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SHORT COMMUNICATION

Multiple paternity in side-neck turtles *Podocnemis expansa*: evidence from microsatellite DNA data

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Abstract

Multiple paternity was found in two clutches of *Podocnemis expansa* using eight microsatellite loci. When loci were analysed separately a minimum of two males was estimated for nest N23, and three for nest C17. When all loci were combined, three patrilines were detected in N23, and six in C17. The distribution of full-sib cluster sizes indicated a disproportionate contribution of one male to clutch C17, consistent with possible sperm competition, or the mixing of leftover and newly acquired sperm. High mutation rates were detected at several loci. Multiple paternity has positive implications for this endangered species as it may slow the loss of genetic variability caused by drift. This is the first report of multiple paternity in the suborder Pleurodira.

Keywords: mating system, microsatellite, multiple paternity, *Podocnemis expansa*, turtles

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Introduction

Although multiple mating and sperm storage are well documented in turtles (e.g. Gist & Jones 1989), evidence of multiple paternity in nature is limited to a few Cryptodiran species: *Caretta caretta* (Harry & Briscoe 1988); *Clemmys insculpta* (Galbraith 1991); *Chelydra serpentina* (Galbraith *et al*. 1993); *Lepidochelys kempi* (Kichler *et al*. 1999); and *Chelonia mydas* (Parker *et al*. 1996; FitzSimmons 1998). Multiple paternity can have important consequences as it increases effective population sizes relative to single paternity (Sugg & Chesser 1994), and decreases estimated genetic correlations used in heritability studies (Rhen & Lang 1995).

Podocnemis expansa is an endangered South American pleurodirian species. Large clutch size (~100 eggs) and sperm storage (Alho *et al*. 1979) make *P. expansa* a good candidate for examination of multiple paternity. Because of the effects that multiple paternity can have on effective population sizes (N_e), genetic variability, and evolutionary potential, I conducted a preliminary survey to determine the presence of multiply sired clutches in Colombian populations of *P. expansa* using microsatellites as genetic markers.

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Materials and methods

Samples were collected from two clutches (C17: $n = 46$, and N23: $n = 19$) chosen at random from *Centro* beach, in the Middle Caquetá River in Colombia (1° 15′ S, 71° 30′ W). Blood (0.5 mL) was sampled from the cervical sinus using a 25-gauge needle inserted at the base of the skull, and was preserved in 'Queen's' lysis buffer (Seutin *et al*. 1991). Standard phenol–chloroform DNA extraction was used (Sambrook *et al*. 1989). Total DNA was digested with Sau3AI, *Bam*HI, *Hin*dIII. Fragments of 200–700 bp were selected, ligated to a Bluescript vector, and transformed into *Escherichia coli*. Dioxigenin-labelled oligos composed of artificial dinucleotide microsatellite sequences $(AT)_n$ $(CT)_n$, were hybridized with bacterial colonies grown on nylon membranes (Boehringer Mannheim™) using DIG Easy Hyb™ (Boehringer Mannheim™). Vector DNA was extracted with a Wizard Miniprep purification system (Promega™), and digested with *Bam*HI and *Hin*dIII. Insert DNA of the correct size was cycle-sequenced and characterized using an ABI-prism automatic sequencer (Perkin-Elmer™). Primers were designed for microsatellite loci containing at least 10 repeats. One primer per variable locus was 5′-end TET- or HEX-fluorochrome labelled. Individual genotypes were screened using ABI GeneScan™ software. Three primer sets for variable loci were obtained (PE344, PE519, and PE1075; Table 1), and

Table 1 Microsatellite primers characterized during the present study (sequences can be found in GenBank, Accession nos: AF141136, AF141137, and AF141138)

Locus ID	Primer sequence (5′–3′)	Tandem motif	No. of alleles	Size (bp)	Annealing temp. (°C)	$MgCl_2$ (mM)
PE344	ATCCTGAGTTTAAAGGTGA AACTCTTCAAACTCCTCTAG	$(AG)_{13}$	10	144–208	50	2
PE519	GCTGAGCTAGACTAACATGC GTAAATTGCCATACTTGGAG	$(CT)_7(CA)_8(CG)_2(CA)_8$	8	239–327	56	3
PE1075	ATGAGCCTGAAGAGTTGGAA AACTTAGGCTGCATGAGTTG	$(AC)_{11}$	6	247–283	54	3

were complemented with five additional microsatellite primers (Pod1, Pod62, Pod79, Pod128, Pod147) designed for Brazilian *P. expansa* (Sites *et al*., unpublished). Number of alleles and allele sizes per locus were confirmed through multiple PCR amplification, and by running all alternative allele sizes in a single scoring gel. Individuals were not scored until allele values were confirmed.

