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The unexpected genetic mating system of the red-backed toadlet (*Pseudophryne coriacea*); a species with prolonged terrestrial breeding and cryptic reproductive behaviour

Short running title: Cryptic reproductive behaviour in a frog

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Abstract

Molecular technologies have revolutionised our classification of animal mating systems, yet we still know very little about the genetic mating systems of many vertebrate groups. It is widely believed that anuran amphibians have the highest reproductive diversity of all vertebrates, yet genetic mating systems have been studied in less than one percent of all described species. Here, we use SNPs to quantify the genetic mating system of the terrestrial breeding red-backed toadlet *Pseudophryne coriacea*. In this species, breeding is prolonged (approximately 5 months), and males construct subterranean nests in which females deposit eggs. We predicted that females would display extreme sequential polyandry because this mating system has been reported in a closely-related species (*P. bibronii*). Parentage analysis revealed that mating success was heavily skewed towards a subset of males (30.6% of potential sires), and that nearly all females (92.6%) mated with one male. In a high percentage of occupied nests (37.1%) the resident male was not the genetic sire, and very few nests (4.3%) contained clutches with multiple paternity. Unexpectedly, these results show that sequential polyandry is rare. They also show that there is a high frequency of nest takeover and extreme competition between males for nest sites, but that males rarely sneak matings. Genetic analysis also revealed introgressive hybridisation between *P. coriacea* and the red-crowned toadlet (*P. australis*). Our study demonstrates a high level of mating system complexity and it shows that closely-related anurans can vary dramatically in their genetic mating system.

KEYWORDS

Amphibian, cryptic breeding, genetic mating system, nest takeover, reproductive behaviour, SNP

1 | INTRODUCTION

Knowledge of mating systems is important for understanding mechanisms of sexual selection and the evolution of reproductive strategies (Emlen & Oring, 1977; Avise et al., 2002; MacManes, 2013). Historically, our understanding of animal mating systems has been based on behavioural observation, with classification of mating systems broadly defined according to the number of mates acquired by each sex (i.e. social mating systems). While this approach has provided fundamental insights into intra- and inter-specific variation in reproductive strategies, social mating systems can be extremely misleading (Hughes, 1998). Behavioural observations will only yield accurate estimates of sex-specific differences in mating frequency if individuals can be continuously monitored, and copulations easily observed. While this might be possible in species with discrete breeding events and conspicuous copulation, it is near impossible in species with prolonged breeding seasons, large home ranges and/or cryptic mating behaviour. Moreover, in systems where females mate with multiple males, post copulatory processes such as sperm competition (Parker, 1970; Simmons, 2001) and cryptic female choice (Eberhard, 1996) can result in fertilisation biases that preclude the reliable assignment of paternity through observation alone (Birkhead, 1998; Pizzari & Wedell, 2013).

Over the past two decades, rapid advances in molecular technologies have revolutionised our classification of animal mating systems by enabling extremely accurate assignment of parentage to offspring (Avise, 1994; Kaiser et al., 2017). The capacity to unambiguously determine the mating success of every individual in a population has unveiled a complex and diverse array of reproductive strategies (Hughes, 1998; MacManes, 2013). Studies of vertebrates in particular have revealed startling discrepancies between social and genetic mating systems (Gagneux et al., 1999; DeWoody & Avise, 2001; Garant et al., 2001; Griffith et al., 2002; Uller & Olsson, 2008). Many species long considered to be monogamous have been revealed to be highly promiscuous, with females actively seeking extra-pair copulations (Griffith et al., 2002). In addition, a diversity of alternative mating tactics have been uncovered, with competitively-inferior males gaining surprisingly high levels of mating success through behaviours such as female mimicry, forced copulation, satelliting and sneaking (Neff & Svensson, 2013). Despite knowledge of vertebrate mating systems increasing exponentially over the past decade, most

genetic work has focussed on birds and mammals (Coleman & Jones, 2011; Dawson et al., 2013). Therefore, we still know comparatively very little about the genetic mating systems of ectothermic vertebrates (Garant et al., 2001).

Anuran amphibians (frogs and toads) have long been a model group for studies of sexual selection and reproductive strategies, and behavioural observations have indicated that anurans display the greatest reproductive diversity of all tetrapods (Duellman & Trueb, 1986). Surprisingly, however, there remains very little known about anuran genetic mating systems. To date, genetic analyses of mating systems have been made for less than twenty species, representing less than one percent of all described species. Nevertheless, considerable diversity has already been uncovered, with reports of mating systems ranging from extreme monogamy and polygyny, to extreme polyandry and polygynandry (Laurila & Seppä, 1998; Lodé & Lesbarrères, 2004; Byrne & Keogh, 2009; Knopp & Merilä, 2009; Brown et al., 2010; Ringler et al., 2012; Cheng et al., 2013; Wang et al., 2014). Critically, however, the vast majority of these studies have only considered a fraction of a species' breeding season, or sampled a very small subset of breeding individuals and offspring. Few studies have undertaken exhaustive sampling and determined patterns of parentage for entire breeding populations, particularly for prolonged breeding species (but see Ursprung et al., 2011; Mangold et al., 2015). The lack of comprehensive genetic analyses of parentage in prolonged breeding species, which constitute a large fraction of all anurans (Wells, 2001), means we still have a very superficial understanding of anuran mating system variation and reproductive ecology.

One group of prolonged breeding anurans that provide an excellent opportunity to investigate genetic mating systems are terrestrial toadlets from the genus *Pseudophryne* (Myobatrachidae). The genus is comprised of 14 species with natural- and life-history traits highly amenable to exhaustive sampling. Specifically, toadlets are characterised by non-aquatic egg deposition, small clutch sizes (typically < 100 eggs) (Anstis, 2017) and extreme breeding-site fidelity (Heap et al., 2014). Males excavate small, concealed chambers in loose soil or leaf litter and use a combination of calls and chemosignals to attract females (Byrne & Keogh, 2007). Mating takes place in nests, and males remain with the eggs until the nest floods and hypoxia triggers hatching. An early study of the breeding biology of three *Pseudophryne* species (*P.*

bibronii, P. dendyi, P. semimarmorata) suggested that females routinely divide their egg clutches between the nests of multiple males. This was based on the observation that clutch sizes in nests were highly variable and often a fraction of the size of clutches held by unmated gravid females (revealed through dissection)(Woodruff, 1976). More recently, a study of the genetic mating system of the brown toadlet *P. bibronii* confirmed that females do indeed mate with multiple males. Using microsatellites to assign parentage to offspring, Byrne and Keogh (2009) revealed that all females were polyandrous, dividing their eggs between the nests of two to eight males. To date, this remains the most extreme level of sequential polyandry reported in a vertebrate. Moreover, the study provided evidence that polyandry is adaptive because it increases female fitness by acting as an insurance against clutch loss resulting from the desiccation of embryos or larvae (as an outcome of nests having suboptimal moisture levels, flooding too early, or failing to flood; Byrne and Keogh, 2009). Notably, *P. bibronii* breeds during autumn and winter, while most *Pseudophryne* species breed in summer (Anstis, 2017). Assuming that the risk of clutch desiccation will be even higher in summer due to higher temperatures and evaporation rates, we predicted that sequential polyandry would be widespread in *Pseudophryne*, and for summer breeders may be even more extreme than previously reported for *P. bibronii.*

In the present study, we quantify the genetic mating system of a natural population of red-backed toadlets (*P. coriacea*) using exhaustive sampling techniques over an entire spring/summer breeding season. To determine the mating success of individuals and the reproductive strategies of each sex, we used single nucleotide polymorphisms (SNPs) to conduct parentage analysis.

