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## Assessment of genetic structure among eastern North Pacific gray whales on their feeding grounds

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### ABSTRACT

Although most eastern North Pacific (ENP) gray whales feed in the Bering, Beaufort, and Chukchi Seas during summer and fall, a small number of individuals, referred to as the Pacific Coast Feeding Group (PCFG), show intra- and interseasonal fidelity to feeding areas from northern California through southeastern Alaska. We used both mitochondrial DNA (mtDNA) and 12 microsatellite markers to assess whether stock structure exists among feeding grounds used by ENP gray whales. Significant mtDNA differentiation was found when samples representing the PCFG ( $n = 71$ ) were compared with samples ( $n = 103$ ) collected from animals feeding further north ( $F_{ST} = 0.012$ ,  $P = 0.0045$ ). No significant nuclear differences were

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detected. These results indicate that matrilineal fidelity plays a role in creating structure among feeding grounds but suggests that individuals from different feeding areas may interbreed. Haplotype diversities were similar between strata ( $b_{\text{PCFG}} = 0.945$ ,  $b_{\text{Northern}} = 0.952$ ), which, in combination with the low level of mtDNA differentiation identified, suggested that some immigration into the PCFG could be occurring. These results are important in evaluating the management of ENP gray whales, especially in light of the Makah Tribe's proposal to resume whaling in an area of the Washington coast utilized by both PCFG and migrating whales.

**Key words:** *Eschrichtius robustus*, gray whale, population structure, mitochondrial DNA, microsatellites, demographic independence.

A single stock of gray whales (*Eschrichtius robustus*) is currently recognized in U.S. waters (Carretta *et al.* 2013). This stock, which is referred to as the eastern North Pacific (ENP) stock, is estimated to contain approximately 19,000 individuals (Laake *et al.* 2009). Most of these whales feed in the Bering, Beaufort, and Chukchi Seas during summer and fall and then migrate south along the coast of North America to overwinter in the lagoons and coastal waters of Baja Mexico. However, a small number of individuals feed in more southern waters from northern California through southeastern Alaska during summer and fall (Gilmore 1960; Pike 1962; Hatler and Darling 1974; Darling 1984; Calambokidis *et al.* 2002, 2012). Photo-identification research, which commenced in the early 1970s and continues to date, has identified a subset of whales that have returned to this southern feeding ground in multiple years and account for the majority of sightings in the area during summer and fall months (Hatler and Darling 1974; Darling 1984; Calambokidis *et al.* 2002, 2012). These whales are referred to as the Pacific Coast Feeding Group (PCFG; IWC 2011a). Recent estimates of annual abundance suggest that the PCFG includes approximately 200 animals (Calambokidis *et al.* 2012). Although PCFG whales account for the majority of sightings on this southern feeding ground during summer and fall, the area is also used by whales that are encountered in the region following the migration (*e.g.*, after 1 June) but are seen in only one year (Calambokidis *et al.* 2012). These individuals are generally seen for shorter time periods and in a more limited area than are PCFG whales, and they may represent stragglers from the larger group of animals that migrate through the southern feeding ground on their way to feeding areas further north (Calambokidis *et al.* 2012).

The PCFG includes some animals that were first identified as calves with their mothers on the southern feeding ground and that have returned to feed in the area in subsequent years (Calambokidis *et al.* 2012). This pattern of behavior, which is often called matrilineal fidelity, likely results from calves learning the location of suitable feeding/calving grounds from their mothers. Matrilineal fidelity to feeding and/or calving areas has been documented in other baleen whales (*e.g.*, Gulf of Maine humpback whales, Clapham and Mayo 1987; southern right whales, Valenzuela *et al.* 2009). Understanding patterns of matrilineal fidelity may be important in shaping management decisions, as it is thought that the lack of recovery or repopulation of baleen whales in some areas heavily impacted by commercial whaling is related to the loss of knowledge of where suitable habitat is located (Clapham *et al.* 2008).

Concern for PCFG whales has arisen in part from recent interest in the resumption of whaling by the Makah Tribe in northwest Washington, an area used by virtually all migrating whales as well as by foraging whales considered part of the PCFG. The current proposal by the Makah Tribe includes time/area restrictions designed to

reduce the probability of killing a PCFG whale by focusing hunt effort on the much larger group of whales migrating to/from feeding areas further north. However, PCFG whales are present during the migratory season, and it is impossible to ensure that no PCFG whales would be killed. The Makah Tribe also proposes to compare photographs of any whales harvested in the hunt to a photo-identification catalog of known PCFG whales and to suspend the hunt if needed to prevent the number of PCFG whales harvested from exceeding the annual allowable bycatch level for that year (IWC 2011*b*).

Evaluating whether any kills would, over time, have the potential to deplete the PCFG requires an understanding of how individuals are recruited into the group. If recruitment into the area is exclusively internal, such that use of the area is driven by calves learning the location of feeding grounds from their mothers, then a PCFG individual that is removed would not be replaced by immigration. However, if recruitment is largely external, then it is possible that any takes from the PCFG could be offset by immigration into the PCFG by whales that in previous years fed in northern areas. As aforementioned, some PCFG individuals were first identified as calves on the feeding ground and have returned to the area to feed in subsequent years. However, the origin of other individuals is unknown, and "new" (previously unidentified) noncalf whales are identified each year, some of which have returned to the southern feeding ground in subsequent years (Calambokidis *et al.* 2012). Although these whales may be individuals who were "missed" as calves (*e.g.*, not identified as a calf or not photographed that season), they could also represent whales that previously fed further north but now demonstrate fidelity to the PCFG range.

Genetic studies have provided some insight into mechanisms of recruitment into the PCFG. Initial work utilizing a simulation-based approach indicated that if the PCFG originated from a single recent colonization event in the past 40–100 yr, with no external recruitment into the group, detectable mtDNA genetic differentiation would be generated (Ramakrishnan and Taylor 2001). Subsequent empirical analysis, however, failed to detect such a signal when comparing 16 samples collected from PCFG whales using Clayoquot Sound, British Columbia, with samples ( $n = 41$ ) collected from individuals presumed to feed in more northern areas (Steeves *et al.* 2001). More recently, Frasier *et al.* (2011) used mtDNA to compare samples collected from 40 individuals considered part of the PCFG with published data generated from 105 samples collected from ENP gray whales, most of which stranded along the migratory route (LeDuc *et al.* 2002). All haplotypes identified among the PCFG samples were also found in the larger ENP sample set, and haplotype diversity found in the PCFG ( $b = 0.93$ ) was lower than, but similar to, that found among the samples representing the larger ENP population ( $b = 0.95$ ). However, significant differences in estimates of long-term effective size and mtDNA haplotype frequencies were identified between the two groups. These results suggest that matrilineally directed fidelity plays a role in use of this area, and the authors concluded that the PCFG should be recognized as a distinct management unit (Frasier *et al.* 2011).