Data consist solely of hatchling genotypes. Maternal samples were not available, but it was possible to infer the partial or complete genotype for several loci. The detection of five alleles per locus among the hatchlings indicated the presence of multiple paternity if no maternal allele was known, four alleles if one maternal allele was detected, and three alleles if the complete maternal genotype was inferred. A maternal allele was detected whenever a homozygous hatchling was found at a given locus. The complete maternal genotype was inferred when homozygous hatchlings for two different alleles were found. If a hatchling had an unexpected allele at only one locus, it was assumed to come from mutation, but if the hatchling exhibited rare alleles at several loci, it was considered to be fathered by a different male (FitzSimmons 1998). Alleles derived from mutation were excluded from the analysis of independent loci, yielding a conservative estimate of the number of fathers. Tests for linkage disequilibrium at all pairs of loci were performed in GENEPOP 3.1 (Raymond & Rousset 1995), using samples from 55 nests, one individual per nest, to assure that the information from each locus was independent. Tests for heterozygote deficiency were performed to detect loci where null alleles may be present.

An overall estimate of the number of patrilines was obtained by clustering hatchlings (UPGMA) using their pairwise relatedness coefficients calculated from their multilocus data (Queller & Goodnight 1989) using Relatedness 5.0 (Goodnight & Queller 1989). Clusters with a relatedness coefficient above 0.5 were considered full-sib groups. To avoid overestimating the number of paternal sibships, single individuals that were sister clades to full-sib clusters were considered part of that cluster. Alternatively, band-sharing coefficients (Lynch 1990) were calculated as estimates of genetic similarity between individuals, and their association with relatedness coefficients determined through matrix correlation. To test for the disproportionate contribution of one male to each nest, the goodness of fit of the frequency distribution of full-sib cluster sizes to a Poisson distribution was calculated using a *G*-test (Sokal & Rohlf 1995). The probability of observing the largest cluster size detected given the sample size was calculated from a Poisson distribution.

Results

When analysed separately, all loci in nest C17 and several loci in nest N23 indicated multiple paternity (Table 2). Many loci indicated that at least two males sired the clutches. Three loci indicated that three males fathered C17. In the population analysis, none of the 28 dilocus comparisons showed significant linkage disequilibrium. Homozygotes for no more than two alleles were detected per locus in each nest. Partial, complete or likely maternal genotype was inferred for all loci. Although heterozygote deficiency was detected in the population samples at loci Pod128, Pod147, and PE1057 after Bonferroni correction ($P < 0.005$), null alleles were not present in either clutch, with the exception of individuals N23–11 and C17–61, who appeared homozygous for paternal alleles (Pod1: 202 bp, and Pod62: 204 bp, respectively).

The relatedness UPGMA tree suggests the presence of three and six full-sib clusters for N23 and C17, respectively (Fig. 1a,b). Seven individuals exhibited unexpected alleles at only one locus, and were considered to derive from mutation and not from a different father [Pod1: N23–6 (186 bp); N23–39 (150 bp); Pod128: N23–25 (171 bp), C17–49 (181 bp); Pod147: C17–20 (233 bp); PE344: C17–53 (203 bp)]. Individual C17–7 showed unique alleles at three loci (Pod62: 214 bp, Pod128: 171 bp, and Pod147; 185 bp) and is considered to be fathered by a different male. The distribution of cluster sizes in each clutch followed a Poisson distribution: $G_{[6]} = 30.4$, $P > 0.39$, and $G_{[3]} = 2.7$, $P > 0.9$ after Williams correction (Sokal & Rohlf 1995). The probability of observing the largest

Table 2 Evidence of multiple paternity from each of eight loci (PE344, PE519, PE1075, Pod1, Pod62, Pod79, Pod128, and Pod147) for nest N23 and C17. Number of alleles detected at each locus are given excluding mutations

