Microsatellite markers developed using a next generation sequencing technique for *Neotrogla* spp. (Psocodea: Prionoglarididae), cave dwelling insects with sex-reversed genitalia

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Abstract

The genus *Neotrogla* (Psocodea: Prinoglarididae) comprises four named species from Brazil. Females of this cave-dwelling insect are characterized by a conspicuous penis-like intromittent organ, termed a gynosome, which is inserted into the vagina-like male genitalia during copulation. Another evolutionarily novel structure, the spermathecal plate, enables a female to simultaneously store two freshly deposited spermatophores (consisting of sperm and possibly nutritious substances) in her sperm storage organ (spermatheca). It is unknown whether the two spermatophores are derived from two different males. To investigate the mating ecology and population genetic structures of these insects with sex-reversed genitalia, 16 novel highly polymorphic microsatellite loci were isolated and characterized based on ~2,275 Mbp genomic sequences from an undescribed *Neotrogla* species. Our first screening detected 99,888 candidate loci. Similar to other hemipteroid insects studied thus far, AAT motif microsatellites were conspicuously dominant. We further screened 99 sequences, for which 50 pairs of PCR primers were successfully designed. Sixteen of these primers successfully amplified products of the expected size in the 11 *Neotrogla* sp. individuals collected from two caves. The number of alleles per loci varied from two to nine, with no significant deviation from Hardy–Weinberg equilibrium in either population. Although the caves sampled were only approximately 1 km apart, significant genetic differentiation was detected between the two populations. In total, 13, 12, 13, and 11 loci were cross-amplified in *N. aurora*, *N. brasiliensis*, *N. curvata*, and *N. truncata*, respectively, indicating the applicability of these microsatellite loci for metapopulation genetic studies in multiple *Neotrogla* species.

Key words: cave populations, genetic differentiation, nuptial gift, sex role reversal, simple sequence repeat (SSR)

INTRODUCTION

The cave insect genus *Neotrogla* (Psocodea: Prinoglarididae) comprises four named species from Brazilian caves (Lienhard et al. 2010; Lienhard & Ferreira 2013). Female insects of this genus are characterized by a conspicuous penis-like intromittent organ, termed a gynosome, which is inserted into the male genitalia during copulation (Lienhard et al. 2010; Lienhard & Ferreira 2013; Yoshizawa et al. 2014). The male genitalia or "vaginas," are relatively simple structures, but possess multiple species-specific pouch-like invaginations, which accommodate the elaborate spines of the gynosome (Yoshizawa et al. 2014). The correlated male and female genital morphologies result in a firm coupling of mating pairs, and copulation lasts ~40–70 h (at least for *N. curvata* Lienhard & Ferreira), during which voluminous materials are transferred to the female sperm storage organ, the spermatheca, to form gigantic spermatophores (Yoshizawa et al. 2014). The spermatophore is formed as a hardened capsule, and females usually possess multiple (up to 11 in *N. brasiliensis* Lienhard) empty capsules in the spermatheca (Yoshizawa et al. 2014). Thus, female *Neotrogla* likely engage in polyandrous mating to obtain multiple spermatophores, not only for fertilization but also as nutrition. Interestingly, *Neotrogla* spp. females have evolved another specialized structure, the spermathecal plate, on their spermatheca. This organ enables females to store two freshly deposited spermatophores simultaneously (Yoshizawa et al. 2014), whereas the females of other psocids (e.g., *Lepinotus patruelis* Pearman) can store only one undigested spermatophore at a time (Wearing-Wilde 1995).

As *Neotrogla* inhabit nutritionally poor cave environments, the evolutionarily novel female structures, the gynosome and spermathecal plate, likely represent

adaptations to obtain as many nuptial gifts from males as possible in an efficient manner. To investigate the mating system of *Neotrogla* underlying the evolution of their sex-reversed genitalia, parentage analysis by means of highly polymorphic genetic markers is indispensable. There are two possible functions of the spermathecal plate. A female may coerce her mating partner to transfer a large volume of ejaculate corresponding to two fresh spermatophores by holding him with the gynosome for a long period of time. A second possibility is that males may provide only one spermatophore at a single bout of copulation, and females may mate with multiple males in succession to obtain more spermatophores. To discriminate between these two possibilities, analyzing the paternal DNA of spermatophores offers a promising approach.