One limitation of previous genetic studies on the PCFG is that they utilized samples primarily collected from gray whales that stranded while on the ENP migratory route as representative of the larger ENP population in their comparisons. Although the likelihood that any of these stranded animals were part of the PCFG is low given the large size of the ENP gray whale population, this possibility could not be ruled out based on the location where most of the ENP samples were collected. More importantly, the limited number of samples available from the feeding ground(s)

north of the Aleutians precluded previous studies from making a direct comparison between animals utilizing different feeding grounds.

At the end of the feeding season, PCFG whales are thought to join the southbound migration to Mexican waters and have therefore been presumed to interbreed with the larger ENP population (Calambokidis *et al.* 2002, 2012). Earlier genetic studies of the PCFG relied exclusively on mtDNA, however, and the assumption that PCFG whales interbreed with gray whales feeding in other areas was not assessed. Conception in gray whales is thought to occur primarily during a 3 wk period between late November and early December (27 November to 13 December), although if no conception occurs during this first period, a second estrus may occur about 40 d later when whales are on or near their wintering grounds (Rice and Wolman 1971). Rugh *et al.* (2001) estimated that the median (peak) sighting date for the southbound migration is 12 December for Unimak Pass, Alaska, suggesting that many gray whales would be north of the PCFG seasonal range during the first mating period and raising the possibility that some segregation in breeding could occur with respect to feeding ground origin.

Here we contribute to the understanding of stock structure of gray whales by (1) comparing samples collected from gray whales feeding north of the Aleutians with samples collected from PCFG whales to directly address whether structure exists among feeding grounds used by ENP gray whales, and (2) using nuclear markers ( $n = 12$  microsatellites) to test the assumption that PCFG whales interbreed with whales from other feeding grounds. We also increased the number of samples collected from PCFG whales and, for those samples linked to photographed individuals, were able to further refine our representation of the PCFG by incorporating sighting histories of known individuals in the comparisons. Although other scenarios are possible, here we test the following three hypotheses:

(1) No population structure (*e.g.*, panmixia) is present among feeding grounds used by ENP gray whales; individuals move between feeding areas and exhibit random mating. This hypothesis would be supported by a finding of no nuclear or mitochondrial differentiation between samples from PCFG whales and those collected from animals feeding further north.

(2) Utilization of feeding areas is influenced by internal recruitment, with calves following their mothers to feeding grounds and returning in subsequent years. Mating is random with respect to feeding ground affiliation. This hypothesis would be supported by a finding of significant differences in mtDNA haplotype frequencies when comparing samples from PCFG whales with those collected from animals feeding further north, but no significant differences in microsatellite allele frequencies between these groups.

(3) Utilization of feeding areas is influenced by matrilineal fidelity and mating is not random with respect to feeding ground affiliation. This hypothesis would be supported by a finding of significant differences in both mtDNA haplotype and microsatellite allele frequencies.

## METHODS

### *Samples*

The initial sample set consisted of 277 samples collected between 1994 and 2010, with collection locations ranging from northern California to Barrow, Alaska and

Chukotka, Russia (Fig. 1, Table S1). Although some samples were collected from individuals taken as part of a subsistence hunt off Chukotka ( $n = 75$  samples) or from stranded individuals ( $n = 17$ ), the majority of samples ( $n = 185$ , including all samples collected between northern California and British Columbia, Canada) were collected as biopsies from free-ranging individuals. During biopsy sample collection, efforts were made to obtain a photograph of each biopsied whale. These photographs were compared to a photo-identification catalog maintained by Cascadia Research Collective and containing photo-identification images primarily collected between 1998 and 2009. This catalog focuses on the PCFG whales but also includes some migrating whales that were photographed in the spring (March through May) during their northward migration.

Linking biopsy samples to photographed whales allowed the sighting history of individuals to be evaluated when determining which samples should be used to represent the PCFG whales. As noted earlier, whales utilizing the PCFG's seasonal range fall into two categories: (1) whales that return frequently and account for the majority of sightings, and (2) apparent stragglers from the migration that are sighted in only one year (Calambokidis *et al.* 2012). To ensure that our PCFG stratum was representative of the first category of whales, samples were screened using two criteria: (1) the sample had to be linked to a photo-identified animal, and (2) the photo-identified

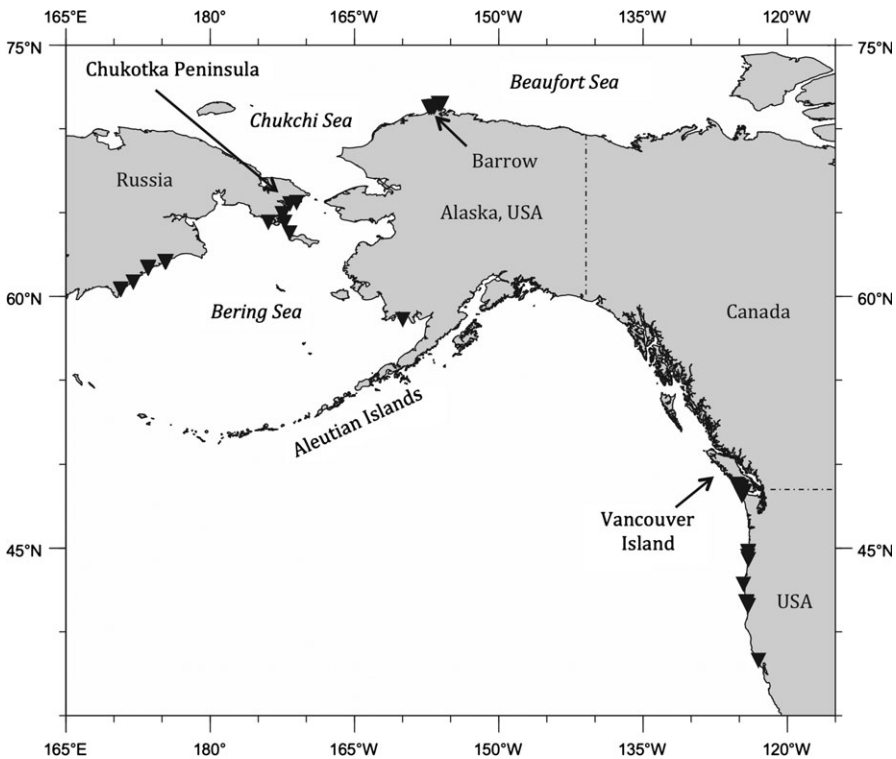


Figure 1. Locations where samples were collected, with key areas mentioned in the text labeled.

