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NOTE

Evidence for multiple paternity in two species of *Orconectes* crayfish

A.F. Kahrl, R.H. Laushman, and A.J. Roles

Abstract: Multiple mating is expected to be common in organisms that produce large clutches as a mechanism by which sexual reproduction can enrich genetic variation. For freshwater crayfish, observation of multiple mating suggests the potential for high rates of multiple paternity, but genetic confirmation is largely lacking from natural populations. We studied paternity within wild-caught broods of two crayfish species in the genus *Orconectes* (Sanborn's crayfish (*Orconectes sanbornii* (Faxon, 1884)) and the Allegheny crayfish (*Orconectes obscurus* (Hagen, 1870))). Although females have been observed mating with multiple males, this is the first genetic confirmation of multiple paternity in broods of these two species. Berried females were collected in the field and maintained in aquaria until their eggs hatched. We amplified and genotyped extracted DNA from maternal and hatchling tissue for several microsatellite loci. For both species, paternity reconstruction (GERUD 2.0) yielded 2–3 sires per brood and no single paternity clutches. We discuss these results from natural populations in light of the body of work on reproductive ecology of decapod crustaceans and in the context of changes in life history following the transition from marine to freshwater habitats.

Key words: Orconectes, crayfish, multiple paternity, *Orconectes sanbornii*, Sanborn's crayfish, *Orconectes obscurus*, Allegheny crayfish, decapod crustacean.

Résumé : L'accouplement multiple devrait être répandu chez les organismes qui produisent de grandes pontes, agissant comme mécanisme par lequel la reproduction sexuée peut enrichir la variation génétique. Pour les écrevisses d'eau douce, l'observation d'accouplements multiples semble indiquer la possibilité de taux élevés de paternité multiple, bien que la confirmation génétique de ce phénomène dans les populations naturelles fasse généralement défaut. Nous avons étudié la paternité au sein de nichées capturées à l'état sauvage de deux espèces d'écrevisses du genre *Orconectes* (l'écrevisse de Sanborn (*Orconectes sanbornii* (Faxon, 1884)) et l'écrevisse obscure (*Orconectes obscurus* (Hagen, 1870))). S'il a été observé que des femelles s'accouplaient avec plusieurs mâles, il s'agit de la première confirmation génétique de la paternité multiple de nichées chez ces deux espèces. Des femelles œuvées ont été prélevées sur le terrain et maintenues dans des aquariums jusqu'à l'éclosion de leurs œufs. Nous avons amplifié et génotypé de l'ADN extrait de tissus maternels et des bébés pour plusieurs microsatellites. Pour les deux espèces, la reconstitution de la paternité (GERUD 2.0) a donné de 2 à 3 géniteurs par nichées, aucune des pontes ne présentant une paternité unique. Nous discutons de ces résultats en ce qui concerne les populations naturelles à la lumière des travaux antérieurs sur l'écologie de la reproduction des crustacés décapodes et dans le contexte des modifications du cycle biologique à la suite du passage d'habitats marins à des habitats d'eau douce. [Traduit par la Rédaction]

Mots-clés : Orconectes, écrevisse, paternité multiple, *Orconectes sanbornii*, écrevisse de Sanborn, *Orconectes obscurus*, écrevisse obscure, crustacé décapode.

Introduction

Organisms with high reproductive output living in variable environments frequently engage in mating with multiple partners (Birkhead 2000; Avise et al. 2011), which is hypothesized to improve fitness by maximizing the production of genetically variable offspring (Yasui 1997; Jennions and Petrie 2000). Both sexes may benefit; females may produce a variable brood and males may sire a larger total number of offspring across several broods when females can store sperm. However, the differences in these potential gains create conflicts between males and females over paternity of a single brood. Selection may favor strategies that prevent multiple paternity within a brood in males, while selection may favor strategies that allow for multiple paternity in females (Stockley 1997). Determining the extent of multiple paternity within and among species provides insight into the resolution of this conflict and the evolution of mating systems.