To date, both nymphs and adult *Neotrogla* have been found exclusively in caves. The adults possess fully developed wings and are capable of flight (Lienhard et al. 2010; Yoshizawa & Kamimura, personal observation). However, their wing structures (and observation of living specimens) suggest that they are poor fliers (Ogawa & Yoshizawa 2018), and that migrations may occur only among nearby cave populations. The distributions of the four named species of this genus do not overlap (Lienhard et al. 2010; Lienhard & Ferreira 2013), nor do those of populations that possibly represent undescribed species. Highly polymorphic genetic markers can also be used to reveal their (meta)population genetic structures, providing the basis for their conservation.

Next-generation sequencing techniques are increasingly used to develop large numbers of genetic markers, such as single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs: microsatellites), in non-model organisms (reviewed in Ekblom & Galindo 2011; Guichoux et al. 2011; McCormack et al. 2013; Lemmon & Lemmon 2013; Hodel et al. 2016; Wachi et al. 2017). Here, we report the development of 16

93 microsatellite markers for an undescribed species of *Neotrogla*. Cross-amplification tests
94 were also conducted in all four named species of the genus.
95
96 ## MATERIALS AND METHODS
97 ### Genomic DNA extraction and sequencing
98 Total genomic DNA was extracted from the head and thorax of 11 ethanol-preserved
99 samples (six males and five females) of *Neotrogla* sp. using a DNeasy Blood & Tissue
100 Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The samples
101 were collected on 19 January 2015 from two caves in Varzelândia, Minas Gerais, Brazil:
102 Gruta da Madeira (GM; eight individuals; sample nos. 150119GM1–8) and Lapa do Índio
103 (LI; three individuals; 150119LI1–3). The horizontal projections of these caves are
104 approximately 400 m and 30 m, respectively. The sampled species is apparently very
105 close to *N. aurora* Lienhard, but, owing to their considerable difference in body size, we
106 tentatively treated it as an undescribed species. Some of the DNA extracted from the 11
107 individuals (20 to 40 μL each) was pooled to make a required volume (350 μL) of DNA
108 solution in AE buffer (10.5 ng/μL) for sequencing with an Illumina HiSeq 2500 platform
109 (Illumina, Inc., San Diego, CA, USA). Library construction and sequencing were
110 provided as a custom service of Eurofins Genomics, Inc. (Tokyo, Japan). A paired-end
111 library of ca. 200-bp inserts was sequenced for 125 bp from both ends. After sequencing,
112 low-quality sequences were trimmed using bcl2fastq Conversion Software v.1.8.4
113 (Illumina, Inc.).
114
115 ### Microsatellite mining and primer design
116 Forward and reverse reads of each insert were assembled using BioConductor (Gentleman

et al. 2004) implemented in R v. 3.4.1 (R Core Team 2017), and the text-processing language awk (Aho et al. 1987). Assembled reads shorter than 150 bp were excluded. Our mining strategy of microsatellite markers consisted of two steps. In the first step, all reads were scanned for the presence of microsatellite regions using MSATCOMMANDER 1.08 (Faircloth 2008). Following the methods of several previous studies on other insect groups (Pannebakker et al. 2010; Abe and Pannebakker 2017), we searched for microsatellites with motif lengths of 2–6 bp with minimum repeat numbers of eight, five, five, five, and five for di-, tri-, tetra-, penta-, and hexanucleotides, respectively. Repeats with unit patterns that were circular permutations and/or reverse complements were categorized as one motif type (e.g., an AGC pattern includes AGC, GCA, CAG, GCT, TGC, and CTG: Jurka & Pethiyagoda 1995). In the second step, reads containing microsatellite regions with 13 or more repeats and 70-bp or longer flanking sequences were screened to facilitate subsequent primer designs.

Primer pairs for the amplification of microsatellites were designed using BatchPrimer3 ver. 1.0 (You et al., 2008), using the following search parameters: primer size, 18−22 bp (opt., 20 bp); primer Tm, 58−62°C (opt., 60°C); max Tm difference, 2°C; primer GC%, 40−60%; and other parameters of the default setting.