animal to which the sample was linked had to have been sighted in two or more years within the defined season (1 June to 30 November) and area (between 41°N and 52°N, in concordance with the boundaries used by the International Whaling Commission's Scientific Committee, IWC 2012) representative of PCFG whales. Samples collected on the southern feeding ground but not meeting these criteria ( $n = 36$ ) were removed prior to data analysis, leaving 113 samples collected from whales considered to represent the PCFG in the sample set.

Samples collected from gray whales on the northern feeding area were stratified in two ways. First, all samples collected from whales that were north of the Aleutian Island chain between June and November were included in a "North" stratum ( $n = 128$ ). This stratification assumes that whales use the northern feeding area in a relatively uniform manner, such that sampling location within this area does not matter. However, little is known about whether gray whales exhibit fidelity to smaller regions within the northern feeding area. If multiple feeding aggregations exist north of the Aleutians, then sampling location within that larger area is important. Although the original design of the study was to have a stratum representing Chukotka, Russia, and a stratum representing Barrow, Alaska, the sample size for the latter ( $n = 14$  individuals) was insufficient to characterize genetic frequencies from that area. As such, we were unable to directly address hypotheses about whether additional structure exists north of the Aleutian Islands. However, we did include a comparison of the PCFG stratum to the Chukotka stratum ( $n = 75$  samples) to avoid including unrecognized heterogeneity in our representation of animals feeding in the north.

#### *Laboratory Processing*

*DNA extraction, PCR amplification and sequencing*—Genomic DNA was extracted from samples using either sodium chloride protein precipitation (Miller *et al.* 1988) or silica-based filter purification (Qiagtractor DX reagents, Qiagen, Valencia, CA) following the manufacturers' instructions. Extractions were performed on a JANUS automated work station (Perkin-Elmer, Waltham, MA). MtDNA sequences for eight of these samples had been generated previously for another study (LeDuc *et al.* 2002); however, to provide consistent quality control, these samples were resequenced for our analyses. The 5' end of the hyper-variable mtDNA control region was amplified from extracted genomic DNA, using the polymerase chain reaction (PCR) and the primers used in the LeDuc *et al.* (2002) study (H00034, Rosel *et al.* 1994; L15812, Chivers *et al.* 2005). DNA was amplified using a 25  $\mu$ L reaction of  $\sim$ 100 ng DNA, 1 $\times$  PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1.5 mM MgCl<sub>2</sub>), 0.6 mM dNTPs, 0.3  $\mu$ M primers, and 0.25 units of Taq DNA polymerase (New England BioLabs, Inc.). The PCR cycling profile consisted of 90°C for 2 min, followed by 35 cycles of 94°C for 50 s, an annealing temperature of 60°C for 50 s, and 72°C for 1 min, then a final extension of 72°C for 5 min. Sequencing of amplified products followed standard techniques (Saiki *et al.* 1988, Palumbi *et al.* 1991), and both strands of the amplified DNA product were sequenced independently on an Applied Biosystems, Inc. (ABI) model 3730 sequencer. If a sample was identified as having a mtDNA haplotype that was not found among any of the other samples, mtDNA amplification and sequencing were replicated to confirm the haplotype identity. All sequences were aligned using Sequencher v4.8 (Gene Codes Corp. 2000), resulting in final sequences that were 523 base pairs long.

*Nuclear DNA processing*—Twelve microsatellite loci isolated from other cetacean species were used to genotype the samples (see Table S2): EV14, EV37, and EV94

(Valsecchi and Amos 1996); Gata028, Gata098, Gata417, and Gt023 (Palsbøll *et al.* 1997); RW31 and RW48 (Waldick *et al.* 1999); and SW10, SW13, and SW19 (Richard *et al.* 1996). For all reverse primers except those amplifying Gata098 and EV37 (which failed to amplify with modified primers), the primer sequence was modified from the original design by placing the sequence GTTTCTT on the 5' end to facilitate complete adenylation and thus more consistent scoring (Brownstein *et al.* 1996). Forward primers were fluorescently labeled. Extracted DNA was amplified using a 25  $\mu$ L reaction of  $\sim$ 100 ng of DNA, 1 $\times$  PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1.5 mM MgCl<sub>2</sub>), 0.6 mM dNTPs, 0.3  $\mu$ M primers, and 0.5 units of Taq DNA polymerase (New England BioLabs, Inc.). The PCR cycling profile included 90°C for 2.5 min, followed by 35 cycles of 94°C for 45 s, 1 min at the optimal annealing temperature (see Table S2), and 72°C for 1.5 min, then a final extension of 72°C for 5 min. Only one locus was amplified per reaction, and each PCR product was assessed electrophoretically on a 2% agarose gel for size and quality before loading onto an ABI 3730 Genetic Analyzer. ABI GeneMapper software (version 4.0) was used along with an internal size standard (GeneScan-500 ROX, ABI) to determine allele fragment size. Two positive control samples were included on each plate to ensure consistent sizing between runs.

*Sex determination*—Samples were genetically sexed by amplification and Real-Time PCR (MX3000p, Stratagene Inc.) of the zinc finger (ZFX and ZFY) genes. Samples from one male and one female for which sex had been determined *via* examination of a stranded animal were included as positive controls in all amplifications. Sex was determined by the amplification pattern: males had two products and females had one (Morin *et al.* 2005).

