The development of species-specific, highly polymorphic nuclear markers, such as microsatellites, would facilitate the analysis of multiple paternity (how many fathers contribute to a brood), studies of relatedness within barnacle aggregations (which can be high; Veliz et al. 2006), as well as standard and coalescent-based population genetic analysis of genetic divergence, divergence time, migration (current and historical), and diversity (e.g., effective population size) in *P. elegans*.

Microsatellites, highly variable genetic markers made up of short (1–6 bp) tandem repeats, are the markers of choice for population and evolutionary genetic studies because of their codominant inheritance, high variability, high abundance in the genome, and relative ease of genotyping on a variety of platforms. Despite the increased popularity of single nucleotide polymorphisms as genetic markers, individual microsatellite markers consistently show greater power in analyses of population genetic structure, association studies, and sibling reconstructions (Ohashi and Tokunaga 2003; Rosenberg et al. 2003; Liu et al. 2005; Wang and Santure 2009); thus, they are still widely used across a range of taxa and experiments. Their isolation, detection, and development can, however, be relatively time consuming and expensive (e.g., Hamilton et al. 1999; Zane et al. 2002). With recent advances in next generation sequencing (NGS), 1000s to millions of short reads of random genomic sequence can be obtained quickly and at relatively low cost, and programs that screen for microsatellites in silico can be used to find repeat sequences and design suitable primers for polymerase chain reaction (PCR) amplification (e.g., Abdelkrim et al. 2009; Gardner et al. 2011). The large number of reads produced on NGS platforms also provide a snapshot of the genome, and with these data, one can investigate the genomic frequency and distribution of repeat types, providing important insights into the origin and evolution of microsatellites, and genome structure and organization more generally (Tóth et al. 2000; Ellergren 2004; Meglécz et al. 2012a). No genome-wide repeat data have been generated for any barnacle species, so this analysis provides the first data of this sort for future genomic comparisons across Cirripedia.

Here, we use NGS on the Roche 454 FLX platform to develop and characterize microsatellite loci for use in future studies of paternity, parentage, and population structure in *P. elegans*. We also take advantage of the large amounts of sequence data produced with this shotgun sequencing approach and make genomic comparisons of the repeat density and diversity in *P. elegans* with other arthropods and more distantly related animal taxa, with the expectation that more closely related taxa will exhibit similar patterns of microsatellite density and repeat class frequency. Finally, we perform a preliminary analysis of genetic diversity and genetic structure in 2 populations to show the utility of these markers for population genetic analyses.

Methods

Samples and DNA Extractions

Barnacles from near the southern tip of the Baja California peninsula, Mexico (Gaspareno: 23°10'58.09"N, 110°8'26.51"W)

and Northwestern Peru (Islilla: 5°13'17.85"S, 81°11'18.99"W) were collected in 2009 and 2010, and peduncle tissue was stored in 95% ethanol. DNA was extracted using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle and Dickson 1987), with 2 chloroform extraction steps and two 70% ethanol washes.

454 Sequencing and Quality Control

Shotgun sequencing of a genomic DNA library from a single individual from southern Baja, Mexico was performed in a portion of a single lane run on a 454 FLX Titanium sequencer (Roche 454 Life Sciences, Brandford, CT, USA), at the Genome Sequencing and Analysis Facility at the University of Texas at Austin. A total of 200 000 reads were requested, and library preparation proceeded following the manufacturer's protocol (Margulies et al. 2005). Briefly, DNA was fragmented through nebulization, and fragments in the 500–800-bp range were ligated to adaptors, captured on beads, and clonally amplified through emulsion PCR. After sequencing, raw sequences were filtered and trimmed for quality and length using the FASTQ tool kit on the Galaxy public server (http://main.g2.bx.psu.edu; Blankenberg et al. 2010). Reads were discarded if they were less than 100 bp in length or if they had a mean quality score lower than 30. Low-quality bases from the 3' end of the read were trimmed with a sliding window, discarding bases until at least 2 individual scores were above a quality score of 35 (PHRED). After quality trimming, we performed a de novo assembly of reads using NEWBLER version 2.8 (Roche 454 Life Sciences) to eliminate duplicate reads, which can account for up to 25% of total reads from 454 FLX Titanium run (Niu et al. 2010; Balzer et al. 2013) and can bias upward the quantification of microsatellites in analyses of repeat density. After assembly using default parameters, contigs and unassembled reads were retained for repeat density analysis.