2 | METHODS

2.1 | Study species

The red-backed toadlet (*P. coriacea)* is a small (24-36mm) terrestrial toadlet inhabiting the east coast and ranges of Australia. The species typically prefers sclerophyll forest and low-lying marshy areas (Cogger, 2014), and breeds from November through to March (austral Spring to Summer) in ephemeral pools and water courses which periodically fill following summer rainfall (Anstis, 2017). Gravid females produce an average of 47 eggs (range 26 – 78)(O'Brien, unpublished data), and hatching occurs approximately 14 days post-fertilisation (Anstis, 2017).

Metamorphosis can occur after a minimum larval duration of 46 days with some individuals taking up to 112 days (O'Brien, unpublished data).

2.2 | Study population

The study was conducted on a natural population of *P. coriacea* (adult population size = 371) located within the Jilliby State Conservation Area, New South Wales, Australia (-33.100 S, 151.379 E) over an entire spring/summer breeding season. The breeding site consisted of an ephemeral breeding pond (approximately 60 m long and 4-5 m wide) located along a ridgeline. The study area was situated in moist, open eucalypt forest with soils dominated by lithosols and siliceous sands. Vegetation within the study area was dominated by *Eucalyptus pilularis* (Blackbutt) and *Allocasuarina littoralis* (Black She-oak) with a sparse ground cover containing *Pteridium esculentum* (Bracken) and *Lomandra longifolia* (Spiny-headed Matt Rush).

2.3 | Field methods

Prior to the start of the breeding season the breeding site was enclosed with a drift fence and pit-fall traps. The 127 m long and 30 cm high fence encircled the site with 21 plastic pit-fall traps (diameter: 30 cm; depth: 30 cm) positioned approximately every 6 m along the fence line. Traps were checked every morning from 20 October 2014 to 9 February 2015 (81 continuous trap nights). Toadlets were captured entering the breeding site as they moved in from surrounding bushland and were then toe-clipped, measured, photographed and released inside the enclosure. If individuals were caught again they were released back inside the enclosure, and if they were caught a third time they were released outside the enclosure, with this sequence repeated over any subsequent recaptures. This approach was taken to ensure that frogs were given the opportunity to move in and out of the breeding site to avoid a situation where density was artificially inflated. Each night during the study period, males advertising from nests were located by tracking their calls, and nest sites were flagged on the surface using a unique marker (labelled plastic planter tag). During the study period there were two significant rain events that corresponded with peaks in female arrival and breeding activity, hereafter referred to as 'breeding event 1' and 'breeding event 2'. Breeding event 1 occurred between 19 October and 26 December 2014, and breeding event 2 occurred between 27 December 2014 and 24 January 2015. Nests were checked for eggs

and resident adults during each event after breeding activity had subsided. To ensure no nests were missed, leaf litter in a two metre squared area around each male was systematically searched.

Resident males were weighed and photographed for identification and 15% of tadpoles per clutch were sampled. Within nests, embryos of different developmental stages were considered to belong to different clutches and were sampled equally. Embryos were reared in plastic containers at a field station until larvae reached a late developmental stage (Gosner stage 27-28), at which point hatching was induced via flooding, and tadpoles preserved in 75% ethanol in Eppendorf tubes.

2.4 | Parentage analysis

To assign parentage to offspring, and determine mating success for both sexes, we genotyped all males and females that entered the breeding site, and 15% of all offspring with a large SNP (single-nucleotide polymorphism) dataset. Tissue samples (adult toe-clips and the tails of sampled tadpoles) were sent to the commercial genotyping service of Diversity Arrays Technology that have developed a widely used genotyping technique called DArTseq™. DArTseq™ represents a combination of DArT complexity reduction methods and next generation sequencing platforms (Kilian et al., 2012; Courtois et al., 2013; Cruz et al., 2013; Raman et al., 2014). The background and process has been outlined in detail in a previous study (Head et al., 2017) and we followed the same process for the generation of our SNP data set (also see Booksmythe et al., 2016).

We obtained a data set of approximately 15,746 SNPs with an average call rate of 90.0% and a reproducibility of 98.8 %. From these SNPs we calculated a Hamming Distance Matrix of all 869 successfully genotyped individuals to determine paternity and maternity. Recent studies show that as few as 30 optimized SNPs are sufficient to differentiate among 100,000 individuals using Hamming Distance Values (HDV) (Hu et al., 2015). Each offspring was lined up against the other offspring in the same clutch and also every potential sire and dam, and Hamming Distance Values (HDV) compared. The HDV are a measure of genetic dissimilarity across the full SNP data set. For our data set, HDV for siblings and parents and offspring ranged from

approximately 0.06-0.13, whereas unrelated animals had HDVs that ranged from approximately 0.14-0.19. Comparing values within clutches confirmed that the clutches comprised either full siblings or a mix of half siblings, full siblings or non-siblings from separate mating events. Half-siblings had intermediate HDV values. In a few clutches the HDV values were slightly higher for parent-offspring and sibling-sibling relationships because one of the parents was a hybrid. We compared HDV for every offspring and every potential dam and sire separately. For most clutches we had detailed information on the potential sires that had occupied a particular nest site and we also had this information for many potential dams, which provided us a means of testing the accuracy of our paternity assignments. In almost every case there was a single clear best match for both sire and dam based on the HDV (i.e. parentage could be unambiguously assigned). The only exception was for two offspring from the same nest where there were several potential sires. In this case, paternity was assigned to the male that was closest to the nest and was also present during the breeding event. It was clear from the SNP data that the sire or dam for some offspring had not been sampled because no potential sire or dam had HDVs in line with these relationships.

2.5 | Statistical analyses

Shapiro-Wilk tests were used to determine whether the body size distributions of males and females (measured as body mass and snout-vent length) deviated from normality. Wilcoxon signed-rank tests were used to test for differences in the body size (mass and SVL) of: i) mated and unmated males, ii) single-mated males and polygynous males, and iii) mated and unmated females.

2.6 | Ethics statement

This work followed protocols approved by the University of Wollongong's Animal Ethics Committee (AE14/17) in accordance with the "Australian Code for the Care and Use of Animals for Scientific Purposes 2013"; and was authorised by New South Wales National Parks & Wildlife Service - Office of Environment and Heritage (SL101436).

3 | RESULTS

3.1 | Population size, sex ratio, and body size variation

Over the 81 day study period a total of 371 adult frogs were captured, with the population displaying a slightly male-biased adult sex ratio (59% males: 219 males, 152 females). The average adult male body length was 29.8 mm (range = 27 to 33 mm), and the average male body weight was 2.4 g (range = 1.8 to 3.1 g). Distributions of both male body weight and SVL deviated significantly from normality (Shapiro-Wilk test, body weight: $W = 0.98$, $P < 0.01$; SVL: $W = 0.93$, $P < 0.01$, Fig. 1 and 2). The average adult female body length was 33.2 mm (range = 29 to 37 mm), and the average adult female weight was 3.7 g (range = 2.4 to 6.0 g). Distributions of both female body weight and SVL deviated significantly from normality (Shapiro-Wilk test, body weight: $W = 0.98$, $P < 0.01$; SVL: $W = 0.95$, $P < 0.01$, Fig. 1 and 2).