	Nest N23 (n = 20)				Nest C17 (n = 46)			
Locus	Number of alleles detected	Homozygous hatchlings found	Multiple paternity evidence provided	Minimum number of fathers inferred	Number of alleles detected	Homozygous hatchlings found	Multiple paternity evidence provided	Minimum number of fathers inferred
PE344	3	None	No	1	4	For 1 allele	Yes	2
PE519	3	For 2 alleles	Yes	2	3	For 2 alleles	Yes	2
PE1075	4	For 1 allele	Yes	2	3	For 2 alleles	Yes	2
Pod1	4	None	No	1	6	For 2 alleles	Yes	**3**
Pod62	2	For 1 allele	No	1	4	For 1 allele	Yes	2
Pod79	3	None	No	1	7	For 1 allele	Yes	3
Pod128	4	None	No	1	5	For 2 alleles	Yes	3
Pod147	4	For 1 allele	Yes	2	5	For 1 allele	Yes	2

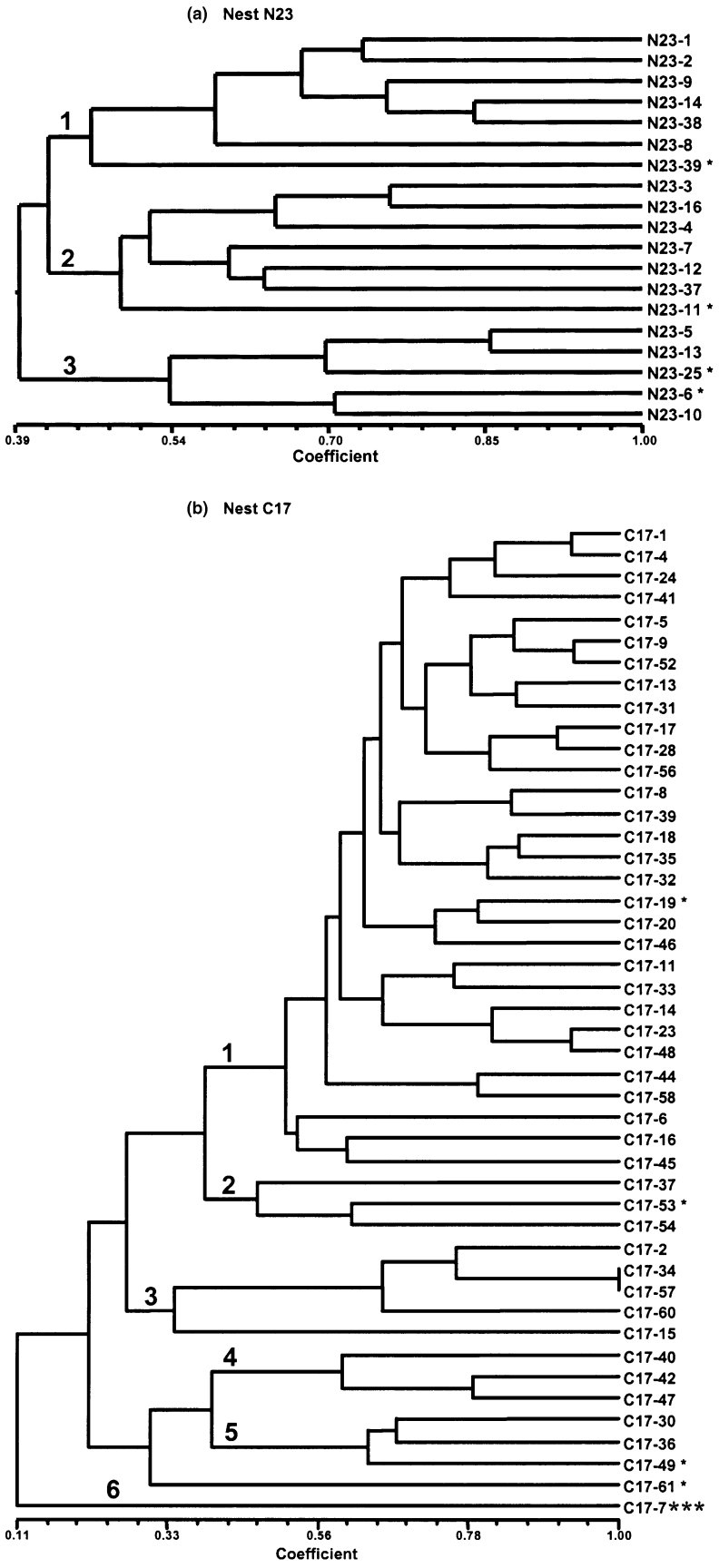

Fig. 1 UPGMA full-sibship dendograms. Estimated full-sib clusters are enumerated in the graph. Mutations detected per individual are denoted by asterisks. Singletons were considered part of the sister full-sib group. (a) Nest N23 presented three patrilines. Hatchlings N23–39 and N23–11 fall outside their full-sib cluster due to the presence of a mutation and are considered part of the same full-sib group. (b) Nest C17 presented six full-sib clusters. Individual C17–7 exhibited three unique alleles and consequently forms a patriline of its own.

cluster size in C17 was less than expected by chance ($P < 0.00001$) but was not significant for N23.

