

# Kin distribution of amphibian larvae in the wild

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## Abstract

According to kin selection theory, the location of an individual with respect to its relatives can have important ramifications for its fitness. Perhaps more than any other vertebrate group, anuran amphibian larvae have been the subject of many experiments on this topic. Some anuran species have been shown in the laboratory to recognize and associate with their siblings and half-siblings. However, due to the difficulty of identifying sibships, no kinship studies with anuran larvae have been conducted in the wild. Here, we use microsatellite analysis to show that wood frog (*Rana sylvatica*) tadpoles were nonrandomly distributed in two ponds with respect to their relatives. In one pond, the tadpoles were significantly clumped with their siblings or half-siblings as expected from other published laboratory studies on this species. However, in another pond, the tadpoles were significantly nonrandomly dispersed from their siblings or half-siblings. This is the first example of kin repulsion of nonreproductive animals in the wild and the first time a species has been shown to display both aggregation and repulsion under different circumstances. These results suggest that kin distribution is context dependent and demonstrate the importance of testing kin selection hypotheses under natural conditions.

*Keywords:* amphibian, inclusive fitness, kin recognition, kin selection, microsatellite, *Rana sylvatica*

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## Introduction

In the four decades since Hamilton (1964a, b) published his theory of inclusive fitness, numerous theoretical and empirical studies have shown that the spatial distribution of relatives can have important fitness consequences. Groups, whether composed of relatives or not, may be more efficient at foraging and competing for resources, better at predator detection, avoidance, or deterrence, and better able to regulate their environment (reviewed in Blaustein & Waldman 1992). However, group living may also have costs in the form of increased competition, cannibalization, predation, susceptibility to disease, and inbreeding (Hamilton & May 1977; Pfennig *et al.* 1993; Dwyer *et al.* 1997; reviewed in Bateson 1983; Shykoff & Schmid-Hempel 1991; Garret & Mundt 1999). When these costs are less than the benefits and are not shared equally,

individuals can increase their inclusive fitness by aggregating with kin, rather than with unrelated conspecifics, because relatives are more likely to share and pass on the same genes. When the costs of aggregation are greater than the benefits, individuals may increase their inclusive fitness if they or their offspring are dispersed from their relatives.

Because it can facilitate the optimal spatial distribution, kin recognition abilities can have important fitness consequences. Numerous experimental and observational studies have shown that kin recognition is common in invertebrates and vertebrates alike (Greenwood 1980; Fellowes 1998). Anuran amphibians have been especially well studied in this regard and wood frogs (*Rana sylvatica*), in particular, have been shown in five different experiments to recognize and preferentially associate with their kin (Waldman 1984; Cornell *et al.* 1989; Fishwild *et al.* 1990; Gamboa *et al.* 1991; Rautio *et al.* 1991; reviewed by Blaustein & Waldman 1992). However, because of the difficulty of identifying relatives using traditional techniques, no kinship studies with wood frogs or, indeed, with any anuran larvae have been conducted in the wild.

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Here, we use microsatellite analysis to estimate kinship and examine its import for the spatial distribution of wood frog larvae in the wild. While confirming the import of relatedness for anuran larval ecology, our results also show that kin selection and recognition may be considerably more complicated than previous laboratory studies suggest. In the first example of nonrandom dispersal (hyperdispersal) of nonreproductive animals in the wild and the first demonstration that a species may have both aggregated and hyperdispersed kin distributions, our study confirms what theory has long predicted — that context and scale may play important roles in kin selection.