The benefits of multiple mating have been studied in many diverse taxa (Jennions and Petrie 2000), and life-history characteristics are hypothesized to influence the prevalence and degree of multiple mating or paternity (Avise et al. 2011). Crustacean decapods typically produce large clutches, store sperm, and mate promiscuously. Multiple paternity is frequently observed in this group, generally with 2–3 sires per brood (range of 1–11; Avise et al. 2011) and variable proportions of paternity across sires (e.g., Walker et al. 2002; Streiff et al. 2004; Toonen 2004; Gosselin et al. 2005; Sainte-Marie et al. 2008; Yue and Chang 2010; Yue et al. 2010). Crustacean decapods have nonmotile sperm encapsulated in single or multiple spermatophores that are transferred to the female externally or internally (Reynolds 2002), enabling manipulation of fertilization by males and females. In crayfish, females produce usually a single brood annually and have the capacity to store sperm in their spermatheca throughout a mating season (Holdich and Reeve 1988; Buřič et al. 2013), permitting multiple paternity

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Table 1. Genotyped sample sizes (*N*; total number of juveniles sampled in parentheses), number of alleles per locus, and paternity estimates for each brood of Sanborn's crayfish (*Orconectes sanbornii*; San) and the Allegheny crayfish (*Orconectes obscurus*; Obs).

		Number of alleles				Percent progeny		
Brood	*N*	*3.1*	*2.12*	*AP2*	*AP3*	1° sire	2° sire	Minimum number of sires per brood
San1	32 (32)	2	—	3	1	93	7	2
San2	13 (40)	5	—	3	1	84	16	2
San3	16 (40)	6	—	2	—	56	44	2
San4	32 (40)	4	—	4	3	48	45	3
San5	18 (40)	—	—	3	2	61	39	2
Mean ± SD		4.25±1.7	—	3.0±0.7	1.75±0.9			2.2±0.5
A		9	—	6	4			
Obs1	36 (40)	3	4	—	3	79	21	2
Obs2	35 (40)	5	2	—	2	75	20	3
Obs3	13 (23)	4	2	—	3	61	31	3
Mean ± SD		4.0±1.0	2.7±1.2	—	2.7±0.6			2.7±0.6
A		8	4	—	6			

Note: The mean (SD) number of alleles and total number of alleles (*A*) reported for each locus are also shown. A dash indicates unsuccessful amplification.

within a single brood when the female is polyandrous and sperm mixing occurs. Male crayfish may bias paternity in their favor via several mating behaviors such as extruding sperm plugs (Holdich and Reeve 1988), interrupting mating pairs (Stein 1976), diluting sperm masses (Rubolini et al. 2007), and removing sperm masses from previous males (Villanelli and Gherardi 1998; Galeotti et al. 2007). Additionally, female crayfish exhibit precopulatory choice for male body size (Aquiloni and Gherardi 2008*a*) and fighting ability (Aquiloni et al. 2008), as well as postcopulatory cryptic female choice (Galeotti et al. 2006; Aquiloni and Gherardi 2008*b*).

While multiple mating has received extensive attention in some taxa, crustacean decapods are relatively understudied with information on genetic parentage available for only about 12 species (summarized in Gosselin et al. 2005; Avise et al. 2011). Of those studies, only three describe freshwater species: a shrimp (Yue and Chang 2010) and two crayfish (Walker et al. 2002; Yue et al. 2010). Brood size, brood care, and development differ substantially between freshwater and marine decapods, with freshwater decapods tending to have small broods of relatively large eggs with direct development that require extensive brood care in contrast to the larger broods of small eggs with an extended planktonic larval cycle common in marine decapods (Vogt 2013). Improved resolution on the frequency of multiple paternity in marine and freshwater species may provide insight into mating system adaptations that arose during the transition from marine to freshwater environments. Our study contributes two additional species to the list of documented crustacean decapods: Sanborn's crayfish (*Orconectes sanbornii* (Faxon, 1884)) and the Allegheny crayfish (*Orconectes obscurus* (Hagen, 1870)).

Using a panel of microsatellite markers, we report patterns of paternity for multiple broods of both species by estimating the minimum sires per brood and the proportion of offspring per male parent for multiple-sire broods. We then compare our results with those found for two other freshwater crayfish species and for other crustacean decapods. This information is critical to improve our understanding of this relatively understudied group and to contribute to the knowledge of variation in crustacean mating systems, especially in freshwater versus marine habitats.

