loci in a population. All p-values were significant ($p<0.05$).

Population	Tgr-A11	Tgr-B11	Tgr-C11	Tgr-C117	Tgr-D134	Tgr-D5	Wilcoxon's test (p-value)
Burgos	*	*	*	*	*	*	**0.008**
Lucero	*	*	*	-	*	*	**0.008**
Victory	*	*	*	-	*	*	**0.016**
Masinloc	*	*	*	*	*	*	**0.008**
Lian	*	*	*	*	*	*	**0.008**
Guimaras	*	-	*	*	*	*	**0.016**

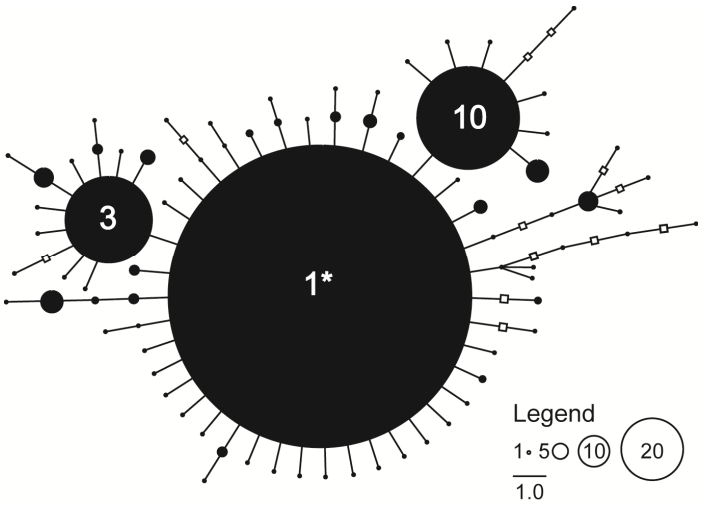

Fig. 1 Minimum spanning tree for the CO1 haplotypes of *T. gratilla*. Filled circle represents a unique haplotype which was sized proportionally to its absolute frequency, ranging from 1– 98. The dominant haplotypes were identified by their sequence number. The asterisk denotes the most probable root haplotype while the square represents unsampled haplotypes as revealed by TCS v1.21 (Clement et al. 2000).

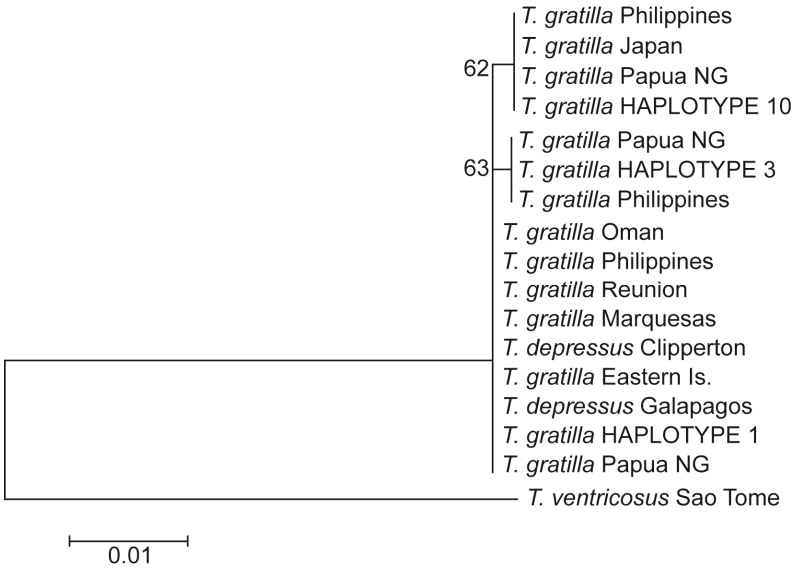

Fig. 2 Neighbor-joining tree illustrating the relationship of the major CO1 haplotypes in this study and the CO1 sequences from Lessios et al. (2003) based on LogDet substitution model. Numbers next to nodes indicate bootstrap support from 1000 iterations and nodes with less than 50% support have been collapsed.