Repeat Density Analysis and Microsatellite Discovery

To quantify the number and frequency of repeat classes and motifs throughout the sequences, we used the repeat scanning software PHOBOS version 3.3.12 (described in Mayer et al. 2010), which can search for perfect and imperfect microsatellites, giving a more accurate picture of repeat density across the genome compared with other repeat finding software. Using the PHOBOS results, we compared the genomic density of repeats and the frequency of each repeat class in *P. elegans* with genomic repeat data from a variety of animal species, including relatively closely related arthropods and the recently sequenced crustacean *Daphnia pulex*. Because repeat discovery rates can vary depending upon the software platform and the search parameters used, we used data from Mayer et al. (2010) and employed identical search parameters for *P. elegans* in PHOBOS version 3.3.12 to compare repeat data across taxa. Though our shotgun 454 reads represent only a fraction of the *P. elegans'* genome, inferences of genome-wide microsatellite frequency and density from low-coverage 454 reads have been shown to be highly correlated

with, and not significantly different from, estimates made from full, assembled genome data (Meglécz et al. 2012b).

To isolate microsatellites and design primers for population genetic analysis, we used the program QDD2.1 (beta) on the Linux platform (Meglécz et al. 2010). QDD2.1 searches for perfect microsatellites with repeat units of 1–6 bp and is able to filter for unique sequences and discard those associated with duplications or mobile elements. Default primer design settings were used except for the minimum flanking distance between a primer and a microsatellite (set to 30 bp) and the minimum PCR product size (set to 90 bp).

Primer Testing

A total of 45 primer pairs from the QDD2.1 output were chosen for further development and analysis in *P. elegans*. Eight Mexican individuals were used initially to test for amplification success by visualizing amplification products on 1.0% low-melt agarose gels (Fisher Scientific, Waltham, MA, USA). Loci that amplified consistently were then genotyped in the 95 individuals sampled from Peru ($n = 47$) and Mexico ($n = 48$) to evaluate polymorphism and population genetic parameters. For genotyping, a 5' sequence tag that was complementary to a fluorescent probe was added either to the forward or to the reverse primer (FAM tag: GGAAACAGCTATGACCAT; VIC tag: CAGTCGGGCGTCATCA; NED tag: ACCAAC CTAGGAAACACAG; and PET tag: GGCTAGGAAAGG TTAGTGGC; see Table 2 and Hauswaldt and Glenn 2003). In some cases, one of the 2 primers was directly labeled with a fluorophore if a suitable sequence tag could not be placed on the primer (see Table 2). PCR reactions were carried out in volumes of 12.5 µL, with 0.5 µL of each 5 mM primer (if direct labeled) or 0.05 µL of the tagged primer, 0.45 µL of fluorophore, and 0.5 µL of reverse primer (if tag labeled: all primers at 5 mM), 2.5 µL 5× GoTaq buffer with 25 mM MgCl$_2$, 0.3 µL of 5 mM dNTPs, 0.125 µL of 1% Triton-X (Fisher Scientific), and 0.1 µL of GoTaq (Promega, Madison, WI, USA). Amplification was carried out separately for each locus on an MJ research Dyad thermocycler (now Bio-Rad, Hercules, CA, USA). Cycle conditions were as follows: an initial 2-min denaturing step at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 53–57 °C (locus dependent; Table 2), and a 45-s extension step at 72 °C, all followed by a final extension step of 15 min at 72 °C. Successfully amplified loci were also tested for cross amplification in *P. pollicipes*, *P. polymerus*, and the recently described *P. caboverdensis/darwini* (Fernandes et al. 2010; Quinteiro et al. 2011). All sequencing was carried out at the DNA laboratory at Arizona State University. Genotypes were scored by eye using LIZ600 as an internal size standard on the Peak Scanner 1.0 software (Life Technologies, Carlsbad, CA, USA).