### Analysis

*Data review*—Quality control and sample tracking procedures, as detailed in Morin *et al.* (2010), were implemented during data generation. A randomly chosen set of samples, representing 13% of all samples processed, was sequenced, sexed, and genotyped a second time, and these records were reviewed for consistency. For the microsatellite data, replicate and original genotypes were compared, and a per-allele error rate was calculated by determining the number of discrepant allele calls divided by the total number of allele calls compared across all loci. In addition, all microsatellite genotypes were scored independently by two experienced genotypers. The allele calls from each genotyper were compared, and calls that did not match were reviewed jointly by both genotypers. Inconsistencies that could not be resolved upon review were treated as missing data.

After genotyping of samples was complete for eight of the twelve loci (EV14, EV94, Gata028, Gata417, Gt023, RW31, SW13, and SW19), the program GENE-CAP (Wilberg and Dreher 2004) was used to calculate the probability that two randomly chosen individuals would share the same multilocus genotype under both the assumption of Hardy-Weinberg equilibrium (PID<sub>HW</sub>, Paetkau and Strobeck 1994) and under the more conservative assumption that full siblings may be present within the data set (PID<sub>SIB</sub>, Waits *et al.* 2001). Samples with identical genotypes, indicating that they may have been collected from the same animal, were flagged for further review. These sample pairs were checked to see if they also shared the same mtDNA haplotype and sex, and, when possible, photo-identification records were used to confirm the genetic match. For all samples that shared identical mtDNA haplotypes, sexes, and nuclear genotypes at the eight loci, one sample from each pair



was removed and then the remaining samples were genotyped at the additional four loci prior to further analysis.

After genotyping at all 12 microsatellite loci was complete, the data set was reviewed to identify samples that were missing data for  $\geq 25\%$  of the markers; these samples were considered to be of poor quality and were removed prior to further analysis. The program MSTOOLS (Park 2001) was used to identify any additional samples whose genotypes matched at eight or more loci (using the full 12 microsatellite data set) and thus might represent duplicate samples that were not detected in the earlier analysis. Deviations from Hardy-Weinberg equilibrium (HWE) were assessed for each locus using Genepop (version 4.0.11, Rousset 2008). Both the probability test (Guo and Thompson 1992) and the test for heterozygote deficiency (Rousset and Raymond 1995) were conducted using the program defaults for the Markov chain parameters (10,000 dememorization steps, 20 batches, 5,000 iterations/batch). Genepop was also used to test for linkage disequilibrium (LD) for each pair of loci. All tests were run for the combined data set as well as for each stratum. The false discovery rate (FDR) adjustment (Benjamini and Hochberg 1995) was used to control for multiple testing when the results of the HWE and LD analyses were assessed.

*Genetic diversity*—For the mtDNA data, nucleotide ( $\pi$ ) and haplotype ( $h$ ) diversities (Nei 1987) were calculated using Arlequin 3.1 (Excoffier *et al.* 2005). To look for phylogeographic patterns among the mtDNA data, the software package Network 4.5.1.0 (available at <http://www.fluxus-engineering.com/sharenet.htm>) was used to generate a median-joining network of haplotypes using the algorithm of Bandelt *et al.* (1999). For the microsatellite data, the number of alleles per locus and observed and expected heterozygosities (Nei and Roychoudhury 1974) were calculated using custom code (eiaGenetics<sup>2</sup>) written in the statistical programming language R (R Core Development Team 2009).

*Genetic structure*—Pairwise estimates of genetic divergence were calculated using both  $F_{ST}$  (Weir and Cockerham 1984) and the AMOVA  $\Phi_{ST}$  (Excoffier *et al.* 1992) for the mtDNA data using Arlequin v3.1 (Excoffier *et al.* 2005). For the  $\Phi_{ST}$  pairwise distance calculations, the program jModelTest v2.1.4 (Guindon and Gascuel 2003, Posada 2008, Durriba *et al.* 2012) was used to select the best nucleotide substitution model based on the Akaike Information Criterion (AIC). Statistical significance was assessed using 10,000 permutations. Fisher's exact test (Raymond and Rousset 1995) was also used to test for mtDNA differentiation between strata using Arlequin 3.1 (Excoffier *et al.* 2005); 10,000 replications were used to test for significance. For the microsatellite data,  $F_{ST}$  (Weir and Cockerham 1984),  $F'_{ST}$  (Hedrick 2005, Meirmans 2006), and a  $\chi^2$  test were used to assess genetic differentiation using custom R-code (eiaGenetics). Statistical significance was determined from 5,000 permutations of each data set.

## RESULTS

### *Data Review*

Fourteen samples (including 11 samples collected from stranded whales) did not produce useable mtDNA sequence data and also failed to amplify at  $>4$  microsatellite

<sup>2</sup>Available on request from E. Archer at [eric.archer@noaa.gov](mailto:eric.archer@noaa.gov).

loci; these samples (identified as “poor quality” samples) were removed from all subsequent analyses and data review (Table S1, S3).

Based on the genotypes of the remaining samples ( $n = 227$ ) at the initial eight loci, the probability of two individuals possessing the same multilocus genotype was  $9.08 \times 10^{-9}$  for unrelated individuals ( $PID_{HW}$ ) and was  $6.97 \times 10^{-4}$  for full siblings ( $PID_{SIB}$ ), indicating that the microsatellite loci were adequate for identifying unique individuals. These samples were screened for duplicates (*i.e.*, samples considered to be from the same animal) after genotyping of the first eight loci was complete. Fifty samples had microsatellite genotypes that were identical to at least one other sample in the data set. In all cases, the mtDNA haplotypes and sexes of each pair also matched. Forty-two of the duplicate samples were identified in the PCFG stratum; 74% of these ( $n = 31$ ) were confirmed to be the same animal using photo-identification records. All 50 duplicate samples were removed from further analysis. No movements of animals between regions representing different strata were identified based on genetic matches (*i.e.*, all samples sharing identical genetic profiles were part of the same stratum). The number of unique individuals ( $n = 177$ ) remaining after removal of duplicates is shown in Table S3.

The proportion of missing genotypes at each locus was  $\leq 2\%$  for all loci (Table S2). Using the samples randomly selected for replication, a per-allele error rate of 0.11% was detected for the full microsatellite data set. After controlling for the FDR, no loci demonstrated significant deviations from HWE for either the probability test or the test for heterozygote deficiency. One pair of loci (EV94-SW19) showed significant linkage disequilibrium (LD) in the Chukotka and the North strata, while three pairs of loci (EV14-Gt023, EV94-RW48, and EV94-Gata098) demonstrated significant LD in the PCFG stratum. All loci were retained in subsequent analyses.