3.2 | Parentage analysis

DNA extraction was successful for 99.2% of all adult frogs and tadpoles. Paternity was assigned to 30.6% of adult males (67/219), and maternity was assigned to 53.3% of adult females (81/152). Of the 505 offspring that were collected and genotyped, 89.9% (454/505) were assigned to both a sire and dam, 1.4% (7/505) were assigned to a dam but not a sire, and 8.7% (44/505) were assigned to a sire but not a dam.

Approximately 6% of *P. coriacea* adults (23/371) were hybrid individuals between *P. coriacea* and the closely related congener *P. australis.* Classification of hybrids was based on both genetic data (hamming distance value >0.23 when compared to population mean) and morphological characteristics (resemblance of a red crown specific to *P. australis*) (Fig. 3). Of the male hybrids ($N = 11$), two individuals gained mating success. Of the female hybrids ($N = 12$), four individuals gained mating success.

3.3 | Patterns of paternity

During breeding events 1 and 2, males constructed a total of 113 and 110 nests respectively. Of the 219 males present at the study site, 180 (82.2 %) were present in breeding event 1, and 203 (92.7%) were present in breeding event 2. A total of 164 males (74.9 %) were present across both

245 breeding events. In breeding event 1, 23.9 % of nests (27/113) contained eggs. In these nests, the
246 number of eggs present was highly variable (range = 18 to 127, mean ± SEM = 57.25 ± 5.56, *N* =
247 27), and the distribution of eggs across nests deviated significantly from normality (Shapiro-Wilk
248 test; $W = 0.9095$, $P = 0.0223$, $N = 27$). Of the 27 nests containing eggs, two nests (N9 and N92)
249 could not be tested for paternity because the eggs were covered in fungus and decomposing. Of
250 the 25 nests where paternity was tested, the identity of the sire was determined in 96.4% of cases
251 (27/28 sires), with paternity assigned to 27 males (Table 1).

252 In most nests (76.0 %, 19/25) a resident male was present, and in one nest (N23) three
253 males were present. Of the nests where resident males were present, the resident male was the
254 genetically deduced sire in 57.9 % of nests (11/19). In the remaining 42.1 % of nests (8/19)
255 (which included the nest containing three males), resident males accompanied offspring that they
256 did not sire, indicating that nest takeover had occurred.

257 A subset of non-attendant genetically deduced sires were captured defending nests
258 between 0.1 and 8 m from the nest where they sired offspring. One of these males (male 149) had
259 sired a second clutch of eggs (providing evidence for polygyny across nests). Overall, 64.0 % of
260 nests with eggs (16/25) had offspring produced by a single male and single, 20.0 % of nests
261 (5/25) contained offspring produced by a single male and multiple females (providing evidence
262 for polygyny within nests), and 12.0 % of nests (3/25) contained offspring produced by multiple
263 males and females (providing evidence for nest takeover as well as repeated nest use by different
264 pairs) (Fig. 4). In addition, one nest (N14) contained offspring produced by two males and a
265 single female, providing evidence for multiple paternity (Fig. 4). Of note, because one of these
266 sires (male 149) was the resident male, and because this male also gained mating success at a
267 second nest in breeding event 1, as well as a third nest in breeding event 2 (see Table 1 and 2), it
268 is likely that multiple paternity was the outcome of the second sire (male 145) sneaking
269 fertilisations.

270 In breeding event 2, 40.9 % of nests (45/110 nests) contained eggs. Similar to breeding
271 event 1, the number of eggs laid in a nest was highly variable (range = 15 to 206, mean ± SEM =
272 64.1 ± 5.16), and the distribution of eggs across nests deviated significantly from normality
273 (Shapiro-Wilk test; $W = 0.7948$, $P = 0.001$, $N = 45$ nests). Of the 45 nests with eggs, the identity

of the sire was determined in all cases, with paternity assigned to 50 males. Of note, 20.0 % of males (10/50) that sired offspring in breeding event 2 also sired offspring in breeding event 1, providing evidence for polygyny across nests.

Resident males were present in most nests (82.2 %, 37/45), and of the nests where resident males were present, the resident was confirmed to be the genetic sire in 62.2 % of cases (23/37). In the remaining 37.8 % of nests (14/37), resident males accompanied offspring that they did not sire, indicating that nest takeover had occurred (Table 2). A subset (7/20) of the non-attendant genetically deduced sires were captured defending nests located between 0.5 and 25 meters away from their original nest site. One of these males (male 344) was also successful in siring offspring in a second nest (providing further evidence for polygyny across nests).

Overall, 71.1 % of nests (32/45) had offspring produced by a single male and single female (Table 2), 17.8 % of nests (8/45) contained offspring produced by a single male and multiple females (providing evidence for polygyny within nests), 8.9 % of nests (4/45) contained offspring produced by multiple sires and multiple dams (providing evidence for nest takeover and repeated nest use by different pairs) and 4.4 % nests (2/45) (N16 & N20a) contained offspring sired by multiple males and a single female (providing evidence for multiple paternity).

Across both breeding events, there was no significant difference between the body size of mated and unmated males, measured as either body weight (mated males: mean ± SEM = 2.42 g ± 0.03, N = 67, unmated males: mean ± SEM = 2.43 g ± 0.02, N = 152)(Wilcoxon test, Z = 0.02, P = 0.98), or snout-vent length (mated males: mean ± SEM = 29.9 mm ± 0.15, N = 67, unmated males: mean ± SEM = 29.8 mm ± 0.09, N = 152)(Wilcoxon test, Z = 1.29, P = 0.20).

There was also no significant difference between the body size of males that mated with one female versus males that mated with multiple females, when body size was measured as either body weight (single mated males: mean ± SEM = 2.39 g ± 0.04, N = 45, polygynous males: mean ± SEM = 2.48 g ± 0.06, N = 22)(Wilcoxon test, Z = 0.99, P = 0.32), or snout-vent length (single mated males: mean ± SEM = 29.8 mm ± 0.19, N = 45, polygynous males: mean ± SEM = 30.1 mm ± 0.21, N = 22)(Wilcoxon test, Z = 1.09, P = 0.27).

3.4 | Patterns of maternity

Of the 152 females present at the study site, 51 (33.6 %) were present in breeding event 1, and 139 (91.4 %) were present in breeding event 2. A total of 38 females (25 %) were present across both breeding events. In breeding event 1, 64.0 % of the nests containing eggs (16/25) had offspring that were assigned to a single dam (and sire), indicating that the nests were only used by one female. An additional nest (N14) also contained offspring belonging to a single female, however, two males were shown to share paternity (providing evidence for simultaneous polyandry). In 32.0% of nests (8/25), offspring were assigned to multiple females (2-3 individuals), indicating that multiple females had used the same nest site. In 62.5 % of these nests (5/8), offspring were assigned to multiple females and a single sire, providing evidence that different females mated sequentially with the same male. In the remaining 37.5 % of nests (3/8), the offspring of different females were each sired by different males, indicating that multiple females mated sequentially with the resident of a nest site, despite changes in male ownership of the nest (i.e. several bouts of nest takeover).