Characterization of Novel Microsatellite Markers

We estimated overall number of alleles and observed and expected heterozygosities for the newly developed markers using FSTAT version 2.9.3.2 (Goudet 1995). Tests for Hardy–Weinberg equilibrium (HWE) were also carried out in FSTAT using 1500 permutations, and *P* values were adjusted with a sequential Bonferroni correction. For those markers that differed significantly from HWE, estimates of null allele frequency were made using the FreeNA software (Chapuis and Estoup 2007). Pairwise tests for linkage disequilibrium (LD) were performed using 10 000 permutations in GENETIX (Belkhir et al. 2004).

Analysis of Genetic Diversity and Population Structure in Peru and Mexico

We used the novel microsatellite markers to perform a preliminary population genetic analysis of the 2 populations sampled (Islilla, Peru and Gaspareno, Baja California, Mexico). Allelic richness was calculated using rarefaction in FSTAT version 2.9.3.2, and significant differences between the 2 populations were tested using the sign test (e.g., Jin et al. 2000). Gene diversity was also calculated in FSTAT, and significant differences between populations were assessed with a Wilcoxon signed-rank test (see Nei 1987, p. 183). To determine the degree of population differentiation, we estimated F_{ST} by calculating Θ (Weir and Cockerham 1984), which accounts for small and unequal sample sizes, in FSTAT. Confidence intervals (95% CIs) for Θ were calculated by bootstrapping across loci and used to assess overall significance.

Results and Discussion

454 Sequencing Results

We obtained 191 746 reads with a mean length of 325 bp from a shared lane run on the Roche 454 FLX sequencing platform (Table 1). All reads have been deposited on the sequence read archive (accession SRP029161; http://www.ncbi.nlm.nih.gov/sra). Raw reads were then filtered and trimmed for length and quality, which resulted in the exclusion of 82 331 reads (47%), leaving 109 417 for downstream analysis. Cleaned reads were then assembled using NEWBLER version 2.8 into 973 contigs (maximum contig length of 8272 bp) and 99 324 unassembled reads, producing a set of 100 296 unique sequences for repeat analysis. These 100 296 sequences (36 805 630 nucleotides) accounted for approximately 2.83% of the genome of *P. elegans*, assuming a genome size of 1.3 gigabases, which was estimated from