Discussion

The results from analyses of each locus independently identified a minimum of two fathers for nest N23, and three for nest C17. The larger number of fathers estimated by cluster analysis is not surprising, because the analysis of all data simultaneously can expose half-sib groups nested within a seemingly full-sib cluster. Although the presence of null alleles would underestimate multiple paternity, they were not detected except for two individuals, where they seem to come from mutation of a maternal allele.

Of the six unique alleles assigned to mutation, four were detected in the population sample and thus may come from undetected fathers. If these six unique alleles are true mutations, then these loci have some of the highest mutation rates observed for dinucleotide microsatellite loci in natural populations: 1.5–3.1%, and up to 4.6% including null alleles).

Differences in the estimates of the number of fathers could reflect true differences in the number of males that sired these clutches, but sampling error could contribute to this difference, because only 19 samples were available from N23 while 46 samples were available from C17. Clutch size (60 and 98 eggs, respectively) may partially explain the differences in number of fathers. In this species, clutch size (60–184 eggs) is positively correlated with female size (von Hildebrand *et al*. 1997; N. Valenzuela unpublished), which is positively correlated with age even after maturity (Ojasti 1971). Therefore, the clutch size difference may reflect age differences, and the smaller number of males found in clutch N23 could result if the younger female N23 mated fewer times than the older female C17. Nonetheless, multiple paternity was found in both nests, suggesting that this phenomenon might be prevalent in this species.

The low probability of observing a full-sib cluster of size 30 in nest C17 indicates the disproportionate success of one male, maybe through sperm competition. However, mixing scarce leftover sperm from previous years with abundant newly acquired sperm could also produce this pattern, even if sperm from each mating season is completely mixed and used in proportion to its abundance. Multiple paternity is a very important finding for this endangered species because it could increase N_e thus reducing the loss of genetic variability through drift (Sugg & Chesser 1994).

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Appendix I Genotypes per locus detected in this study for nests N23 and C17. Mutations are indicated in bold