Of the 25 nests containing eggs, mating was assigned to 32 females (Table 1). Of these females, all but one could be identified (matched to a sampled female). Overall, 96.9 % of identified females (31/32) mated with a single male. Almost all of these females (30/32) mated with a single male in one nest, but one female (female 92) mated with the same male in two separate nests (approximately 2 m apart), providing evidence for clutch partitioning. Only one female (female 109) mated with multiple males (males 26 & 224) within the same nest, providing evidence for simultaneous polyandry.

In breeding event 2, 73.3 % of nests containing eggs (33/45) were assigned to a single mother (and father). In 24.4% of nests with eggs (11/45), offspring were assigned to 2-4 females, suggesting that several females sequentially used the same nest site. Due to nest takeover by males, 40.0 % of those nests (4/10) contained offspring produced by multiple dams and multiple sires. In 4.4 % of nests (2/45), multiple males sired offspring produced by a single female, providing evidence for simultaneous polyandry. Of the 45 nests containing eggs, maternity was

assigned to 59 females (Table 2). Of these females, 86.4 % (51/59 females) were matched to sampled females.

Overall, 92.3% of females (47/51) mated with a single male in a single nest. Of the remaining females, two females (female 221 & 325) exhibited simultaneous polyandry, where each female produced offspring with multiple males in a single nest. Another two females (female 320 & 334) mated with different males in different nests, providing evidence for sequential polyandry. For both of these females, the distance between nests in which they deposited eggs was approximately 7 meters. One female (female 112) mated with multiple males in different nests across breeding periods, providing additional evidence for sequential polyandry.

Across both breeding events, there was no significant difference between the snout-vent length of mated and unmated females (mated females: mean ± SEM = 33.4 mm ± 0.17, N = 81, unmated females: mean ± SEM = 33.0 mm ± 0.19, N = 71)(Wilcoxon test, Z = -1.24, P = 0.21), however there was a significant difference in body mass, whereby mated females were heavier (mated females: mean ± SEM = 3.84 g ± 0.08, N = 81, unmated females: mean ± SEM = 3.56 g ± 0.08, N = 71)(Wilcoxon test, Z = -2.23, P = 0.03).

3.5 | Description of the mating system

Over the entire study period, 68.7 % (46/67) of mated males sired offspring with a single female (Fig. 5). Most of these males attracted females to a nest (95.5 %, 64/67), but a small subset of males (4.5 %, 3/67) gained mating success by sneaking fertilisations. Of the males that gained mating success, 31.3 % (21/67) sired offspring with multiple females (2 to 4 females), and were deemed to be polygynous. Of the polygynous males, individuals either mated with multiple females in the same nest (52.4 %, 11/21), or mated with multiple females across multiple nests (47.6 %, 10/21). Interestingly, polygynous males that mated in multiple nests were never recorded to have fertilised eggs in different nests within the same breeding period. Of the mated females, 92.6 % (75/81) mated with a single male, while 7.4 % (6/81) mated with multiple males (2 to 3 males) and were deemed to be polyandrous (Fig. 5). Of the polyandrous females, 50 % (3/6 females) mated with multiple males within the same nest, exhibiting simultaneous

polyandry, while the other 50 % (3/6 females) mated with multiple males at different nests, exhibiting sequential polyandry.

4 | DISCUSSION

Knowledge of the genetic mating systems of ectothermic vertebrates remains limited, particularly for species with prolonged breeding and cryptic mating behaviour. The present study quantified the genetic mating system of the terrestrial breeding red-backed toadlet (*P. coriacea*), a small frog in which breeding lasts several months, and mating takes places in concealed subterranean nests. A single population was exhaustively sampled over an entire breeding season and SNPs were used to assign parentage to offspring. We found that females typically either did not lay eggs, or laid a single clutch of eggs over a breeding season, and that nearly all females (92.6 %) mated with one male. The small percentage of females (3.7 %) displaying sequential polyandry mated with no more than three males. Male mating success was heavily skewed towards a small subset of individuals (30.6 %), and the majority of successful males (70.1 %) mated with one female. Within nests, eggs were typically accompanied by a resident male, but in nearly one third of cases (31.7 %) the resident was not the genetic sire, suggesting a very high incidence of nest takeover. Despite a heavy mating skew, only 4.2 % of nests contained clutches that were sired by multiple males, indicating that sneaking behaviour was either extremely uncommon, or rarely resulted in fertilisations.

Our finding that almost all females mated with a single male, and that male mating success was heavily skewed, was unexpected. We predicted an extremely high level of sequential polyandry because early observational work with three closely related *Pseudophryne* species (*P. bibronii, P. dendyi and P. semimarmorata*) suggested that clutch partitioning may be widespread in terrestrial toadlets (Woodruff, 1976). Furthermore, a long term study exploring the genetic mating system of one of these species, (the autumn breeding brown toadlet *P. bibronii*) uncovered the most extreme level of sequential polyandry reported in a vertebrate (every female mated with 2-8 males) (Byrne & Keogh, 2009). Sequential polyandry in *P. bibronii* was shown to be adaptive because it reduced the risk of nest failure caused by eggs desiccating in nests with low moisture, or nests that either failed to flood, or flood at suboptimal times (Byrne & Keogh,

2009). Given that *P. coriacea* breed in summer, we assumed that nests and ponds would dry more rapidly, and that an increased risk of brood failure would favour an even more extreme level of sequential polyandry. Why then sequential polyandry was so rare remains unclear.

One explanation for the low incidence of sequential polyandry is that clutch partitioning is a highly plastic behaviour and that re-mating was repressed during our study period. Plasticity in polyandrous behaviour has been reported in other vertebrate systems and is often related to stochastic environmental conditions and fluctuating costs of mate searching (Rossmanith et al., 2006; Mobley & Jones, 2009). In toadlets, it is conceivable that climatic factors such as temperature and rainfall will constrain promiscuous activity, or alter the costs of mate searching. The expected outcome of such environmentally determined constraints and costs is that the frequency of sequential polyandry will fluctuate within and between breeding seasons. Notably, however, even though climatic conditions varied considerably over our 81-day study period, the incidence of sequential polyandry remained constant. Therefore, it seems unlikely that females were refraining from re-mating due to unfavourable environmental conditions.