PCR amplification and genotyping

PCR amplification of microsatellite loci was performed in the 11 *Neotrogla* sp. individuals using an ABI 2720 thermal cycler (Life Technologies, Carlsbad, CA, USA). We adopted Schuelke's (2000) procedure of nested PCR with the M13 universal primer. For this purpose, the 5′ ends of forward primers were attached to M13-tails (5′-TGTAAAACGACGGCCAGT-3′) for annealing with fluorescence-labeled M13

141 universal primers. Nested PCR reactions were conducted in a 10 μL volume containing
142 0.1 μL forward and 0.4 μL reverse primers (10 μM each), 0.4 μL M13 primer (10 μM)
143 labeled with a fluorescent dye (6-FAM, VIC, NED, or PET), 1 μL 10× PCR buffer, 0.8
144 μL 25 mM $MgCl_2$, 0.8 μL dNTPs (2.5 mM each), 0.05 μL AmpliTaq Gold 360 DNA
145 polymerase (Applied Biosystems, Carlsbad, CA, USA), and 1 μL of genomic DNA. The
146 PCR temperature profile consisted of 5 min at 94°C, then 30 cycles of 30 s at 94°C, 45 s
147 at 60°C, and 45 s at 72°C, followed by 8 cycles of 30 s at 94°C, 45 s at 53°C and 45 s at
148 72°C, and a final extension for 10 min at 72°C. Following electrophoresis with an ABI
149 3130 capillary sequencer (Life Technologies), the amplified fragments were analyzed
150 using the Peak Scanner software v. 1.0 (Life Technologies).
151 For cross-amplification tests, DNA was also extracted from the head and thorax
152 of an ethanol-preserved individual of each of the four named species of *Neotrogla*: *N.*
153 *aurora*, *N. brasiliensis*, *N. truncata* Lienhard, and *N. curvata*. The *N. truncata* sample
154 was collected for observing the genital coupling during copulation (Yoshizawa et al.
155 2014), and thus initially fixed in hot water (~80°C) and then transferred to 100% ethanol.
156
157 **Genotypic analysis**
158 We calculated the observed and expected heterozygosity (H_O and H_E) and tested
159 deviations from Hardy–Weinberg equilibrium (HWE) for each cave population (GM and
160 LI) and for the combined data set using GENEPOP 4.6.9 (Rousset 2008). Although the
161 sample sizes were small (eight and three individuals), genetic differentiation between the
162 cave populations was evaluated as a preliminary analysis. For this purpose, F_{ST} and Rho_{ST},
163 which take the allelic identity or the difference between microsatellite allelic sizes,
164 respectively, into account (Weir & Cockerham 1984; Michalakis & Excoffer 1996), were

calculated using GENEPOP 4.6.9 software, and the former was tested using the method of Raymond and Rousset (1995). Linkage disequilibrium was also calculated and tested for all possible combinations of the examined loci. For the GM cave population, non-exclusion probability (NEP), the probability that two unrelated males cannot be discriminated as sperm donors, was calculated for each locus using CERVUS version 3.0.7 (Kalinowski et al. 2007). Significance thresholds in multiple comparisons were corrected using the false discovery rate (FDR; Benjamini & Hochberg 1995).

RESULTS AND DISCUSSION

By sequencing with an Illumina platform, we obtained 32,317,868 reads of 125 bp, corresponding to a 4,040-Mb genomic sequence of the *Neotrogla* sp. The raw sequence data are deposited in the NCBI Sequence Read Archive (accession no.: SPR6871534). After connecting of the corresponding forward and reverse reads (with more than 25 bp overlap), we obtained 13,376,080 reads (with an average length of 170.1 bp; a range of 150−225 bp; and a total of 2,274,799,820 bp) for mining microsatellites.