Further review of the microsatellite data set did not identify any samples that were identical for  $\geq 7$  loci. Two samples amplified at  $\leq 8$  loci and were removed from the microsatellite analyses, leaving a total of 175 unique individuals for the microsatellite analyses. These samples did produce useable mtDNA sequence data and were thus retained in that data set.

No discrepancies were identified when the replicated and original mtDNA haplotype sequences were compared. The mtDNA haplotype could not be resolved for three of the 177 individuals, and these individuals were removed from the mtDNA data set but retained in the microsatellite data set. Sex was determined for all of the 177 individuals.

### *Genetic Diversity*

Thirty-six mtDNA haplotypes defined by 36 variable sites were identified among the 174 individuals for which mtDNA haplotypes were resolved (Table 1). Thirty-two (NCBI Accession numbers AF326789–326824) of these haplotypes had been previously identified in LeDuc *et al.* (2002). The frequency of each haplotype in the defined strata (including Barrow) is shown in Table 2. Nineteen haplotypes were shared between the North and the PCFG strata, with four haplotypes found only in the PCFG. For all strata, many haplotypes were found in only one individual ( $n = 13$  haplotypes in the North,  $n = 12$  haplotypes in Chukotka, and  $n = 8$  haplotypes in the PCFG, including three of the haplotypes found only in the PCFG). Haplotype diversity ( $h$ ) was high in all strata defined for the analysis (0.945–0.953). Nucleotide diversity ( $\pi$ ) was also similar among the three defined strata (0.0144–0.0154). The median-joining network shows the relationship among mtDNA haplotypes and their

Table 1. Number of mtDNA control region haplotypes, haplotype diversity ( $\pm$  SE), and nucleotide diversity ( $\pm$  SE) within each stratum.

Strata	No. of samples	No. of haplotypes	Haplotype diversity ( $b$ )	Nucleotide diversity ( $\pi$ )
North <sup>a</sup>	103	32	0.952 ( $\pm$ 0.008)	0.0144 ( $\pm$ 0.008)
Chukotka	69	27	0.953 ( $\pm$ 0.011)	0.0145 ( $\pm$ 0.008)
PCFG	71	23	0.945 ( $\pm$ 0.010)	0.0154 ( $\pm$ 0.008)

<sup>a</sup>Samples from Chukotka are included as part of the North stratum.

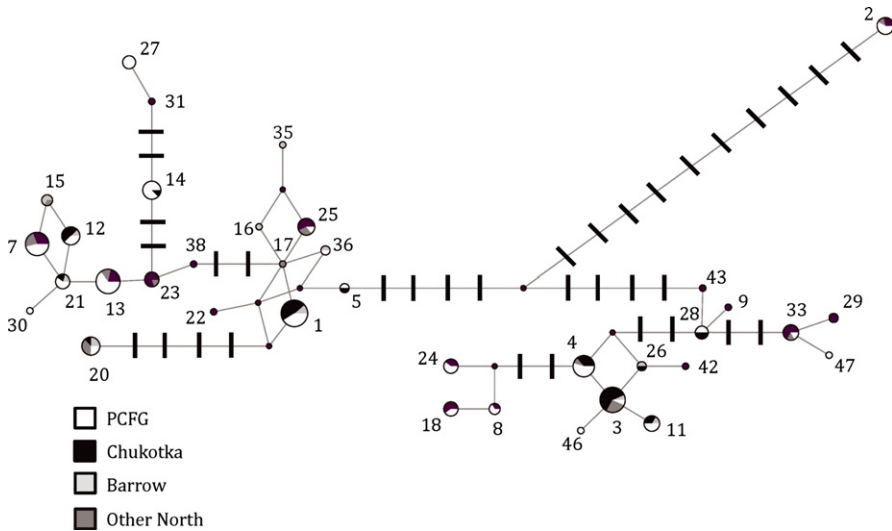
Table 2. The mtDNA haplotypes identified in the study, their corresponding NCBI accession numbers, and the number of individuals with each haplotype in each stratum.

MtDNA haplotype ID	NCBI accession number	North <sup>a</sup> ( $n = 103$ )	Chukotka ( $n = 69$ )	Barrow ( $n = 14$ )	PCFG ( $n = 71$ )
1	AF326789	10	8	2	7
2	AF326790	3	2	0	4
3	AF326791	14	9	1	1
4	AF326792	5	4	0	6
5	AF326793	1	1	0	1
7	AF326795	7	4	0	6
8	AF326796	1	1	0	2
9	AF326797	1	1	0	0
11	AF326799	3	2	1	3
12	AF326800	5	4	1	3
13	AF326801	5	3	0	9
14	AF326802	1	1	0	7
15	AF326803	3	0	2	0
16	AF326804	1	0	1	0
17	AF326805	1	0	0	0
18	AF326806	3	3	0	2
20	AF326808	6	1	2	2
21	AF326809	2	1	1	3
22	AF326810	1	1	0	0
23	AF326811	5	4	0	0
24	AF326812	2	2	0	3
25	AF326813	6	4	0	1
26	AF326814	2	1	1	0
27	AF326815	0	0	0	4
28	AF326816	2	2	0	2
29	AF326817	2	2	0	0
30	AF326818	0	0	0	1
31	AF326819	1	1	0	0
33	AF326821	5	4	0	1
35	AF326823	1	0	1	0
36	AF326824	1	0	1	1
38	KC917326	1	1	0	0
42	KC917327	1	1	0	0
43	KC917328	1	1	0	0
46	KC917329	0	0	0	1
47	KC917330	0	0	0	1

<sup>a</sup>Samples from Chukotka are included as part of the North stratum.

frequency in each stratum (Fig. 2). MtDNA haplotypes from both Chukotka and the PCFG are dispersed throughout the network, and no phylogeographic pattern was apparent.

A summary of nuclear diversity for each microsatellite locus is shown in Table S2. Measures of nuclear diversity for each stratum after averaging across loci are shown in Table 3. As in the comparisons of mtDNA haplotype and nucleotide diversity, nuclear diversity was similar across all strata. Nine alleles were found only among whales that were part of the North stratum (six of these were from Chukotka), and three alleles were identified only among PCFG whales.