Materials and methods

Crayfish collection

We collected crayfish in April of 2008 from allopatric locations within each species' native range in Ohio (Thoma and Jezerinac 2000) by disturbing rocks upstream of a handheld seine. We collected six *O. sanbornii* berried females (i.e., with eggs attached to her pleopods) from the Vermilion River (41°21′N, 82°21′W) and eight *O. obscurus* berried females from the Mahoning River (41°21′N, 83°30′W). Each of these females was transported to the laboratory, kept in isolation in 2.5 gallon tanks (1 gallon = 3.785411784 dm^3), and fed Crab Cuisine (Hikari, Hayward, California, USA) until the eggs hatched. After the eggs hatched and juveniles reached stage 2 (had their first molt and were no longer attached by a telson thread to the dam), 23–40 offspring were sampled from each brood (Table 1). We were unable to determine total clutch size brooded by females, but it is likely that clutch size falls into the range of 20–514 (mean 250) observed for the congener *Orconectes placidus* (Hagen, 1870) (placid crayfish) (Walker et al. 2002). Maternal tissue samples consisted of several pleopods (abdominal swimmerets) from each individual. All tissue samples were stored in 300 μL Cell Lysis Buffer (Qiagen, Valencia, California, USA) at 4 °C prior to DNA isolation.

DNA isolation

DNA was isolated from the samples using the Gentra Puregene DNA Purification Kit (Qiagen). Samples were homogenized in 300 μL Cell Lysis Buffer with a mortar and pestle. Proteins were removed from the homogenized sample with 100 μL of Puregene Protein Precipitation Solution (Qiagen) then pelleted by centrifugation. The DNA was precipitated using 300 μL of 100% isopropanol, pelleted by centrifugation, and then washed with 300 μL of 70% ethanol. DNA pellets were allowed to dry completely and were then rehydrated overnight at room temperature with 50 μL of Puregene DNA Hydration Solution (Qiagen). DNA quality was checked by agarose gel electrophoresis.

Microsatellite amplification and paternity analysis

Several studies indicate reasonable cross-species amplification among crayfishes (Belfiore and May 2000; Gouin et al. 2000). A.J. Roles, R.H. Laushman, and E.B. Kohler (unpublished data, 2008) optimized polymerase chain reaction (PCR) conditions, tested reliability, and characterized polymorphism for several markers developed for *O. placidus* (Walker et al. 2002) and the white-clawed crayfish (*Austropotamobius pallipes* (Lereboullet, 1858)) (Gouin et al. 2000) in several populations of *O. sanbornii* and *O. obscurus*. For this study, we amplified four dinucleotide repeat microsatellite loci (Table 2). Isolated DNA was amplified by PCR with a final reaction volume of 25 μL: 19 μL of sterile water, 2.5 μL 10× RED*Taq* buffer (Sigma-Aldrich, St. Louis, Missouri, USA), 0.375 μL of forward and reverse primers (10 μmol/L), 0.25 μL dNTPs (10 mmol/L), 0.5 μL of Failsafe 2× Premix J (Epicentre Biotechnologies, Madison, Wisconsin,

USA), 1 μL of DNA, and 1 μL of RED*Taq* (1 unit/μL). All reactions contained 1.1 mmol/L magnesium chloride. We used the following PCR cycling conditions: initial denaturation step of 2 min at 95 °C; 30 cycles of denaturation (1 min at 95 °C), annealing (1 min at annealing temperature), and extension (1 min at 72 °C); with a final extension of 3 min at 72 °C. This program was adapted from the procedure outlined by Walker et al. (2002) with number of cycles and temperature modifications made as necessary. For *O. sanbornii*, annealing temperatures were 55 °C for locus *3.1*, 45 °C for locus *Ap3*, and 42 °C for locus *Ap2*; amplification was unsuccessful for locus *2.12*. For *O. obscurus*, annealing temperatures were 65 °C for loci *2.12* and *3.1* and 42 °C for locus *Ap3*; amplification was unsuccessful for locus *Ap2*. Primer sequences are found in Walker et al. (2002) and Gouin et al. (2000). Amplified PCR products were run on an Applied Biosystems 3730xl DNA Analyzer with the LIZ 500 GeneScan Size Standard (Applied Biosystems, Grand Island, New York, USA). Fragment sizes were analyzed using Peak Scanner version 1.0 software (Applied Biosystems). If amplification failed for a particular individual, we attempted at least once to reamplify the sample. Given our small sample sizes, we did not test for Hardy–Weinberg proportions in these samples. However, the unpublished work mentioned above reported that all of the loci used in this study were in Hardy–Weinberg proportions across multiple populations.