## Methods

### *Larvae sampling*

Two different ponds known as Quarry and Little Thing, hereafter called LT pond, in the Yale-Myers forest in northeast Connecticut were used in this study. Quarry is the closest wood frog breeding pond to LT pond; it lies less than 50 m away. LT pond is a smaller (pond-full surface area = 79 m<sup>2</sup>) and shallower (pond-full maximum depth = 65 cm) pond than Quarry (pond-full surface area = 197 m<sup>2</sup>; pond-full maximum depth = 110 cm). LT pond receives somewhat more direct sunlight than Quarry because it has less forest canopy cover, but the difference is small (Halverson *et al.* 2003). More wood frog larvae predators were found living in Quarry; marbled salamander larvae (*Ambystoma opacum*), dragonfly larvae (*Libellula* spp.), predaceous diving beetle larvae (*Dytiscus* spp.), and backswimmers (*Notonecta* spp.) were all found during multiple dipnet surveys in the years 2000 and 2001. Of these predators, only dragonfly larvae were found in LT pond during this time (Halverson & Freidenburg, unpublished).

We surrounded LT pond with a drift fence in February 2002, before the breeding season began. Pit traps were located every 5 m around the inside and outside of the fence. We checked the traps and searched the pond exhaustively every day from 8 March 2002 to 7 April 2002. We also searched and tallied the number of egg masses in Quarry during this time interval. There was no more calling and no more egg masses appeared in either pond after this time. We weighed, measured snout-vent length, and took a toe clip from all adult wood frogs captured entering the pond and released them on the inside of the fence. The toe clips were stored in 70% alcohol for later analysis. Wood frogs captured on the inside of the fence during this time were released on the outside of the fence. Other vertebrates captured in the traps were released on the opposite side of the fence from where they were found. We opened sections of the fence after the last wood frog egg mass appeared to allow free access to the pond by other organisms.

To count the eggs in every egg mass in LT pond, we gently sandwiched each mass *in situ* between a white background and a clear piece of plastic and took a photograph with a digital camera. We counted the eggs in the photographs by marking each one by hand in ARCVIEW 3.2. Sampling from at least three nonadjacent parts, we collected a total of 20–30 embryos as soon as the egg mass was discovered and raised them in the laboratory until they hatched. The hatchlings were euthanized and stored in 70% alcohol for later analysis.

On 8 May 2002, we pushed a pipe sampler (35-cm diameter) through the water column and into the substrate in three discrete locations in LT pond and five discrete locations in Quarry (Shaffer *et al.* 1994). To minimize disturbance to the tadpoles, all sample sites were approached from the shore and were located 1 m from the perimeter of the pond. The first sampling location in each pond was chosen haphazardly and subsequent samples were 3 to 5 m from their nearest neighbour. Because the rocks and sticks scattered around the pond made pipe sampling infeasible in some locations, there was some variation in the distance between samples. We used a dip net to collect all the tadpoles within the pipe. We recorded the depth, water temperature 5 cm beneath the surface, and presence or absence of direct sunlight at each sampling location. The tadpoles were stored in 70% ethanol. The snout-vent length of every tadpole collected was measured and the developmental stage was assessed (Gosner 1960).

### *Microsatellite analysis*

We extracted DNA from the toes of the adults and portions of the tails of the larvae using the guidelines and materials in the Puregene DNA extraction kit (Gentra systems) and suspended the product in 50 µL of the DNA Hydration Solution.

For the microsatellite analysis, we used five tetranucleotide microsatellite loci described by Julian & King (2003) (RsyC11, RsyC23, RsyC41, RsyD20, RsyD88). Polymerase chain reactions (PCR) were carried out in 10-µL volumes containing 1× Applied Biosystems PCR buffer, 2 mM MgCl<sub>2</sub>, 5× New England Biolabs Purified BSA, 0.4 µM of each primer, 0.2 mM of each dNTP, and 0.5 U of Applied Biosystems AmpliTaq® DNA Polymerase. The products were resolved on a 4% polyacrylamide sequencing gel on an ABI 377 automated sequencer and analysed and scored using ABI GENESCAN® and GENOTYPER® software.