*Figure 2.* Median-joining network showing relationships among the mtDNA haplotypes. The numbers next to the nodes correspond to the haplotype IDs listed in Table 4. The size of the nodes is proportional to the frequencies of the haplotypes, and each node is shaded to indicate the fraction of individuals with that haplotype from each stratum. The small black diamonds (unlabeled) indicate haplotypes that were inferred by the program but were not found among our samples. The length of lines connecting nodes is proportional to the inferred number of mutations separating haplotypes; for all haplotypes separated by more than one mutation, hash marks are used to represent the number of mutational events.

*Table 3.* Estimates of the number of alleles, observed heterozygosity ( $H_o$ ), and expected heterozygosity ( $H_e$ ) averaged across loci within each stratum for the microsatellite data. The genotypes of two samples that were used in the mtDNA analysis were removed because they amplified for  $\leq 8$  loci.

Strata	No. of samples	Mean number of alleles	Mean $H_o$	Mean $H_e$
North <sup>a</sup>	105	8.75	0.72	0.73
Chukotka	70	8.33	0.73	0.73
PCFG	70	8.00	0.74	0.73

<sup>a</sup>Samples from Chukotka are included as part of the North stratum.

### Sex Ratio

All strata were comprised of more females than males, with ratios of 1.4 females per male in each stratum (Table S3). This female bias is similar to that (1.47 females per male) described in Frasier *et al.* (2011). Although the female bias was not significantly different from the expected 1:1 ratio in any of the strata, when all samples were combined the female bias was significantly different from parity ( $\chi^2 = 5.43$ ,  $P < 0.05$ ).

### Genetic Structure

The results of the mtDNA comparisons are shown in Table 4a. The Tamura and Nei model of nucleotide substitution (Tamura and Nei 1993) with invariant sites (TrN + I) was selected as the most appropriate model of sequence evolution and was used in calculating  $\Phi_{ST}$ . When the PCFG stratum was compared with the North stratum, significant differences in mtDNA haplotype frequencies were detected using  $F_{ST}$  and the exact test ( $F_{ST} = 0.012$ ,  $P = 0.0045$ ; Fisher's exact test  $P = 0.0067$ ), but no significant differences were found in the  $\Phi_{ST}$  comparison ( $\Phi_{ST} = 0.012$ ,  $P = 0.0740$ ). Statistically significant differences were detected in all mtDNA comparisons of the PCFG stratum with the Chukotka stratum ( $\Phi_{ST} = 0.020$ ,  $P = 0.0386$ ;  $F_{ST} = 0.010$ ,  $P = 0.0348$ ; Fisher's exact test  $P = 0.0254$ ). None of the comparisons across strata utilizing the microsatellite data were significant (Table 4b).

## DISCUSSION

Given that PCFG whales share the same migratory routes and wintering grounds used by other ENP whales, it has generally been thought that PCFG whales interbreed with whales that feed further north (*e.g.*, Calambokidis *et al.* 2002, 2012). Here we were able to test that assumption directly by using microsatellite markers to compare PCFG whales with whales feeding north of the Aleutians. No significant nuclear differences between the two groups were identified, indicating that gray whales feeding in these areas likely represent a single interbreeding population. Significant differences in mtDNA haplotype frequencies were identified between the PCFG and northern feeding whales, however, suggesting that some structure exists among

Table 4. Results of pairwise comparisons across strata using (a) mtDNA and (b) 12 microsatellites. Comparisons that are statistically significant are shown in bold.

Pairwise comparison	$\Phi_{ST}$	<i>P</i> -value	$F_{ST}$	<i>P</i> -value	Fisher exact test <i>P</i> -value
(a)					
North <sup>a</sup> (103) vs. PCFG (71)	0.012	0.0740	0.012	<b>0.0045</b>	<b>0.0067</b>
Chukotka (69) vs. PCFG (71)	0.020	<b>0.0386</b>	0.010	<b>0.0349</b>	<b>0.0254</b>
Pairwise comparison	$F_{ST}$	<i>P</i> -value	$F_{ST}'$	<i>P</i> -value	$\chi^2$ <i>P</i> -value
(b)					
North <sup>a</sup> (105) vs. PCFG (70)	0.000	0.5269	0.000	0.5271	0.3491
Chukotka (70) vs. PCFG (70)	0.001	0.2539	0.003	0.2539	0.3503

<sup>a</sup>Samples from Chukotka are included as part of the North stratum.

feeding grounds used by ENP gray whales. Within the PCFG, this finding is concordant with photo-identification records that indicate that many animals first identified as calves return to the PCFG feeding area in subsequent years (Calambokidis *et al.* 2012). When combined, these findings are consistent with the second proposed hypothesis, and suggest that while mating is random with respect to feeding ground affiliation, utilization of feeding areas is influenced by internal recruitment.

The results of our mtDNA comparisons are similar to those presented in Frasier *et al.* (2011), who also found evidence of maternally driven structure when comparing samples from whales that were considered to represent the PCFG with a sample set comprised primarily of animals that stranded along the migratory route in the ENP. All of the samples utilized in the Frasier *et al.* (2011) study to represent the PCFG were collected from whales in Clayoquot Sound, which is located off the central west coast of Vancouver Island, British Columbia. In contrast, 89% of the samples representing the PCFG in this study were collected from animals in the waters off northern California, Oregon, and Washington, with only 12 samples (11%) collected off southern Vancouver Island. While the majority of PCFG whales photographed off southern Vancouver Island (52%) and northern Washington (60%) have also been sighted off western Vancouver Island, interchange between more distant areas (*e.g.*, comparison of northern California and western Vancouver Island) has been documented less frequently (Calambokidis *et al.* 2012). In addition, while some whales are known to move throughout the range of the PCFG, sightings of other whales are concentrated within subareas (Calambokidis *et al.* 2012), suggesting that individual gray whales may not use the range of the PCFG randomly. Thus while there is likely overlap among the individuals sampled in Frasier *et al.* (2011) and the current study, neither represents random sampling across the range of the PCFG. In the future, the collection of additional samples from whales in the northern portion of the PCFG range and/or integration of our sample set with that utilized by Frasier *et al.* (2011) would provide more evenly distributed sample coverage throughout the range of the PCFG and could provide insight into whether additional substructuring within the PCFG exists.