We estimated the minimum number of sires per brood from the genotype data using GERUD 2.0 (Jones 2005). This program uses a multilocus minimum method, which totals the multilocus paternal gametotypes and divides that number by two. The program subtracts the maternal genotype (for all loci) and then calculates the minimum number of paternal genotypes required to produce the progeny in that array. Paternity was estimated from a minimum of two loci for each brood; broods that were not successfully genotyped for at least two loci were excluded.

Results

In total, we successfully isolated DNA from five of six *O. sanbornii* and from three of eight *O. obscurus* berried females from the original capture. The other six broods were excluded due to mortality of the dam (one of eight *O. obscurus* dams) or of eggs. We successfully amplified a total of four loci: loci *3.1* and *AP3* for both species, as well as locus *2.12* for *O. obscurus* and locus *AP2* for *O. sanbornii* (Table 1). We experienced some amplification failure with these microsatellite primers, which may have been due to yolk-protein contamination in the offspring DNA sample. Within broods, some samples from juveniles consistently failed to amplify for one or more primer pairs as indicated by the number of genotyped samples versus the total number of juveniles sampled (Table 1). For successfully amplified samples, the alleles of the offspring showed independent assortment following simple Mendelian inheritance. Locus *3.1* was the most polymorphic, with a total of 9 alleles in *O. sanbornii* (mean 4.25 alleles per brood) and 8 alleles in *O. obscurus* (mean 4.0 alleles per brood; Table 1). GERUD 2.0 requires a minimum of two loci with offspring genotypes for all loci analyzed; therefore, for each brood some juveniles and loci were excluded in the paternity analysis.

GERUD 2.0 calculates the most likely combinations of the paternal genotypes, providing estimates of each sire's contribution to the brood. The mean number of sires per brood was 2.2 (range 2–3, $N = 5$; Table 1) for *O. sanbornii* and 2.7 (range 2–3, $N = 3$; Table 1) for *O. obscurus*. No broods exhibited patterns consistent with single paternity. These findings agreed with direct exclusion methods. The sire contributions for each brood were variable with contributions ranging from 48% (brood with three sires) to 93% (brood with two sires; Table 1). None of the reconstructed paternal genotypes were shared across broods, suggesting that all of the sires were unique.

Discussion

Observations of multiple mating attempts of female crayfish by two or more male crayfish have been made in captivity (Berrill and Arsenault 1982; Reynolds 2002; Buřič et al. 2013), and many laboratory studies have addressed the reproductive ecology of crayfish (e.g., Snedden 1990; Rubolini et al. 2007; Aquiloni and Gherardi 2008*a*, 2008*b*; Galeotti et al. 2009). However, few studies have examined genetic paternity; here, we infer that females not only copulate with multiple males, but that they use the sperm from more than one male to fertilize their eggs. In fact, none of the eight broods that we studied exhibited single paternity (Table 1). The range of 2–3 sires per brood observed for both of our species is very similar to findings in the congener *O. placidus* (1.8 sires per brood, $N = 15$; Walker et al. 2002) and in the red swamp crayfish (*Procambarus clarkii* (Girard, 1852)) (2.6 sires per brood, $N = 30$; Yue et al. 2010). Additionally, our data provide no evidence of polygyny within the sampled populations, as the predicted sire genotypes were not replicated within more than one brood. However, this finding is most likely a limitation of sample size; increased sampling of berried females may reveal male polygyny.