Table 1. Raw read length statistics from Roche 454 sequencing

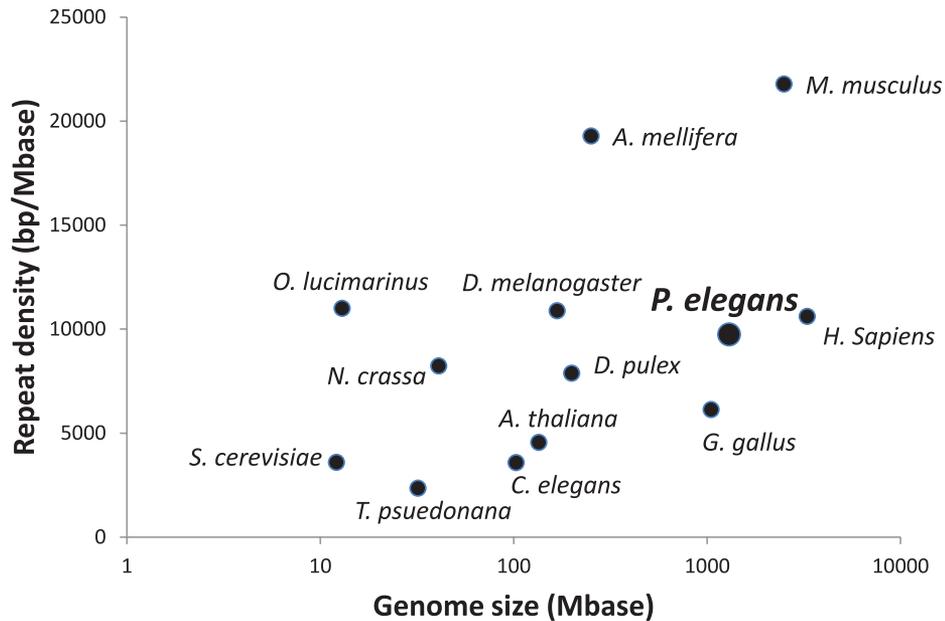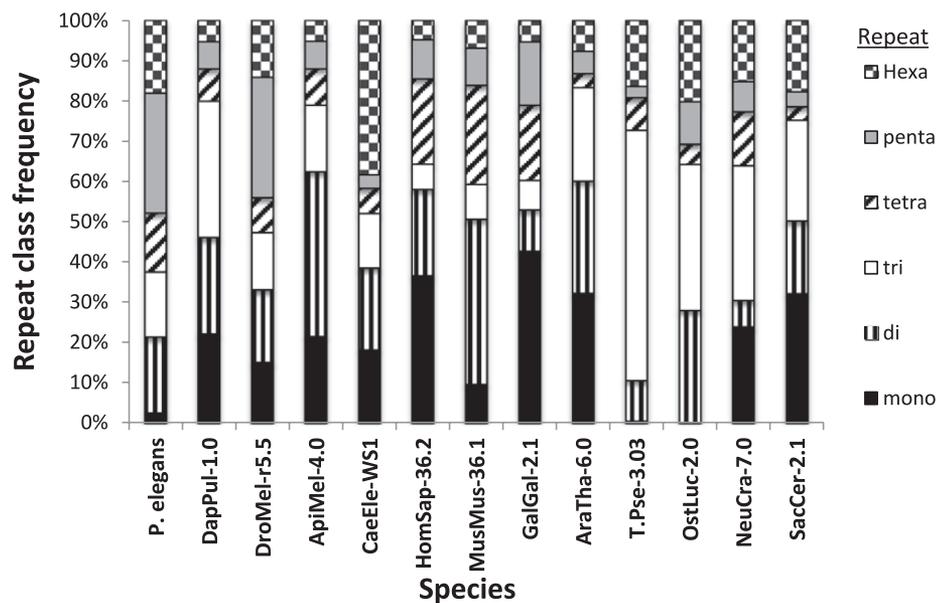| Median read length	358
Mean read length	325.07
Length	Number of sequences
100	16 267
200	17 536
300	26 544
400	72 831
500	56 240
600	2308
700	20
Total	191 746

A

B

Figure 1. (**A**) Genomic density of 1–6-bp repeat motifs for *Pollicipes elegans* and 12 other taxa versus genome size (log transformed). (**B**) Percentage distribution of repeat classes for each species. Following each taxon name on the *x* axis is the build, release, or version number of the genome used for the repeat analysis (for more information, see Table 1, Mayer et al. 2010).

the average sizes of 9 barnacle genomes from the animal genome database (Gregory 2012).

Genome-Wide Microsatellite Characterization

We found 13 809 repeats of 1–6 bp comprising 358 619 bp of total repeat sequence. We calculated that *P. elegans* has a repeat density of 9744 bp of microsatellite repeats per

megabase (Mbase), which is slightly higher than that of the recently sequenced crustacean, *D. pulex* (7879 bp/Mbase) and similar to the fruit fly *Drosophila melanogaster* (10 875 bp/Mbase; Figure 1A). Repeat density appeared to scale somewhat with genome size, but this relationship was not significant (Pearson's correlation: 0.46, $P = 0.11$). Comparing the frequency (bp/Mbase) of repeat classes in *P. elegans*, pentanucleotide repeats were the most common (29%), above that of