For this data set, our first screening detected 29,132 (29.2%), 66,086 (66.2%), 3,151 (3.2%), 1,049 (1.1%), and 470 (0.5%) reads (99,888 total) containing di-, tri-, tetra-, penta-, or hexanucleotide repeats, respectively, as candidate sequences for developing microsatellite markers. Pannebakker et al. (2010) mined microsatellites in the genomes of 10 insect species using essentially the same strategy, and found that the compositions of microsatellites varied extensively both between and within insect orders. For example, dinucleotide motifs are more than twice as abundant as trinucleotide motifs in Hymenoptera, whereas trinucleotide motifs are usually more abundant than dinucleotide

189 motifs in other groups (Pannebakker et al. 2010; Abe & Pannebakker 2017). The
190 microsatellite composition of *Neotrogla* sp. was characterized by the almost two-fold
191 overrepresentation of trinucleotide repeats (66.2%) compared with dinucleotide repeats
192 (29.2%), and the low representation of longer motifs (less than 5% total). Of the
193 trinucleotide repeats, the AAT motif was notably dominant over the other motifs (Fig. 1B).
194 Among the insects studied thus far, these characteristics are shared by the aphid,
195 *Acyrthosiphon pisum* Harris (Homoptera: Aphidoidea: Aphididae) (Pannebakker et al.
196 2010). For the brown planthopper (*Nilaparvata lugens* (Stål): Homoptera: Delphacidae),
197 Jing et al. (2012) also reported that AAT repeats were the most abundant, but represented
198 only 15.1% of the trinucleotide motifs. Hemipteroid insects, or Paraneoptera (Psocodea
199 + Hemiptera + Thysanoptera) showed an almost consistent A-T bias, especially in the
200 mitochondrial genes of many Psocodea and some Hemiptera species (Yoshizawa &
201 Johnson 2003, 2013). In accordance with this tendency, the CCG motif was the rarest
202 among the trinucleotide repeats (Fig. 1B). The GC-motif is especially rare among
203 dinucleotide motifs in *Neotorgla* sp. (Fig. 1A) as well as in *A*. *pisum* (Pannebakker et al.
204 2010).

205 We further screened 99 reads containing 13 or more repeats and 70-bp or longer
206 flanking sequences, for 50 of which a pair of primers was successfully designed (47 and
207 3 primer pairs for di- and trinucleotide repeats, respectively; expected PCR product size:
208 138−198 bp; Table S1).

209 As we required markers potentially amplifiable with only a small amount of low
210 quality DNA (e.g., genotyping sperm in female storage organs), we selected 16 primer
211 pairs among 50 primer pairs tested that successfully amplified products of expected sizes
212 in all 11 *Neotrogla* sp. individuals for further characterization. All 16 loci were

213 polymorphic, with two to nine alleles (Table 1). The observed heterozygosity was
214 significantly lower than those expected from the HWE at two loci (Neosp18 and
215 Neosp23) when the data from the two cave populations were merged, but not when they
216 were analyzed separately (Table 1). Although the two caves are only approximately 1 km
217 apart and only a small number of individuals (eight and three) were sampled, these two
218 populations showed significant genetic differentiation (F_{ST} = 0.043, Chi2, 34 = 75.3, P =
219 0.000059; Rho_{ST} = 0.048). Thus, the observed deviations from HWE, in the direction of
220 overabundance of homozygotes, in the combined data set are likely due to the presence
221 of population-specific alleles: in the LI population, for which only three individuals were
222 sampled, we detected a total of 22 microsatellite alleles at 14 loci specific to this cave
223 population (Table 1).

224 Based on the analysis of multiple microsatellite loci, a comparative value of F_{ST}
225 (0.042) was reported for the fruitfly *Drosophila americana* Spencer (Diptera:
226 Drosophilidae) between Howell Island (Missouri, USA) and Lake Ashbaugh (Arkansas,
227 USA) populations, which are more than 200 km apart (Schäfer et al. 2006). Similarly, in
228 the ant *Formica exsecta* Nylander (Hymenoptera: Formicidae) Switzerland and Ural
229 populations, which are more than 3,000 km apart, show genetic differentiation measured
230 as F_{ST} = 0.043 (Goropashnaya et al. 2007). Thus, although results obtained by different
231 sets of molecular markers cannot be simply compared, the observed genetic
232 differentiation between the *Neotrogla* populations supports the hypothesis that the adults
233 are poor fliers (Ogawa & Yoshizawa 2018) and migration between caves is limited.
234 However, both caves (LI and GM) are located in a continuous limestone outcrop, and
235 several shelters with suitable microclimate conditions certainly exist in between those
236 caves. Accordingly, the moderate genetic differentiation observed may alternatively

237 indicate that populations present high allegiance in relation to the caves, and dispersion
238 may occur only (or especially) if the environmental conditions of their habitat become
239 unsuitable or hazardous (e.g. by extreme oligotrophic conditions).