Despite the fact that the estimated abundance of the PCFG is roughly 1% of that of the ENP population as a whole, the haplotype diversity identified in the PCFG is similar to that found among strata representing the larger ENP population. This high haplotype diversity seems inconsistent with what might be expected if the PCFG was founded by a small number of individuals and has remained isolated (*e.g.*, all recruitment into the group is internal) for many generations. Under such a scenario, the mtDNA haplotypes carried by founders that were males or nonreproducing females would be lost over time, while haplotypes found in successfully reproducing females and their returning offspring would build to higher frequencies, resulting in reduced haplotype diversity in the group. However, the mtDNA haplotype diversity found within the PCFG, as well as the significant but relatively low level of mtDNA differences identified between the PCFG and northern feeding whales, could suggest that colonization of the PCFG range occurred relatively recently. Under this scenario, strong mtDNA differences between PCFG whales and individuals feeding further north may have had insufficient time to develop, and the number and distribution of haplotypes in the PCFG would not have been strongly affected by genetic drift. Little is known about the history and origin of the PCFG. Gray whales have been recorded feeding in the southern portion of the PCFG range as early as 1926, when a single gray whale, which was reported to have been feeding with four other whales, was taken by the Trinidad whaling station off the entrance to the Crescent City Harbor

in July (Howell and Huey 1930). Additional sightings of whales within the PCFG range during summer and fall were reported in the 1940s, 50s, and 60s (Gilmore 1960, Pike and MacKaskie 1969, Rice and Wolman 1971). The repeated return of individual whales to the area was first documented starting in the 1970s (Hatler and Darling 1974, Darling 1984). This time period marked the beginning of photo-identification studies for gray whales, and thus it is unknown if fidelity to the PCFG area occurred prior to this time or if the sightings recorded earlier were of animals that only visited the area during a single feeding season.

It is unclear what oceanographic conditions would have been present during the last century that would have precipitated use of the PCFG feeding area. Pyenson and Lindberg (2011) reconstructed the carrying capacity of gray whales over the past 120,000 yr by quantifying what feeding habitats would have been available during that time. They hypothesized that gray whales survived glacial fluctuations during the Pleistocene by employing generalist filter-feeding strategies that allowed them to take advantage of alternative food sources and feeding areas, similar to foraging strategies and areas used by PCFG whales today (*e.g.*, Darling *et al.* 1998, Dunham and Duffus 2001). More recently, access to the Bering Sea feeding areas would have been limited by heavy ice during parts of the “Little Ice Age” (*ca.* 1450–1850). Even if the PCFG seasonal range was colonized prior to the start of commercial whaling, this group of animals may have been greatly depleted or eliminated prior to the end of commercial whaling. Thus, it is plausible that the PCFG range may have been colonized multiple times in the past as a response to environmental changes and/or to depletion due to whaling.

The low level of mtDNA differentiation and high diversity are also consistent with a scenario in which matrilineal fidelity plays a role in determining use of the PCFG area but in which external recruitment also occurs. Given that the migratory route for whales traveling to the northern feeding ground(s) passes through the PCFG range, such recruitment could take place if migrating whales encounter a productive source of food within the PCFG range, remain in the area for the remainder of the season, and return in subsequent years (Calambokidis *et al.* 2002, 2012). External recruitment would slow the accumulation of genetic differences between PCFG whales and individuals feeding further north. Also, external recruits (at least initially) would likely carry haplotypes not previously identified among PCFG individuals, increasing the number and diversity of haplotypes found as well as the proportion of haplotypes currently shared between the PCFG and the animals feeding north of the Aleutians. Examination of the photo-identification data provides some information relevant to evaluating whether external recruitment into the PCFG could be occurring. Although photo-identification studies of the PCFG started in the early 1970s (Hatler and Darling 1977, Darling 1984), consistent efforts covering a larger portion of the PCFG seasonal range did not begin until 1998 (Calambokidis *et al.* 2012). Between 1998 and 2010, “new” (*i.e.*, previously unidentified) noncalf whales continued to be identified in the PCFG area each year, and many of these whales returned to the area in subsequent years (mean = 11 whales per year, 2002–2009, northern California to northern British Columbia; Calambokidis *et al.* 2012). It is unknown what proportion of these new whales could be immigrants into the group (*e.g.*, external recruits) and what proportion may be animals that were internally recruited but were not identified as calves during their first year (*e.g.*, “missed calves”). Although the number of calves identified on the PCFG range each year is low (mean = 3 calves per year, range 0–9, 2002–2009, northern California to northern British Columbia), calves may wean from their mothers as early as June or July, making them difficult to

identify as calves (*vs.* yearlings or young animals) and leading to underestimates of the number of calves present (Calambokidis *et al.* 2012). Indices of gray whale calf production based on estimates of the number of northbound calves past Piedras Blancas, California, are highly variable and averaged 4.3% (calf estimate/total population estimate, range 1.55%–6.8%) between 1994 and 2000 (Perryman *et al.* 2002). These estimates are likely high relative to the total number of gray whale calves that survive the full migration, as mortality of calves due to killer whale predation is known to occur in areas north of Piedras Blancas, including Monterey Bay, California (see summaries in Jefferson *et al.* 1991, Ford and Reeves 2008), an area that both PCFG and ENP whales traverse while migrating. While it is unknown how these estimates relate to calf production among PCFG whales, applying these indices to a group of 200 animals would result in a mean of 9 calves per year (range 3–13 calves per year).

In addition, comparison of nine whales photographed off Barrow, Alaska in 2006 and 2010 with the photo-identification catalog of animals identified within the PCFG range resulted in two matches (Calambokidis *et al.* 2012). One of these animals was photographed off Vancouver Island during March on a single occasion and thus may have been migrating through the area and would not be considered part of the PCFG. The second animal, however, had previously been sighted in multiple years during summer/fall in the PCFG area. While the significance of this match is difficult to interpret given the limited photo-identification data available from Barrow, it does indicate that at least this one individual has utilized more than one feeding ground during its lifespan.