An alternative explanation for the low incidence of sequential polyandry is that this behaviour is an ancestral state, and that *P. coriacea* is in the evolutionary phase of shifting away from clutch partitioning (Holman & Kokko, 2013). In principle, sequential polyandry inflates mating costs such as the energetic expense of mate searching and the risk of predation, disease contraction or desiccation (Byrne & Roberts, 2012). Consequently, unless there are significant constraints on mate choice, or breeding occurs in an environmental context where mate choice is highly unreliable, sexual selection is expected to strongly favour stringent mate preferences and mating with one male (Kokko et al., 2002). Based on our knowledge of the mating system of *P. bibronii* we assumed that female *P. coriacea* have a limited capacity to reliably evaluate the probability of nest failure, but this might not be the case. Compared to *P. bibronii*, *P. coriacea* have larger eggs with much thicker egg capsules, a trait known to buffer embryos against water loss in other terrestrial breeding frogs (Mitchell, 2002). Furthermore, the developmental rate of embryo's and tadpoles is much faster, meaning that eggs remain in nests for shorter periods, and that tadpoles are less reliant on the persistence of temporary pools to complete larval development. Such differences might substantially reduce the overall risk of embryo or larval

desiccation and increase the capacity for females to discriminate between males based on nest qualities. That selection of high quality nests is an important aspect of the *P. coriacea* mating system is supported by our finding that nest takeover was prevalent. Nest takeover has rarely been reported in anurans (but see Hudson & Fu, 2013), but it is widespread in fish (DeWoody & Avise, 2001; Alonzo, 2004), and experimental studies have shown that frequent nest takeover occurs when males are in strong competition for a limited number of high quality nests (Lindström, 2001; Lindström & Pampoulie, 2004). In other terrestrial frog species in which males construct nests it has been demonstrated that females have the ability to reliably assess nest qualities that influence offspring performance and survival. For example, in the terrestrial breeding ornate nursery frog (*Cophixalus ornatus)*, males construct burrows on creek banks and females prefer deeper more elongated and chambered nests that provide offspring with greater protection from biotic or abiotic disturbances (Felton et al., 2006). If female *P. coriacea* have a similar ability to reliably evaluate nest qualities, mating with one male might be strongly favoured over polyandry.

Assuming that female *P. coriacea* are discriminating between males, and that males (or their nest sites) vary markedly in quality, a curious finding was the low level of polygyny. Over both breeding events, less than one third of successful males mated with multiple females. Moreover, male-mating success was unrelated to body size, indicating that larger males were neither more attractive nor competitively superior (Gerhardt & Huber, 2002; Rausch et al., 2014). A number of factors may have restricted the mating success of resident males. First, given the high incidence of nest takeover, it may have been difficult for males to retain high quality nests, or to quickly acquire new nests after being usurped, restricting opportunities for multiple matings. This is supported by our finding that no males gained matings across multiple nests within the one breeding episode. Nest takeover might also be a time consuming and exhaustive activity. Although no form of male-male combat has been observed in the field, males occupying the same nest typically engage in protracted bouts of threat calling (often lasting several hours), suggesting that nest takeovers are predominately mediated by endurance rivalry. Another possibility is that males became less attractive to females once they had mated multiple times. This could happen for a number of reasons, including the possibility that males become sperm

depleted after successive matings, and females avoid highly successful males to ensure clutch fertilisation. The potential for sperm depletion has not been explored in toadlets, however, this explanation remains plausible because it is not uncommon to encounter nests with large numbers of unfertilised eggs (Byrne, unpublished data). Furthermore, there is evidence that mating history affects sperm concentration in anurans. For example, in gray treefrogs (*Hyla versicolor*), sperm stores are severely reduced after one mating (Doyle, 2011). Recently, it has also been shown that fish can discriminate between males based on mating history. In Trinidad guppies (*Poecilia reticulata*), where males deplete 92 % of their sperm stores after one mating, mate choice tests have shown that females avoid mating with males they have observed sexually interacting with other females (Scarponi et al., 2015). Another possibility is that female toadlets avoid mating with highly successful males because it is costly to deposit clutches in nests that contain a large number of eggs. Large egg masses might restrict effective gas exchange and lead to embryo failure, a problem reported in the Australian moss frog (*Bryobatrachus nimbus*), another terrestrial breeding frog with large egg capsules (Mitchell & Seymour, 2003). Furthermore, tadpoles that hatch in nests containing multiple clutches might face stronger competition for limited food resources in shallow temporary pools. Heightened competition might extend the length of the larval period and reduce body size at metamorphosis, which in anurans can have major negative lifetime fitness consequences (Wilbur & Collins, 1973; Denver, 1997).

Low levels of polygyny might also reflect the fact that a significant proportion of females in our study population did not breed, reducing opportunities for males to re-mate. Why so many females didn't breed remains unclear. One possibility is that some females bred in other choruses before entering the study site. However, this seems unlikely because the nearest breeding site was located several kilometres away (O'Brien, unpublished data) and toadlets have a locomotory mode (crawling rather than hoping) that limits their ability to move quickly through the landscape. Furthermore, toadlets display extreme site fidelity, returning to the same breeding sites between years (Byrne, unpublished data). As such, a more likely explanation is that females varied in their readiness to oviposit, and that females who were not carrying mature eggs missed the opportunity to breed due to unfavourable climatic conditions. Indeed, while mated and unmated females did not differ in snout-vent length, mated females were significantly heavier

when they entered the breeding site, indicating they were carrying mature eggs and were primed for breeding. Furthermore, many of the females that didn't mate arrived late in the breeding season, at which time a lack of late summer rainfall precluded a final bout of breeding. Toadlets are very long lived, having a lifespan that can exceed 10 years in the wild (Byrne, unpublished data) and 24 years in captivity (Thumm, unpublished data). Toadlets also have the capacity to resorb eggs if they haven't mated by the end of a breeding season (Byrne, unpublished data). Therefore, it might be common for females to skip breeding years, and for numbers of breeding females to fluctuate considerably year to year, as reported for other prolonged breeding anurans (Rastogi et al., 1983; Reyer et al., 1999). In years where a higher proportion of females have the opportunity to breed, levels of polygyny might be much higher.

Another curious finding was the low incidence of multiple paternity (i.e. simultaneous polyandry), which indicates that sneaking behaviour was extremely uncommon. Sneaking is widely documented in anuran amphibians and often leads to multiple-male amplexus (simultaneous polyandry) and multiple paternity (d'Orgeix & Turner, 1995; Roberts et al., 1999; Lodé & Lesbarrères, 2004). Furthermore, observational and experimental studies have provided good evidence that sneaking and simultaneous polyandry is driven by intense intra-sexual selection (Byrne & Roberts, 2004; Lodé et al., 2004). Given the strong mating bias we found in our study population, as well as the shortage of breeding females and the apparent intense competition between males for nest sites, it is surprising that the level of multiple paternity was not higher. The breeding habits of toadlets might restrict opportunities for sneaking. Specifically, due to mating occurring in concealed burrows, it might be difficult for sneaks to remain close enough to residents to visually monitor female arrival, yet avoid detection and aggression. In some fish, nest site concealment and nest site architecture are known to influence opportunities for sneaking (Sargent & Gebler, 1980; Oliveira et al., 2002). Moreover, a recent comparative study in frogs indicates that terrestrial breeding has evolved to reduce the risk of sneaking and sperm competition (terrestrial breeding frogs with less exposed amplexus have smaller testes) (Zamudio et al., 2016). However, toadlets display strategic calling behaviour whereby males dramatically increase their calling effort when a female enters a burrow (Byrne, 2008), so sneaks should be able to acoustically monitor mating activity and join pairs opportunistically.