Table 2. Summary marker statistics and population genetic data

		Marker data							Population genetic data				
	Locus	GenBank number	Repeat	Allele number	Allele size[a]	H_o[b]	H_e	Null[c]	H_e Baja[d]	H_e Peru	AR Baja[e]	AR Peru	F_{ST}[f]
1	Pole01	KF562712	AC	16	160–195	0.72	0.82		0.86	0.79	15.32	9.63	−0.001
2	Pole07	KF562713	AC	15	108–145[i]	0.51	0.80	0.14	—	—	—	—	—
3	Pole08	KF562714	GAAT	8	141–177	0.41	0.49		0.56	0.41	6.48	6.34	0.007
4	Pole16	KF562715	AC	3	209–213[ii]	0.25	0.28		0.34	0.22	3.00	2.00	0.001
5	Pole18	KF562716	TG	7	136–148[iiii]	0.50	0.51		0.52	0.49	5.83	5.82	0.006
6	Pole25	KF562717	GGC	5	203–215[i]	0.53	0.57		0.64	0.53	7.31	3.73	0
7	Pole28	KF562718	GCAC	2	173–177[iiii]	0.17	0.28	0.04	—	—	—	—	—
8	Pole29	KF562719	CCCAGA	22	140–210	0.83	0.86		0.87	0.77	8.94	17.41	0.097
9	Pole30	KF562720	TG	6	149–159[ii]	0.27	0.67	0.19	—	—	—	—	—
10	Pole34	KF562721	TGA	4	109–124[iv]	0.63	0.67		0.66	0.41	3.97	3.00	0.09
11	Pole41	KF562722	AG	19	108–180[ii]	0.59	0.80	0.10	—	—	—	—	—
12	Pole43	KF562723	AC	6	117–129[iv]	0.44	0.45		0.65	0.31	5.94	3.93	0.087
13	Pole44	KF562724	CT	17	162–220[iiii]	0.82	0.90		0.84	0.91	10.60	15.09	0.041
14	Pole45	KF562725	TC	7	122–136[ii]	0.66	0.66		0.64	0.68	5.87	5.61	−0.006
15	Pole46	KF562726	GAT	4	114–123[iiii]	0.20	0.21		0.16	0.25	3.91	2.00	0.023
	Average			9.4		0.5	0.597		0.61	0.53	7.02	6.78	0.039 (.01–.06)[g]

[a] For the allele size range of each marker, the following indicates that a universal, 5' tag (see Methods and Hauswaldt et al. 2003) was added to the primer, adding *X* number of base pairs to the PCR product: [i]NED tag: ACCAACCTAGGAAACACAG; [ii]FAM tag: GGAAACAGCTATGACCAT; [iii]VIC tag: CAGTCGGGCGTCATCA; [iv]PET tag: GGCTAGGAAAGGTTAGTGGC.

[b] H_o and H_e represent observed and expected heterozygosities for each locus, respectively (both populations combined, $N = 95$).

[c] Estimated null allele frequency from FreeNA (Chapuis and Estoup 2007) is reported for markers with significant deviation from HWE.

[d] Expected heterozygosity for the Peru ($N = 47$) and Baja ($N = 48$) populations separately.

[e] Allelic richness, estimated with rarefaction in FSTAT (Goudet 1995) for the Peru ($N = 47$) and Baja ($N = 48$) populations.

[f] F_{ST} was calculated as described by Weir and Cockerham (1984).

[g] Overall F_{ST} numbers in parentheses represent the lower and upper bounds of the 95% CI obtained via bootstrapping. Markers that deviated from HWE were not included in the population genetic analysis.

di- and hexa-nucleotide repeats (both at 18%), whereas mononucleotide repeats were found at the lowest frequency, just 2.7% (Figure 1B). Looking across species in Figure 1B, it is quite clear that there is substantial variation in the frequencies of repeat classes, even among the related arthropods. Though overall repeat densities were fairly similar between *D. pulex* and *P. elegans*, the distribution of repeat class frequencies were not—patterns for *P. elegans* resembled *D. melanogaster* to a much greater degree than *D. pulex* (Figure 1B). Though broad taxon-specific variation in repeat frequency has been observed (e.g., Tóth et al. 2000), the discrepancy between these crustaceans likely reflects the complexity of repeat evolution (Ellergren 2004; Meglécz et al. 2012a) and the hundreds of million years of divergence between these species (e.g., Regier et al. 2005).

Microsatellite Screening, Primer Design, and Primer Testing

Perfect microsatellite sequences were identified for marker development by screening the unique sequences for di-, tri-, tetra-, penta-, and hexa-nucleotide repeats using QDD2.1. In total, we identified 2922 microsatellites of at least 5 repeat units. Primers were successfully designed for 1471 microsatellites (48%), and 45 primer pairs were chosen for testing and further characterization using PCR. In total, 24 of the 45 primers designed produced PCR products with reliable amplification, 15 of which were polymorphic and are

characterized here. A total of 17 primer pairs demonstrated poor amplification, produced PCR products that had more than 2 alleles per individual, or showed unreliable amplification across individuals. Only 4 primer pairs produced no amplification whatsoever. Table 2 displays the results for the 15 successfully designed primer pairs. None of the isolated markers amplified reliably in *P. pollicipes*, *P. polymerus*, or *P. caboverdensis*.