We detected substantial variation in the proportion of offspring sired, from cases of a single male siring 93% of the offspring to a three-sire brood in which two sires were responsible for 48% and 45% of the offspring. While our data do not shed light on the source of this variation, a variety of postcopulatory behaviors are known in crayfish that may bias paternity success. Male crayfish may bias paternity by controlling the size of their sperm deposit (Rubolini et al. 2006), using sperm plugs to seal the spermatheca (Bauer and Min 1993), and removing or displacing previously deposited sperm and sperm plugs (Villanelli and Gherardi 1998; Galeotti et al. 2007). Additionally, females may exert cryptic choice after copulation by adjusting their reproductive investment; for example, in single matings, female crayfish can alter investment in brood size and egg size depending on mate quality (Galeotti et al. 2006; Aquiloni and Gherardi 2008*a*, 2008*b*). However, in experimental no-choice multiple matings with *O. rusticus*, Snedden (1990) found that 92% of eggs were fertilized by the last male to mate. Our results demonstrate far greater variation in the proportion paternity than observed by Snedden (1990); in natural populations, paternity bias may be driven by both male and female mechanisms. This could explain cases of extreme primacy by a single male (e.g., 93% by one sire, 7% by the other) and the high variation in the proportion of paternity across broods. Further work is required to determine the possible postcopulatory mechanisms and reproductive strategies causing variation in the pattern of paternity in two natural populations.

Of broader interest may be the comparison of genetic parentage patterns in freshwater versus marine Decapoda. Marine species may release clutches numbering in the thousands to millions and amphidromous Decapoda may have clutches in the tens of thousands (Vogt 2013). In contrast, true freshwater taxa produce clutches of no more than a few hundred and terrestrial Decapoda typically have the smallest clutches, in the tens (Vogt 2013). This pattern may be related to the developmental sequence and amount of parental care that the brood receives (Vogt 2013). We might expect clutch size and multiple mating to be positively correlated in invertebrate brooders because larger broods provide more opportunity for multiple patrilines and the genetic benefits to the incubating parent of multiple patrilines may be amplified in a larger brood. However, in a review of the data for invertebrates, Avise et al. (2011) found no relationship between multiple mating and clutch size, which they suggested may be due to variation in ecological and natural history factors that affect mate acquisition (e.g., population densities, duration of mating season, female trans-molt sperm retention). In examination of the 12 decapod taxa included in Avise et al.'s (2011) review, we note that in comparison with the 3 freshwater taxa, the 9 marine taxa, on

average, exhibit fewer mates per brood (mean 1.6 versus 3.2) and a reduced frequency of multiple paternity clutches (mean 42% versus 86%). The inclusion of our two freshwater taxa slightly reduces the mean for the freshwater taxa to 2.9 mates per brood and increases the observation of multiple paternity broods to 91%. Although three mates per brood is still far below the theoretical detection limit of the developed markers' capabilities, the scant available data suggest a trend contrary to the expected positive relationship between clutch size and multiple paternity (Avise et al. 2011). Comparison of marine decapods to freshwater taxa can give insight into the evolution of mating systems in the transition from marine to freshwater habitats.

Perhaps the more fragmented geographic ranges and resulting smaller effective population sizes of freshwater taxa versus their marine relatives (DeWoody and Avise 2000) increases the benefits of multiple paternity. Coleman and Jones (2011) found no support for this hypothesis in freshwater versus marine fishes and instead suggest that the type of parental care is a stronger predictor of patterns of parentage. This pattern may be especially difficult to tease apart in decapod crustaceans because the degree of parental care and habitat are generally confounded (Vogt 2013). Clearly, additional case studies are needed to determine whether a pattern exists and to establish what factors may explain the observed variation in clutch size and paternity in freshwater and marine species.

Conclusions

Our results are comparable with those of other studies on genetic parentage in crustaceans and are especially similar to other crayfish. Even with a small sample size, it is clear that multiple paternity is a common occurrence in *O. obscurus* and *O. sanbornii*, as all eight broods contained multiple patrilines. In addition, our results indicate that there is considerable variation in the proportion of offspring sired by each male, calling for additional work to identify mechanisms affecting the prevalence and persistence of multiple paternity in these species. This study also contributes to the small number of studies examining multiple paternity in Decapoda, where a pattern is emerging of higher rates of multiple paternity in freshwater taxa than in marine taxa.

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