240 After correcting for multiple comparisons using the FDR, no significant linkage
241 disequilibrium was detected among the 16 loci in both the combined data set and in the
242 GM cave population. Due to the small sample size, linkage disequilibrium could not be
243 tested in the LI population.

244 SNPs are widely used as molecular markers in contemporary genetic studies,
245 including parentage analyses, of non-model organisms. Microsatellites and SNPs both
246 have advantages and disadvantages (Hodel et al. 2016). Each locus of a SNP has up to
247 four alleles (A, T, C, or G). Thus, microsatellites, which usually show much higher allelic
248 diversity, can provide a much more efficient and economical method for analyzing mixed
249 DNA samples (Clayton et al. 1998; Gill 2001). Genetic analyses of sperm stored in a
250 female body, which are likely subject to contamination with female DNA, is such an
251 example. Among the 16 microsatellite loci developed in this study, we observed 6.4 ± 1.9
252 alleles (mean ± standard deviation; Table 1). This higher allelic diversity compared to
253 SNPs (usually only two alleles per site; e.g., Lai 2001) resulted in notably low NEPs:
254 0.1513 on average for the 15 loci (excluding Neosp41, which is monomorphic for the GM
255 population; Table 1). When conjointly used, these markers yield NEP of 4.33×10^{-14},
256 enabling efficient estimation of the number of males that inseminated the focal females.

257 Of the 16 primer pairs, five successfully amplified fragments within the expected
258 size range in all four related species examined. The other eight, two, and one primer pairs
259 exhibited successful amplification in three, two, or only one species, respectively (Table
260 2). The applicability of microsatellite markers usually correlates with the relatedness of

261 the species (e.g., Jarne & Lagoda 1996). Our preliminary molecular analysis of their
262 phylogeny suggests that the *Neotrogla* sp. sampled in this study is closer to *N. aurora*
263 than to *N. brasiliensis*, *N. curvata*, and *N. truncata* (K. Yoshizawa, unpublished data).
264 However, the number of applicable primers did not vary much among species (from 11
265 to 13), suggesting their rapid divergence. The lowest amplification success in *N. truncata*
266 (Table 2) may have been due to DNA deterioration owing to fixation and short-term
267 preservation in hot water (see Materials and Methods). The microsatellite markers
268 developed in this study show promise as powerful tools for analyzing the inter- and
269 intraspecific genetic structures of *Neotrogla*.
270

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REFERENCES

Abe J, Pannebakker BA (2017). Development of microsatellite markers and estimation of inbreeding frequency in the parasitoid wasp *Melittobia*. *Scientific Reports* **7,** Article ID 39879. https://doi.org/10.1038/srep39879

Aho AV, Kernighan BW, Weinberger PJ (1987). *The AWK programming language.* Addison-Wesley Longman Publishing, Boston.

Benjamini Y, Hochberg Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* **B57,** 289–300.

Clayton TM, Whitaker JP, Sparkes R, Gill P (1998) Analysis and interpretation of mixed forensic stains using DNA STR profiling. *Forensic Science International* **91,** 55–70.

Ekblom R, Galindo J (2011) Applications of next generation sequencing in molecular ecology of non-model organisms. *Heredity* **107,**1–15.

Faircloth BC (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* **8,** 92–94.

Gentleman RC, Carey VJ, Bates DM *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology* **5,** Article ID R80. https://doi.org/10.1186/gb-2004-5-10-r80

Gill P (2001) An assessment of the utility of single nucleotide polymorphisms (SNPs) for forensic purposes. *International Journal of Legal Medicine* **114,** 204–210.

Goropashnaya AV, Fedorov VB, Seifert B, Pamilo P (2007). Phylogeography and population structure in the ant *Formica exsecta* (Hymenoptera, Formicidae) across Eurasia as reflected by mitochondrial DNA variation and microsatellites. *Annales*

Zoologici Fennici **44,** 462–474.

Guichoux E, Lagache L, Wagner S *et al.* (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* **11,** 591–611.

Hodel RG, Segovia-Salcedo MC, Landis JB *et al.* (2016) The report of my death was an exaggeration: A review for researchers using microsatellites in the 21st century. *Applications in Plant Sciences* **4,** Article ID 1600025. https://doi.org/10.3732/apps.1600025

Jarne P, Lagoda PJ (1996) Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution* **11,** 424–429.