Based on the genetic results presented here, it is not possible to determine the extent of immigration into the PCFG that could occur while still allowing mtDNA differences to be detected. While dispersal can be indirectly estimated from  $F_{ST}$  values (Wright 1931), the assumptions (*e.g.*, equal population sizes, equilibrium) of the underlying model are unlikely to be valid in wild populations (Whitlock and McCauley 1999). In addition, if the PCFG was isolated from the rest of the ENP population in the past, then the underlying level of genetic divergence would be related to the length of time the two groups had been separated and their effective sizes (Nei and Chakravarti 1977). As the underlying level of genetic divergence increases, the amount of recent immigration that could occur without obscuring the signal of mtDNA differentiation also increases. This highlights the fact that there are multiple scenarios (*e.g.*, colonization histories, number of founders, and immigration rates) that could lead to the pattern of mtDNA differentiation seen in the comparisons of the PCFG and the ENP samples. Given the information that is currently available, we are not able to discriminate among these possibilities.

A remaining question is whether additional structure exists within the northern feeding area. If there is no structure on the feeding grounds north of the Aleutians, then the northern strata (both “North” and “Chukotka”) could be considered representative of the genetic diversity of whales feeding throughout the northern feeding area and the mtDNA differences observed here would be driven by fidelity of individuals to the PCFG seasonal range. However, if structuring is present among northern feeding areas, then the differences demonstrated here may be influenced by fidelity of individuals in either or both areas (PCFG and Chukotka). While the results of photo-identification studies of the PCFG are consistent with the occurrence of some internal recruitment, the collection of additional samples from northern feeding areas would be valuable in further elucidating the mechanisms creating the observed differences and in evaluating whether structuring is present among whales utilizing the northern feeding grounds.



### *Implications for Management*

Understanding recruitment into the PCFG is relevant to management under the Marine Mammal Protection Act (MMPA). The goal of the MMPA is to maintain population stocks as functioning elements of their ecosystem. The National Marine Fisheries Service (2005) considers stocks to be demographically independent units, such that the population dynamics of the affected group is more a consequence of births and deaths within the group (internal dynamics) rather than of immigration or emigration (external dynamics). This definition is similar to that described for management units by Palsbøll *et al.* (2007) and for a population under the ecological paradigm by Waples and Gaggiotti (2006).

Traditionally, the most commonly used approach to evaluate demographic independence using genetic data has been null hypothesis testing, in which significant divergence of allele frequencies between groups is considered evidence supporting the delineation of separate management units (Moritz 1994). This approach assumes that if the migration rate is large enough to lead to demographic dependence, then genetic comparisons will not be able to reject the null hypothesis. Under this criterion, our findings support recognition of the PCFG of gray whales as demographically independent based on the significant differences in mtDNA between the PCFG and whales feeding further north.

Critical to our understanding of whether two groups are demographically independent, however, is the rate of dispersal between them. As noted in Waples and Gaggiotti (2006), there is no general framework for determining at what dispersal rate populations become demographically correlated, although it has been suggested that demographic correlation occurs when the proportion of immigrants in a group is greater than 10% (Hastings 1993). However, simulations have shown that, at least in cases where multiple microsatellite loci are used, it may be possible to reject panmixia even when dispersal rates are higher than this level (Palsbøll *et al.* 2006, Waples and Gaggiotti 2006). These results suggest that while genetic comparisons like those conducted here can provide insight into demographic connectivity, they should be interpreted carefully and integrated with other available information on the demography of the groups being considered (Lowe and Allendorf 2010).

When the significant mtDNA differences identified between the PCFG and the northern feeding strata are put into context with the other available evidence, questions arise about the balance between internal recruitment and external immigration. The significant mtDNA differences, as well as the observations of animals first identified as calves returning to the PCFG (Calambokidis *et al.* 2012), indicate that internal recruitment into the group occurs. However, the low level of mtDNA differences identified, the similarity in haplotype diversities between the PCFG and other groups thought to represent the larger ENP population, and the continued identification of "new" whales each year (Calambokidis *et al.* 2012) suggest that external immigration into the group may also be taking place. While other explanations (*e.g.*, recent colonization and a high rate of "missed" calves) exist that could be consistent with demographic independence of the PCFG, discriminating between these explanations is not currently possible.

Although uncertainty remains, our results indicate that it is plausible that the PCFG represents a demographically independent group and suggest that caution should be used when evaluating the potential impacts of the proposed Makah harvest on this group of animals. Continued monitoring of the PCFG, including the collection of additional photographs and genetic samples, is warranted. Future work

should focus on estimating dispersal rates and levels of internal recruitment in the PCFG. The lack of differentiation in nuclear markers identified in our study limits the use of some approaches (*e.g.*, assignment tests) commonly used to estimate dispersal. However, with the collection of additional samples from PCFG whales, a parentage-based approach, similar to that used by Peery *et al.* (2008), may be valuable in documenting internal recruitment into the group and thus in assessing the demographic independence of the PCFG.

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## SUPPORTING INFORMATION

The following supporting information is available for this article online at <http://onlinelibrary.wiley.com/doi/10.1111/mms.12129/supinfo>.

*Table S1.* Samples used in the study, including the SWFSC accession number, GeneticID, collection method (B = biopsy, H = harvest, S = stranding), date of collection, location of collection, strata, and whether the sample was retained in the final analysis. Samples were removed because they were considered duplicates (code 1), due to poor quality (code 2), or because they could not be assigned to a stratum (code 3, which includes whales that were sampled in the PCFG range but did not meet the criteria for being included in the PCFG stratum). GeneticID represents a unique identifier for individuals, such that samples that were considered to be from the same individual were assigned the same GeneticID. The strata specified include: North, CHK (Chukotka), PCFG, and South. Samples considered part of the CHK stratum were also included in the North stratum in the analyses. The South stratum includes samples collected from whales within the PCFG seasonal range but which did not meet the criteria for being classified as PCFG whales (see text for further explanation).

*Table S2.* Characteristics of the microsatellite loci used in the study, including the species for which primers were initially designed, the size of repeats, the annealing temperature used in the study ( $T_a$ ), the reference listing primer sequences, the number of alleles per locus, the proportion of missing genotypes, the expected heterozygosity ( $H_e$ ), the observed heterozygosity ( $H_o$ ), and the results of the test for heterozygote deficiency (HWE; Rousset and Raymond 1995).

*Table S3.* The total number of samples in each stratum, the number of samples removed from the study due to poor quality (see criteria described in text), the number of duplicate samples removed, and the number of individuals remaining in each stratum for each analysis. Duplicate samples (*i.e.*, samples from the same individual) were identified based on genotyping of eight microsatellite loci. Samples collected on the southern feeding ground but not considered to represent the PCFG ( $n = 36$ ) are not included in the table.