Additionally, sneak males might also be able to locate mating pairs using non-volatile odours laid by resident males as preference tests have shown that *P. bibronii* can recognise and locate conspecifics using chemosignals (Byrne & Keogh, 2007). As such, a more plausible hypothesis for the low incidence of sneaking might be that there is a reduced probability of fertilisation success in the terrestrial environment. For aquatic frogs, sperm can remain viable in water for extended periods and sneaks do not need to be in close physical proximity to pairs to gain fertilisations (Prado & Haddad, 2003; Sherman et al., 2008; Ron et al., 2014). In terrestrial burrows, sperm might die quickly in the soil medium and sneaks may only be successful if they can release sperm directly onto eggs. If this is the case, sneak attempts might rarely result in fertilisations and multiple paternity. Finally, due to costs associated with losing paternity to undesirable sires (Bourne, 1993), or the risk of eggs going unfertilised when multiple males compete for fertilisations (Byrne & Roberts, 1999), there may be strong selection on females to withhold egg release when amplexed by multiple males. The ability to bias paternity by controlling egg release when amplexed by undesirable males has been demonstrated in European waterfrogs (Reyer et al., 1999). If *P. coriacea* have a similar capacity, this could limit the success of sneaks, and reduce the incidence of simultaneous polyandry. Furthermore, if females that are disturbed by sneaks occasionally terminate matings and re-mate at different locations, this could provide a non-adaptive explanation for the instances of sequential polyandry reported.

A final unexpected result was the occurrence of hybridisation between *P. coriacea* and *P. australis*. Hybridisation has been reported in other *Pseudophryne* species (Woodruff, 1973, 1977; McDonnell et al., 1978; Payne, 2014), but this is the first evidence for hybridisation in *P. coriacea*. Hybridisation might arise due to mating mistakes resulting from a high level of species similarity and limited divergence in traits that facilitate species recognition (Nagel & Schluter, 1998). *Pseudophryne coriacea* and *P. australis* are anatomically similar (both species are small and lack obvious sexual size dimorphism), and they also share a similar breeding biology (both species breed over spring and summer, have short pulsatile advertisement calls, use shallow terrestrial nests, and have inguinal amplexus). In the present study we caught female *P. australis* at the breeding site of *P. coriacea*, but never any males. Therefore, we speculate that hybridisation has stemmed from matings between *P. australis* females and *P. coriacea* males.

Female *P. australis* might be strongly attracted to the advertisement calls of male *P. coriacea* due to an ancestral sensory bias. The calls of *P. coriacea* are slightly longer and more pulsatile than *P. australis*, and might act as a hyperstimulus. While it is not known whether female *P. australis* prefer longer more pulsative calls, such preferences are widespread in anurans (Wells & Schwartz, 2007). Male *P. coriacea* might readily accept heterospecific matings because intense male-male competition has favoured indiscriminate clasping behaviour, a widely reported phenomenon in anurans (Pearl et al., 2005). Critically, however, if hybridisation is explained by mating mistakes, we should expect to see post-mating isolation mechanisms in operation, evidenced by extremely high levels of embryo failure, and/or inviable or infertile F1 adults (Woodruff, 1979). Instead, we found that hybrid matings generated viable offspring, and that hybrids were phenotypically indistinguishable from pure-species frogs (except for differences in colouration). Moreover, we found that hybrid males and females that mated with pure-species frogs generated viable larvae, indicating that backcrossed hybrids gain reproductive success. While it is possible that effects of hybridisation are neutral or slightly maladaptive (and that hybrids are not strongly selected against), the possibility that hybridisation is adaptive should also be considered. Specifically, *P. australis* females might prefer heterospecific mates because hybrids perform better under challenging environmental conditions, as has recently been reported in American spadefoot toads (Pfennig, 2007). Following hybridisation, backcrossing and introgression might then be favoured if *P. coriacea* females gain fitness benefits by mating with more genetically variable males. Investigating mechanisms of mate choice in *P. coriacea*, and whether genetic benefits underpin adaptive hybridisation, could provide key insights into the evolution of the *P. coriacea* mating system.

Overall, the findings of our study make an important contribution to our understanding of amphibian mating systems. It is widely believed that anurans have the highest reproductive diversity of all vertebrate groups, yet genetic mating systems have been studied in less than one percent of all described species. Moreover, most genetic studies have only performed paternity analyses on small number of clutches representing a fraction of all breeding individuals, and/or targeted specific mating contexts (e.g. multiple male amplexus), creating a perspective bias. While this research has confirmed that anurans display a diversity of reproductive tactics, it has

only provided a snapshot of anuran genetic mating systems. Our study is one of the first to exhaustively sample a population of a prolonged breeding anuran and demonstrates a high level of mating system complexity. Terrestrial breeding with parental care is widespread in anuran amphibians (spanning at least 206 species from 27 families, representing 51 % of families) (Wells, 2010; Gómez-Hoyos et al., 2012), so there are excellent opportunities to explore mating system variation across a diversity of anuran groups that employ this reproductive mode. More broadly, our findings advance our understanding of vertebrate mating systems variation by showing that closely related species with a similar reproductive biology can differ markedly in their genetic mating system. Even though *P. bibronii* and *P. coriacea* have similar life histories and share the same reproductive mode (mode 17/39, Eggs and early tadpoles in excavated nests; subsequent to flooding, exotrophic tadpoles in ponds or streams) (Haddad & Prado, 2005), they appear to have vastly different genetic mating systems. Demonstrating extreme mating system differences between closely related species provides a valuable opportunity for comparative studies that directly test hypotheses regarding the causes and consequences of sexual selection, and the role of sexual selection in mating system evolution. Saying this, an important limitation of our study was that we only studied one population. Within species, environmental and demographic differences between populations can affect the strength and intensity of sexual selection and drive among population variation in genetic mating systems (for examples see Rispoli & Wilson, 2008; Mobley & Jones, 2009). Therefore, caution must be exercised when extrapolating the patterns we report here to the *P. coriacea* species in general. An accurate assessment of the species mating system will only be possible once genetic mating system studies have been conducted for multiple populations across the species range. Despite this limitation, our findings underscore the importance of using molecular tools to gain initial insights into mating system variation between groups. Ongoing assessment of vertebrate mating systems, particularly for groups with cryptic and prolonged breeding, is likely to reveal that mating systems are far more variable and complex than currently realised. Such work will improve our capacity to discern mechanisms of sexual selection and understand the evolution of reproductive strategies in ectothermic vertebrates.

In conclusion, molecular tools are increasingly being used to quantify animal mating systems, yet we still know very little about the genetic mating systems of amphibians and other ectothermic vertebrates, particularly species with prolonged breeding and cryptic mating behaviour. In this study we quantified for the first time the genetic mating system of the terrestrial breeding red-backed toadlet (*P. coriacea).* We predicted that females would display extreme sequential polyandry because this mating system has been reported in a conspecific. Unexpectedly, we found that almost all females mated with a single male, displaying stringent mate preferences, and that most males mated with single female. We also found a very a high frequency of nest takeover and extreme competition between males for nest sites, but that males rarely gained fertilisations by sneaking. Finally, we discovered that *P. coriacea* hybridises with a congener, resulting in introgression. Our findings highlight that closely related species with the same reproductive mode can differ markedly in reproductive behaviour, and reiterate the importance of using molecular tools to elucidate mating system complexity. Ongoing assessment of the genetic mating systems of ectothermic vertebrates will continue to advance our understanding of mating system variation and provide a conceptual platform for understanding mechanisms of sexual selection and the evolution of reproductive strategies.