Jurka J, Pethiyagoda C (1995) Simple repetitive DNA sequences from primates: compilation and analysis. *Journal of Molecular Evolution* **40,** 120–126.

Jing S, Liu B, Peng L *et al.* (2012) Development and use of EST-SSR markers for assessing genetic diversity in the brown planthopper (*Nilaparvata lugens* Stål). *Bulletin of Entomological Research* **102,** 113–122.

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* **16,** 1099–1106.

Lai E (2001) Application of SNP technologies in medicine: lessons learned and future challenges. *Genome Research* **11,** 927–929.

Lemmon EM, Lemmon AR (2013) High-throughput genomic data in systematics and phylogenetics. *Annual Review of Ecology, Evolution, and Systematics* **44,** 99–121.

Lienhard C, Do Carmo TO, Ferreira RL (2010). A new genus of Sensitibillini from Brazilian caves (Psocodea: 'Psocoptera': Prinoglarididae). *Revue suisse de Zoologie* **117,** 611–635.

325 Lienhard C, Ferreira RL (2013). A new species of *Neotrogla* from Brazilian caves
326 (Psocodea: 'Psocoptera': Prinoglarididae). *Revue suisse de Zoologie* **120,** 3–12.
327 McCormack JE, Hird SM, Zellmer AJ, Carstens BC, Brumfield RT (2013) Applications
328 of next-generation sequencing to phylogeography and phylogenetics. *Molecular*
329 *Phylogenetics and Evolution* **66,** 526–538.
330 Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using
331 distances between alleles with special reference for microsatellite loci. *Genetics*
332 **142,** 1061–1064.
333 Ogawa N, Yoshizawa K (2018) Origin and transformation of the in-flight wing-coupling
334 structure in Psocodea (Insecta: Paraneoptera). *Journal of Morphology* **279,** 517–
335 530.
336 Pannebakker BA, Niehuis O, Hedley A, Gadau J, Shuker DM (2010) The distribution of
337 microsatellites in the Nasonia parasitoid wasp genome. *Insect Molecular Biology*
338 **19,** 91–98.
339 R Core Team (2017). *R: A language and environment for statistical computing.* R
340 Foundation for Statistical Computing, Vienna, Austria. Available from URL:
341 https://www.R-project.org/.
342 Raymond M, Rousset F (1995) An exact test for population differentiation. *Evolution* **49,**
343 1283–1286.
344 Rousset F (2008) Genepop'007: a complete re-implementation of the Genepop software
345 for Windows and Linux. *Molecular Ecology Resources* **8,** 103–106.
346 Schäfer MA, Orsini L, McAllister BF, Schlötterer C (2006) Patterns of microsatellite
347 variation through a transition zone of a chromosomal cline in *Drosophila*
348 *americana*. *Heredity* **97,** 291–295.

Schuelk M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* **18,** 233–234.

Yoshizawa K, Johnson KP (2003) Phylogenetic position of Phthiraptera (Insecta: Paraneoptera) and elevated rate of evolution in mitochondrial 12S and 16S rDNA. *Molecular Phylogenetics and Evolution* **29,** 102–114.

Yoshizawa K, Johnson KP (2013) Changes in base composition bias of nuclear and mitochondrial genes in lice (Insecta: Psocodea). *Genetica* **141,** 491–499.

Yoshizawa K, Ferreira RL, Kamimura Y, Lienhard C (2014) Female penis, male vagina, and their correlated evolution in a cave insect. *Current Biology* **24,** 1006–1010.

You FM, Huo N, Gu YQ *et al.* (2008) BatchPrimer3: a high throughput web application for PCR and sequencing primer design. *BMC Bioinformatics* **9,** 253.

Wachi N, Matsubayashi KW, Maeto K (2017) Application of next-generation sequencing to the study of non-model insects. *Entomological Science* Published online: 17 Sep 2017; https://doi.org/10.1111/ens.12281

Wearing-Wilde J (1995) The sclerotized spermatophore of the barklouse *Lepinotus patruelis*. *Tissue & Cell* **27,** 1006–1010.