Because they are highly polymorphic and codominant, microsatellites are an ideal marker with which to analyse relatedness among individuals. The various methods and algorithms for estimating relatedness can be grouped into those that estimate pairwise relatedness among all individuals and those that partition individuals into groups (e.g. Queller & Goodnight 1989; Smith *et al.* 2001; reviewed in

Blouin 2003). The latter technique, which is used here, compares an individual's genotype to a group rather than all the individuals separately and thus makes use of the emergent information that is generated during the partitioning process. It is especially suitable to situations in which all individuals are known to be members of one cohort, as they are here, and need to be divided into sibships or half-sibships.

We used CERVUS software to analyse the allele frequencies of the sampled adults from LT pond for Hardy–Weinberg equilibrium, observed and expected heterozygosities, and exclusion probabilities (Marshall *et al.* 1998).

We used the computer program KINSHIPPC, which implements the algorithm developed by Smith *et al.* (2001), to analyse the microsatellite data and partition the larval samples into sibships. Because the number of possible partitions is enormous for data sets like the one described here, this program uses a Markov chain Monte Carlo method to sample the possible partitions and find an optimal solution. To test the ability of the program to divide the samples into groups correctly, six embryos from each of the five egg masses in LT pond were included in the analysis. Because there is no evidence for brood parasitism in this species (and the structure of the egg masses and the mechanics of oviposition make it extremely unlikely), we assumed that all individuals from a given egg mass were siblings or maternal half-siblings. If the program put all of the embryos from an egg mass into the same group and put all of the embryos from different egg masses in different groups, it was considered strong evidence that the program was able to correctly divide the larvae into kin groups composed of siblings or half-siblings. The program was then used to analyse the data from the tadpoles collected in Quarry.

We used two different Mantel tests to analyse the relationship between kin groups and sample locations (Mantel 1967). In both tests, we constructed a pairwise matrix of relationships as determined by KINSHIPPC; each pair from the same kin group had a value of 1 and all others had a value of 0. In the first test, hereafter referred to as the binary Mantel test, the second matrix was composed of 1s and 0s with each pair from the same sample having a value of 1 and all others having a value of 0. In the second test, hereafter referred to as the geographic distance Mantel test, the second matrix contained the geographic distances of each pair. In both cases, a two-tailed test was used to analyse the significance of the results when compared to 1000 randomly generated permutations.

We analysed the sensitivity of all of the Mantel tests by randomly selecting eight individuals (more than 10% of the larvae collected in either pond), randomly assigning them to a new sibship, recalculating the test statistics, and comparing the results to the 1000 randomly generated permutations used in the original tests. These sensitivity analyses were conducted 100 times for each of the Mantel tests.

## Results

The three sampling locations in LT pond were all shallower (mean depth = 20.3 cm, SE = 4.8) and warmer (mean temperature = 18.3 °C, SE = 0.3) than the five sampling locations in Quarry (mean depth = 44.2 cm, SE 6.35; mean temperature = 15.9 °C, SE = 0.25). An average of 22.3 (SE = 3.8) wood frog larvae were captured in the LT samples and an average of 15.4 (SE = 2.1) wood frog larvae were captured in the Quarry samples.

There were five wood frog egg masses found in LT pond between 24 March and 1 April and 23 egg masses found in Quarry between 31 March and 1 April. The initial density of wood frog larvae appears to have been higher in Quarry than in LT pond in 2002. The five wood frog egg masses found in LT pond contained an average of 664 eggs for an initial density of about 42 embryos per m<sup>2</sup> surface area. If the 23 egg masses found in Quarry contained the same average number of embryos, there was an initial average of about 77 wood frog embryos per m<sup>2</sup> surface area.

With an average snout-vent length of 0.96 cm ( $n = 67$ , SE = 0.05), the tadpoles from the pipe samples from LT pond were significantly larger (ANOVA  $P < 0.01$ , total d.f. = 143) than the tadpoles captured in Quarry (average = 0.83 cm,  $n = 77$ , SE = 0.08). The tadpoles from LT pond (average Gosner stage = 27.32,  $n = 67$ , SE = 0.083) were also slightly but significantly more developed (ANOVA:  $P < 0.001$ , total d.f. = 143) than the tadpoles from Quarry (average Gosner stage = 26.97,  $n = 77$ , SE = 0.071).