ID	PE344	PE519	PE1075	Pod1	Pod62	Pod79	Pod128	Pod147
N23-1	171/193	317/325	255/283	170/182	194/194	248/262	135/179	219/211
N23-2	171/193	317/325	255/283	170/182	194/196	248/262	157/179	181/211
N23-3	171/165	325/291	255/227	170/176	194/194	248/262	157/147	219/211
N23-4	171/193	325/291	255/255	170/182	194/194	248/262	157/147	181/211
N23-5	171/193	325/291	255/251	170/182	194/194	234/262	157/179	219/219
N23-6	171/193	317/291	255/251	**186**/182	194/196	248/262	157/179	181/219
N23-7	171/165	317/291	255/251	170/182	194/196	234/262	157/147	181/211
N23-8	171/165	325/325	255/283	170/182	194/196	234/262	157/179	181/211
N23-9	171/193	317/325	255/283	170/176	194/194	234/262	157/179	181/211
N23-10	171/193	325/291	255/227	170/182	194/196	248/262	157/179	181/219
N23-11	171/165	325/291	255/255	**202**/202	194/194	234/262	135/147	181/211
N23-12	171/165	317/291	255/251	170/176	194/194	234/262	135/147	181/253
N23-13	171/193	317/325	255/251	170/182	194/194	234/262	135/179	219/219
N23-14	171/193	317/325	255/283	170/182	194/194	234/262	157/147	181/211
N23-16	171/165	317/291	255/255	170/176	194/194	248/262	157/147	219/253
N23-25	171/193	325/291	255/251	170/202	194/196	234/262	157/**171**	219/219
N23-37	171/165	325/291	255/227	170/176	194/196	234/262	157/147	181/253
N23-38	171/193	317/317	255/283	170/182	194/194	234/262	135/147	181/211
N23-39	171/165	317/317	255/255	170/**150**	194/194	248/262	135/179	219/211
C17-1	171/171	293/293	251/255	164/188	194/204	238/260	147/157	207/245
C17-2	165/171	317/293	255/281	170/182	194/194	236/260	157/185	181/207
C17-4	171/171	293/293	251/255	164/188	194/204	238/260	157/157	207/245
C17-5	171/171	293/293	251/255	170/188	188/194	222/260	157/157	207/207
C17-6	171/171	293/293	251/255	164/164	194/194	230/238	157/183	207/207
C17-7	171/183	317/293	251/255	170/182	194/**214**	**238**/**248**	**171**/183	**185**/241
C17-8	165/171	293/293	251/255	164/182	194/204	236/260	147/157	207/245
C17-9	171/171	293/293	251/255	164/170	188/194	222/260	157/157	207/245
C17-11	165/171	317/293	251/255	164/170	184/188	222/260	149/157	207/207
C17-13	171/171	317/293	251/255	170/172	188/194	236/260	157/157	207/245
C17-14	171/171	325/293	251/255	164/170	184/204	236/260	147/157	207/207
C17-15	165/171	317/325	255/255	170/182	194/194	248/260	149/149	207/211
C17-16	165/171	317/293	251/251	164/164	184/188	238/260	147/157	207/207
C17-17	165/171	317/293	251/255	170/188	188/194	238/260	157/157	207/245
C17-18	171/171	293/293	251/255	170/182	188/194	222/260	149/157	207/245
C17-19	171/171	293/293	251/255	170/172	184/188	238/260	157/157	207/**233**
C17-20	171/171	293/293	251/255	170/172	184/204	238/260	149/157	207/245
C17-23	171/171	317/293	251/255	164/170	194/204	236/260	157/157	207/207
C17-24	165/171	293/293	251/255	164/188	194/204	222/260	157/157	207/245
C17-28	165/171	293/317	251/255	164/188	188/194	238/260	157/157	207/245
C17-30	171/171	325/317	251/255	170/182	194/204	230/230	157/157	181/207
C17-31	171/171	293/317	251/255	170/170	188/194	238/260	157/157	207/245
C17-32	165/171	293/293	251/255	164/170	188/194	222/260	149/157	207/245
C17-33	165/171	317/293	251/255	170/172	184/188	236/260	157/157	207/207
C17-34	165/183	317/293	251/281	170/170	194/194	236/260	157/185	181/207
C17-35	171/171	293/293	251/255	164/182	184/188	222/260	149/157	207/245
C17-36	171/171	325/293	251/255	170/194	194/204	230/234	157/183	181/207
C17-37	171/171	293/293	251/255	164/182	184/204	248/260	149/183	207/211
C17-39	165/171	293/293	251/255	164/182	188/194	236/260	157/157	207/245
C17-40	165/193	325/325	251/251	170/172	184/204	230/248	149/183	207/241
C17-41	171/171	293/317	251/255	164/188	184/204	222/260	157/157	207/245
C17-42	171/193	325/325	251/255	170/170	184/204	230/242	149/157	207/207
C17-44	165/171	293/317	251/255	164/188	184/204	236/260	149/157	207/245
C17-45	171/171	317/293	251/255	164/188	188/194	222/260	147/185	207/207
C17-46	171/171	317/293	251/255	164/170	184/188	238/260	149/157	207/245
C17-47	171/193	293/325	251/255	170/182	184/204	230/248	149/157	207/207
C17-48	171/171	293/317	251/255	170/188	194/204	236/260	157/157	207/207
C17-49	165/171	325/325	251/255	170/194	194/194	230/230	157/**181**	181/207
C17-52	171/171	293/293	251/255	164/170	188/194	238/260	157/157	207/245
C17-53	171/**203**	293/317	255/255	164/182	194/204	230/242	149/157	181/207
C17-54	171/183	293/317	251/255	164/182	194/204	230/260	149/157	207/211
C17-56	165/171	293/293	251/255	164/188	188/194	238/260	157/157	207/207
C17-57	165/183	317/293	251/281	170/170	194/194	236/260	157/185	181/207
C17-58	165/171	317/293	251/255	164/188	188/194	236/260	149/157	207/245
C17-60	183/193	317/293	251/251	170/182	194/194	236/260	149/157	181/207
C17-61	171/183	317/325	251/255	170/172	**204**/204	230/242	149/157	181/207