Across the 15 microsatellites, the number of alleles per locus varied from 2 to 22, with an average of 9.4. Observed and expected heterozygosities averaged 0.501, and 0.590, respectively. Most markers consisted of dinucleotide repeats ($n = 9$), but tri-, tetra-, and hexa-nucleotide repeats were also represented in the successfully developed loci (Table 2). One pair of markers, Pole01 and Pole07, showed evidence of significant LD ($P < 0.00041$); the remaining markers showed no significant LD ($P > 0.07$). Four markers exhibited significant deviations from HWE, notably in the direction of fewer heterozygotes than expected (Table 2). Testing these markers for null alleles with FreeNA (Chapuis and Estoup 2007), we found that heterozygote deficiency was likely a result of null alleles, present at low frequency in all 4 of these markers (Table 2). Null alleles are reported often in population genetic literature (reviewed by Dakin and Avise 2004) and are even more common in microsatellite-based analyses of marine invertebrates (e.g., Hare et al. 1996; Hedgecock et al. 2004; Brownlow et al. 2008; Bailie et al. 2010; McInerney

et al. 2011). The low level of null alleles detected here is similar to what has been reported in a population genetic study of the acorn barnacle *Balanus balanoides* (Flight et al. 2012).

Analysis of Genetic Diversity and Population Structure

With the 11 microsatellite loci that demonstrated HWE, we examined genetic diversity and population structure between the Baja (California, Mexico) and Islilla (Peru) populations. Indices of genetic diversity were consistently lower in the Peruvian population, with 9 of the 11 loci showing lower allelic richness (Sign test, $P = 0.0654$) and 8 of the 11 loci showing lower gene diversity (Wilcoxon signed-rank test, $P = 0.053$; Table 2). The pronounced disparity in diversity could be associated with increased fishing pressure in Peru, but other explanations are likely, including differences in colonization history and effective population size. High variability in the abundance and distribution of *P. elegans* populations in Peru has been reported and is thought to be influenced by strong, episodic El Nino events that disrupt recruitment patterns and affect food availability (Kameya and Zeballos 1988). Substantial fluctuations in population sizes would likely have a large and negative effect on genetic diversity and effective population size in the Peruvian population; however, more complex analyses of effective size and colonization history are required to investigate this result further.

We also found relatively low but significant genetic differentiation between the 2 populations ($F_{ST} = 0.039$, 95% CI 0.016–0.061; Table 2). This level of differentiation is a bit lower than expected given the relatively high (1.2%) CO1 divergence and the near reciprocal monophyly of CO1 haplotypes reported by Van Syoc (1994). However, F_{ST} varied substantially among loci, with some loci showing 0 differentiation (e.g., Pole01 and Pole25) and others exhibiting much higher differentiation (max $F_{ST} = 0.097$, Pole29). The relatively low level of overall differentiation suggests that there may be some gene flow between the populations despite the 4000-km distance that separates them. Alternatively, gene flow may be restricted, but not enough time has elapsed for greater divergence in allele frequencies to have accumulated via genetic drift.

Conclusions

We used a simple and efficient protocol with NGS data to identify and characterize 15 novel polymorphic microsatellite markers for the Pacific gooseneck barnacle *P. elegans*, the first markers of this type developed in the genus *Pollicipes*. These markers had a high rate of amplification success, showed relatively high polymorphism, and revealed differences in genetic diversity and population structure in a preliminary population genetic analysis of 2 populations. Analysis of repeat density demonstrated that *P. elegans* had a fairly high density of microsatellites for its genome size and that there was high variability in repeat class frequency among related taxa, highlighting the complex nature of microsatellite evolution. These markers will be useful for future studies of multiple paternity and

parentage, and a more complete, range-wide investigation of population genetic structure and diversity in *P. elegans*.

Data Accessibility Policy

The 454 reads are archived in the sequence read archive (accession SRP029161), sequences used to design the 15 microsatellite primers have been assigned GenBank numbers (KF562712–KF562726), and microsatellite data are deposited in the Dryad repository: doi:10.5061/dryad.b7855.

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