The microsatellite loci used here were all polymorphic, with 5 to 16 alleles each among the 28 adults sampled (Table 1). Expected heterozygosities ranged from 0.416 to 0.900. None of the loci showed a significant departure from Hardy–Weinberg equilibrium. The exclusion probabilities for a parent given no information about the other parent ranged from 0.087 to 0.626 for individual loci, with a multilocus exclusion probability of 0.990. The exclusion probabilities for a parent when the other parent is known ranged from 0.222 to 0.778 for individual loci, with a multilocus exclusion probability of 0.999.

We successfully amplified and scored the microsatellite loci for almost all the larval samples. For one of the 74 samples from Quarry, PCR failed to generate any product at any locus. This failure was probably due to an error in the extraction process and the sample was not used in the analysis.

For LT pond, the microsatellite data from the subset of the hatched embryos that were analysed and the tadpoles were pooled and analysed together. KINSHIPPC partitioned the 67 tadpoles and the 30 hatchlings into five groups (hereafter referred to as kin groups), which was the same as the number of egg masses that were found in the pond at the beginning of the breeding season. Each kin group included a different set of six hatchlings from the same egg

Locus	No. of alleles	$H_O$	$H_E$	Exclusion probability 1	Exclusion probability 2	HWE probability
C23	5	0.321	0.416	0.087	0.222	0.335
C41	9	0.857	0.842	0.493	0.665	0.854
C11	12	0.964	0.895	0.610	0.759	0.777
D20	12	0.857	0.883	0.583	0.738	0.777
D88	16	0.964	0.900	0.626	0.770	0.777
All loci				0.990	0.999	

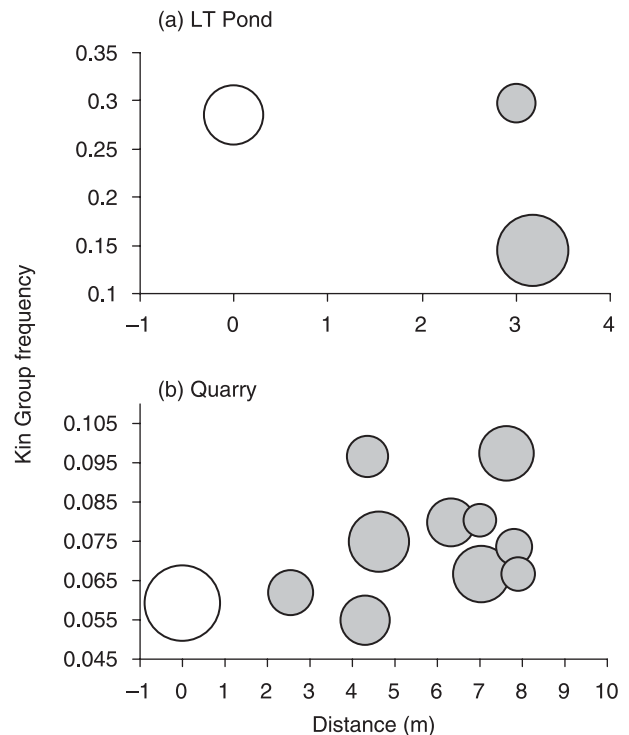
**Table 1** Summary statistics for the microsatellite loci

$H_O$  is the observed heterozygosity.  $H_E$  is the expected heterozygosity based on the number of alleles. Exclusion probability 1 reflects the probability that a given locus will be able to exclude an individual as the parent when there is no information about the other parent. Exclusion probability 2 reflects the probability that a given locus will be able to exclude an individual as the parent when one parent is known. HWE probability is the probability that a locus at Hardy–Weinberg equilibrium would generate the observed allele combinations in the population for a given locus. None of the HWE probability values were significant after a Bonferroni correction was applied.

mass, showing that the algorithm could correctly partition the sampled embryos into full- or half-sib groups. Results from a concurrent experiment suggest there is little multiple paternity in this population; however, we have chosen to be conservative and refer to the individuals from the same egg mass as kin groups rather than sibships (Halverson, unpublished data). Egg mass samples were not available from Quarry to validate the algorithm with that population. However, KINSHIPPC found an optimal solution that partitioned the 74 analysed tadpoles from Quarry into 18 different groups, less than the number of egg masses that were counted in that pond at the beginning of the season. This result would make sense if we did not sample an individual from every egg mass in Quarry, either due to sampling effects or because some egg masses were destroyed by disease, predation, or abiotic factors before hatching.