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DATA ACCESSABILITY

The SNP data, and the Hamming Distance Values generated from the SNP data, is available from the Dryad repository (https://doi.org/10.5061/dryad.m0s82c3).

AUTHOR CONTRIBUTIONS

DO, PB, and AS conceived the study. DO collected and analysed all field data. SK ran and interpreted the genomic analyses. PB and DO wrote the manuscript with input from AS and SK.

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FIGURE LEGENDS

FIGURE 1 Graph showing the distribution of snout-vent length in millimetres for male and female red-backed toadlets (N = 219 males and 152 females).

FIGURE 2 Graph showing the distribution of mass in grams for male and female red-backed toadlets (N = 219 males and 152 females).

FIGURE 3 Photos of a) a pure species *P. coriacea*, b) a *P. coriacea - australis* hybrid, and c) a pure species *P. australis* (right).

FIGURE 4 Percentage of nests during two breeding events (event 1 and event 2) with offspring assigned to four possible sire and dam combinations.

FIGURE 5 A summary of the number of mating partners for each sex across both breeding events.

TABLES

TABLE 1 Details of parentage assignment in *P. coriacea* for breeding event 1. Detailed for each nest where eggs were found are the identities of the resident male(s) found accompanying eggs, the genetically deduced sire(s) of offspring, the genetically deduced dam(s) of offspring, the location/nest of the sire (if captured) and the distance of sire from offspring.

Nest	ID of resident male(s)	ID of genetically deduced sire(s)	ID of genetically deduced dam(s)	Location of sire	Nest of sire capture	Distance of sire from eggs
N5a	no resident	190	85	in different nest	N5b	~ 0.2m
N9	no resident	unknown	unknown	-	-	-
N11	5	280	59	not captured	-	-
N13	214	201	104	not captured	-	-
N13b	29	189	113	not captured	-	-
N14	26	26	109	in nest	N14	0
		224	109	not captured	-	-
N18	215	215	100, 117	in nest	N18	0
N19	187	187	86	in nest	N19	0
		unmatchedM1	208	not captured	-	-
N20	no resident	136	53	in different nest	N5a	~ 8m
N23	27, 58, 125	22	52	in different nest	N20a	~ 1m
		174	64	in different nest	N12	~ 0.5m
N20a	22	148	114	not captured	-	-

N38a	no resident	142	332	not captured	-	-
N41a	1	1	112	in nest	N41a	0
N42	no resident	140	99, unmatchedF1	not captured	-	-
N46	38	38	111	in nest	N46	0
N48	no resident	176	357, 365	not captured	-	-
N49	33	33	61	in nest	N49	0
N50	217	214	35, 50	in different nest	N13	~ 0.3m
N53	152	152	81	in nest	N53	0
N55	265	265	88	in nest	N55	0
N63	145	149	92	in different nest	N76	~ 2m
N74	no resident	133	96	not captured	-	-
N76	149	145	116	in different nest	N63	~ 1.5m
		149	92	in nest	N76	0
N81	186	186	120, 121	in nest	N81	0
N83	no resident	200	94	in different nest	N57	~ 5m
N89	51	51	95	in nest	N89	0
N92	no resident	unknown	unknown	-	-	-

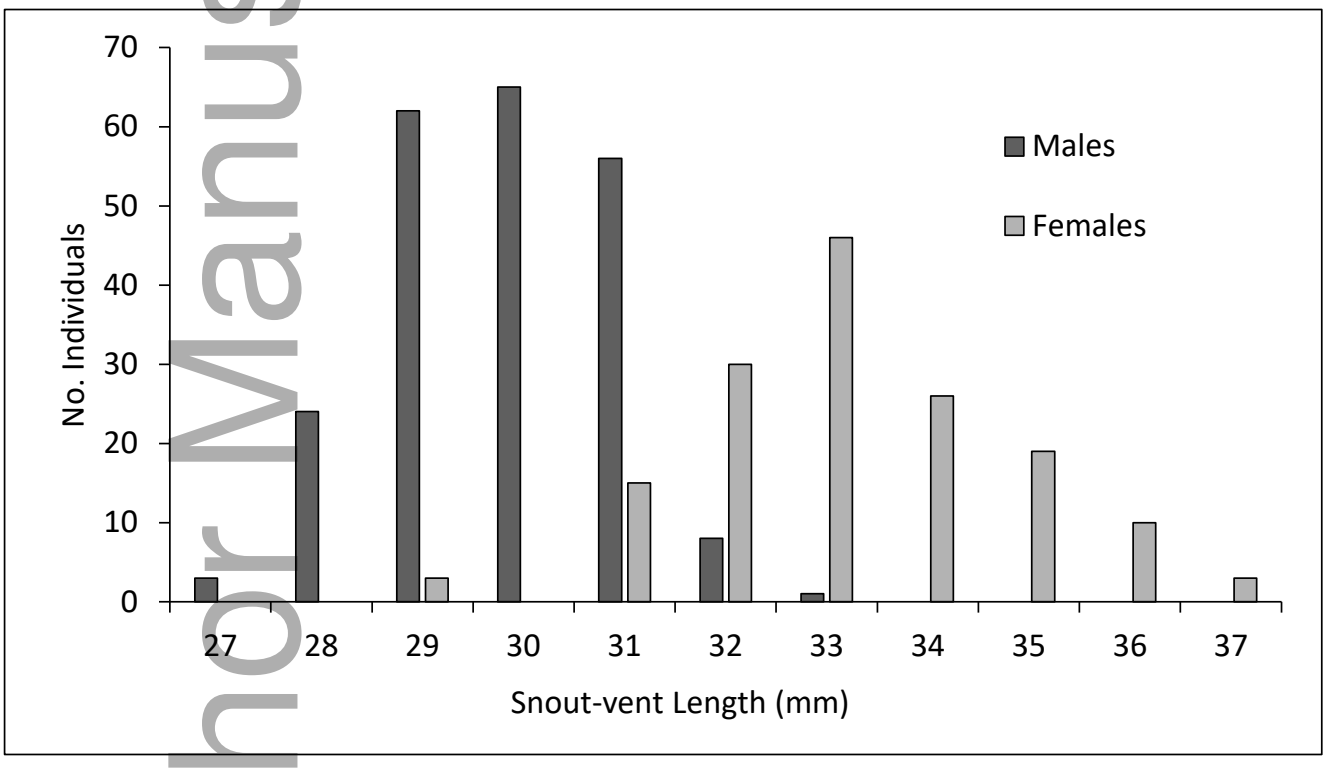
Unknown: offspring could not be genotyped; Unmatched: identity of male/female could not be matched to a sampled adult.