Weir BS, Cockerham CC (1984) Estimating F–statistics for the analysis of population structure. *Evolution* **38,** 1358–1370.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

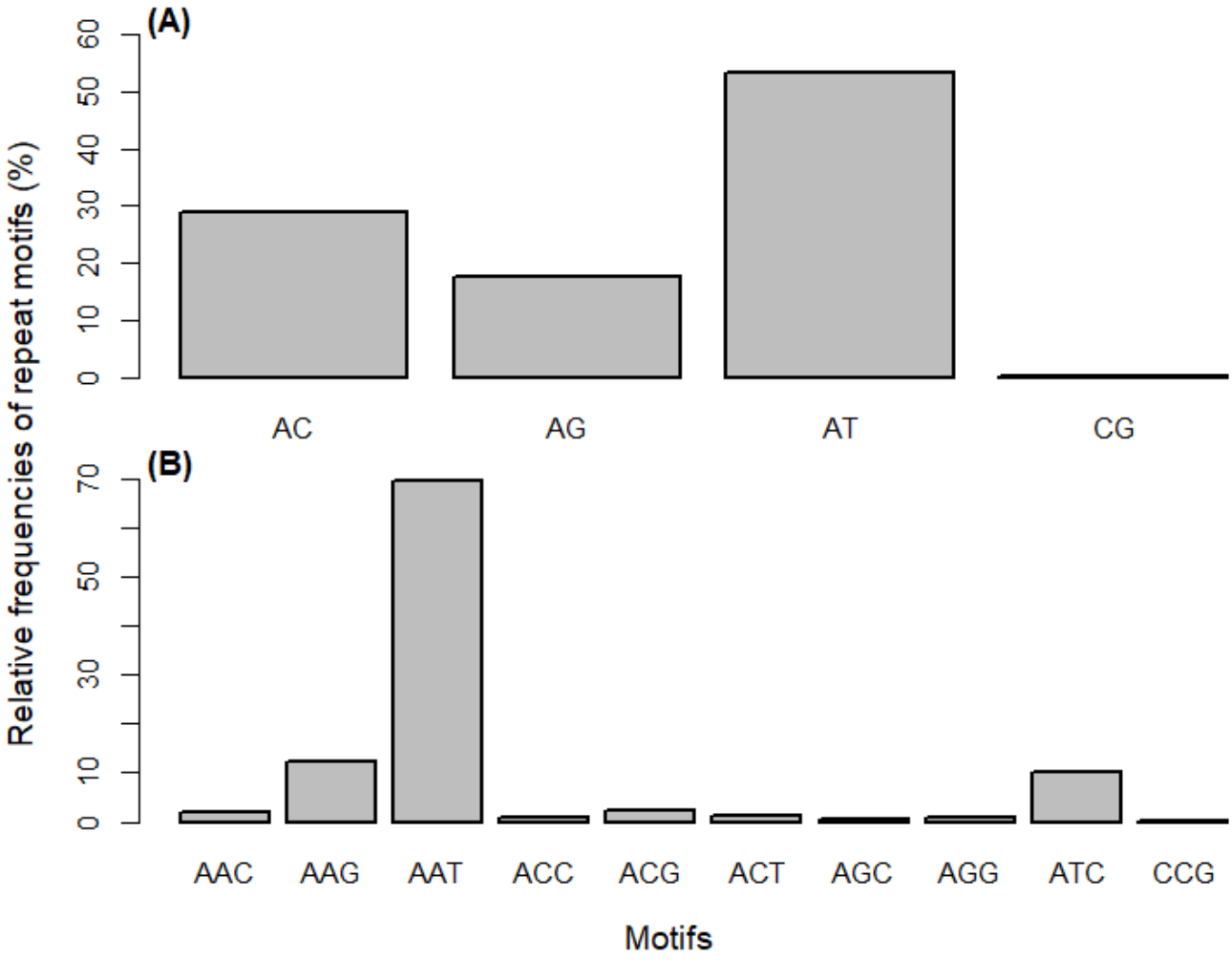
Table S1 The primer pairs designed and tested in *Neotrogla* sp.

374 **Figure Legends**

375

376 **Figure 1.** Relative abundance of (A) di- and (B) trinucleotide repeat motifs found in ca.

377 2,275 Mbp genome sequences of *Neotrogla* sp.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Relative frequencies of repeat motifs (%)
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