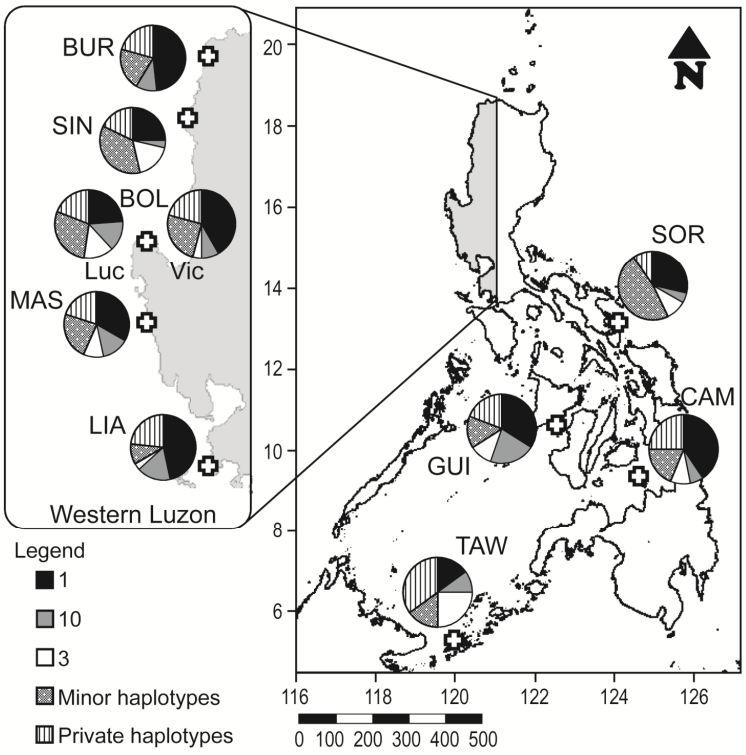

Fig. 3 Relative frequency of mitochondrial haplotypes per population. Represented in the pie graph are the major haplotypes (sequence 1, 10, and 3), minor haploytpes (pooled haplotypes with frequency of 2 to 7), and private haplotypes (haplotypes found in only one site).

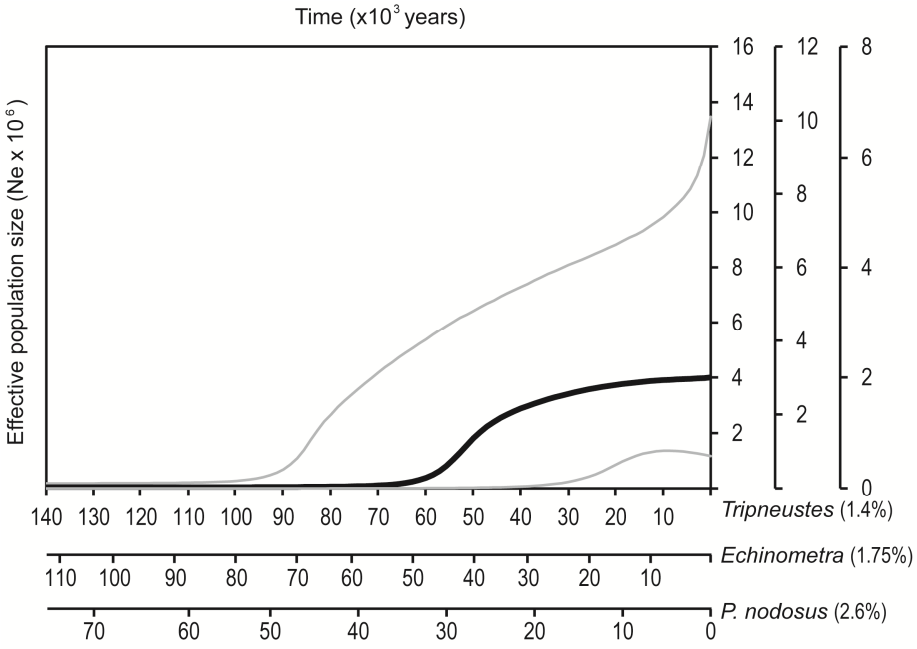

Fig. 4 Bayesian skyline plots of effective population size (N_e) scaled by generation time for CO1 mitochondrial DNA. The plots run from the present to their median time to most recent common ancestor (T_{MRCA}). Grey dotted lines represent the 95% CI.