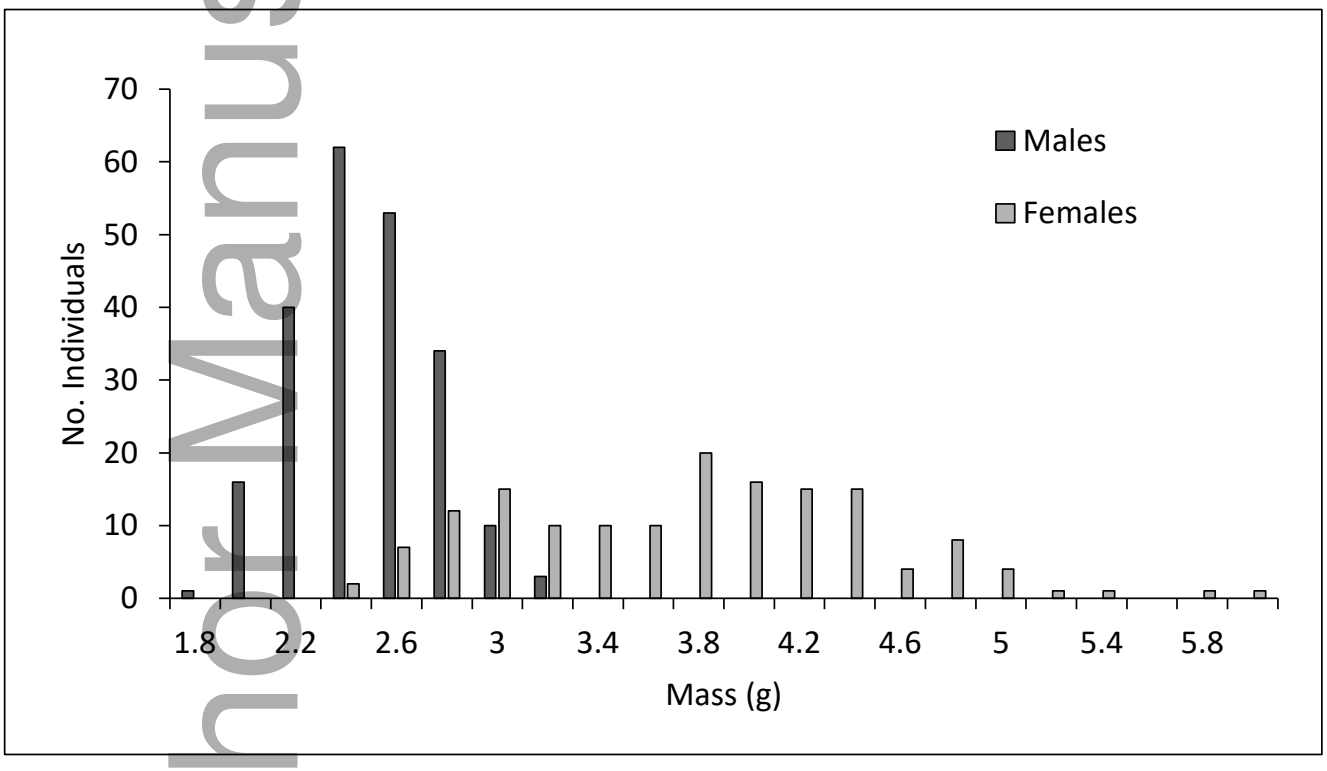
TABLE 2 Details of parentage assignment in *P. coriacea* for breeding event 2. Detailed for each nest where eggs were found are the identities resident male(s) found accompanying eggs, the genetically deduced sire(s) of offspring, the genetically deduced dam(s) of offspring, the location/nest of the sire (if captured) and the distance of sire from offspring.

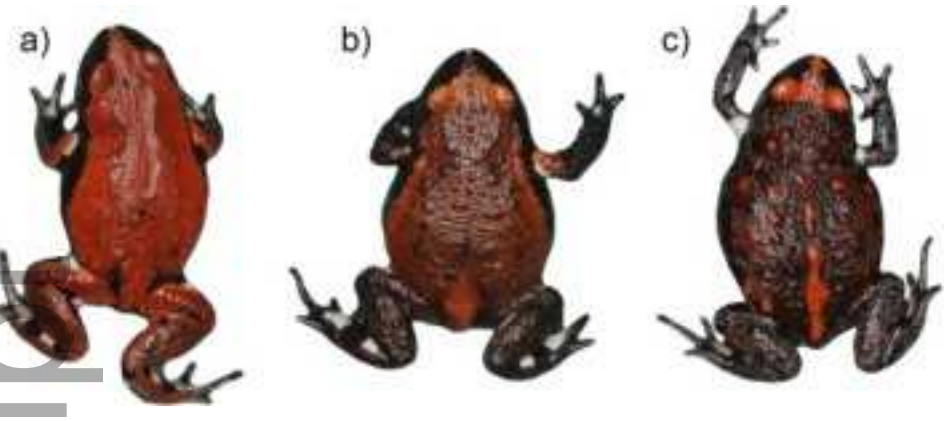
Nest	ID of resident male(s)	ID of genetically deduced sire(s)	ID of genetically deduced dam(s)	Location of genetic sire	Nest of sire capture	Distance of sire from eggs
N3	22	22	324†, unmatchedF3	in nest	N3	0
N5b	190	190	283	in nest	N5b	0
N11	4	4	316, 323	in nest	N11	0
N13	169	169	285	in nest	N13	0
N15b	272	97	303, 320	in different nest	N20	~0.5m
N16	253	12	221	not captured	-	-
		344	221	in different nest	N133	~3m
N19b	191	308	unmatchedF2	not captured	-	-
N20a	337	68	325	in different nest	N38	~15m
		140	325	not captured	-	-
		263†	unmatchedF4	not captured	-	-
N26a	no resident	47	unmatchedF5	in different nest	N108	~1.5m
N32	no resident	21	337	not captured	-	-
N36	196	196	unmatchedF6	in nest	N36	0
N38	68	118	294	not captured	-	-

		170	322	not captured	-	-
N42	164	164	336	in nest	N42	0
N49a	218	218	367	in nest	N49a	0
N55	174	265	266	not captured	-	-
N60	no resident	344	unmatchedF7	in different nest	N133	~1.5m
N65	130	130	327	in nest	N65	0
N69	31	31	321	in nest	N69	0
N73	43	217	295	not captured	-	-
N76	no resident	149	unmatchedF8	not captured	-	-
N77	145	310	112	not captured	-	-
N78	204	204	289	in nest	N78	0
N89	226	226	331	in nest	N89	0
N89a	201	201	282	in nest	N89a	0
N93	194	194	334	in nest	N93	0
N99	229	229	181	in nest	N99	0
N100	14	14	286, 291, 304, 320	in nest	N100	0
N101	62	161	243, 334, 374	not captured	-	-
N103	26	91	261†	not captured	-	-
N104	no resident	210	269	not captured	-	-
N105	219	219	326	in nest	N105	0
N109	200	192	311	not captured	-	-
		200	317	in nest	N109	0
N111	no resident	150	258	not captured	-	-
N116	no resident	63	293, 335	in different nest	N134	~25m
N117	no resident	60	287†	not captured	-	-
N119	197	142	249	not captured	-	-
N121	176	176	298	in nest	N121	0
N122	40	40	299	in nest	N122	0
N123	168	191	328†	in different nest	N19b	~10m
		280	334	not captured	-	-
N127	58	58	247, 330	in nest	N127	0
N132	148, 344	163†	107, 260, 270	in different nest	N115	~10m
N133	344	225	274	not captured	-	-
N135	69	69	288	in nest	N135	0
N137	343	343	338	in nest	N137	0
N138	340	340	281	in nest	N138	0

840 †: hybrid individual; Unmatched: identity of male/female could not be matched to a sampled adult.







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