In the binary Mantel test, the tadpoles from LT pond were significantly clumped with their kin groups ( $P < 0.01$ ). However, the tadpoles from Quarry showed just the opposite pattern; they were significantly hyperdispersed (more dispersed than is likely to occur from chance alone) from their kin groups ( $P = 0.01$ ). In the geographic distance Mantel test, the tadpoles from LT pond were also significantly clumped with their kin groups ( $P < 0.01$ ). However, although the geographic distance Mantel test showed the same trend as the binary test, the results of the geographic distance test in Quarry were not significant by a small margin ( $P = 0.07$ ) (Fig. 1).

The results from LT pond were robust; the results from Quarry were less so. When eight larvae from LT pond were randomly selected and reassigned to other kin groups, the binary Mantel test was significant 87% of the time and the geographic distance Mantel test was significant 100% of



**Fig. 1** Kin group frequency as a function of distance. All possible pairs of larvae were classified as kin or non-kin and into distance classes based on the location in which they were sampled. These figures represent the proportion of pairs in (a) LT pond and (b) Quarry that were classified as kin as a function of a distance class. Bubble size is proportional to the total number of pairs compared at that distance. White bubbles represent the zero distance class or, in other words, all pairs from the same pipe sample. Filled bubbles represent pairs from different pipe samples. There are only two non-zero distance classes because the distance from one of the sampling locations to each of the other two sampling locations was identical.

the time. When eight larvae from Quarry were randomly selected and reassigned to other kin groups, the binary Mantel test was significant 38% of the time and the geographic distance Mantel test was significant 17% of the time.

## Discussion

Prior studies of kin selection in artificial settings have shown that the larvae of wood frogs and other amphibian species often recognize and associate with their kin (reviewed in Blaustein & Waldman 1992). Here we show that studies such as these, conducted in a simplified environment, may not reflect what actually occurs in the wild. Using microsatellite analysis to assign kinship, we show that while kin appeared to be aggregating in one pond, they appeared to be nonrandomly dispersed from their kin in another. Such results are consistent with theory suggesting that animals should either aggregate or be repulsed from their kin, depending on localized factors including competition, predation, parasitism, and abiotic factors (Hamilton 1964a, b).

The mechanism by which such spatial distributions are generated may be passive or active. If the individuals are born in nonrandom locations, their future distribution may also be nonrandom. However, such a mechanism is unlikely in this case because wood frogs typically, and in the ponds studied here, oviposit very close to one another. In addition, by the time the pipe samples were taken, the larvae appeared to have dispersed throughout the pond. Previous studies have shown that wood frog larvae typically move at least a metre a day at this time of year in ponds at the Yale-Myers Forest (Freidenburg 2003).

Individuals may also share environmental preferences with their kin or with individuals of a similar size or developmental stage and thus seek out the same location (Pfennig 1990). In fact, wood frog larvae from different ponds in the Yale-Myers Forest have been shown to have different thermal preferences (Freidenburg & Skelly 2004). However, while shared environmental preferences may be partly responsible for the observed patterns, previous laboratory experiments with this species suggest kin recognition is probably also involved. Five different laboratory studies have been published on wood frog larvae kin recognition and association (Waldman 1984; Cornell *et al.* 1989; Fishwild *et al.* 1990; Gamboa *et al.* 1991; Rautio *et al.* 1991; reviewed by Blaustein & Waldman 1992). In every case, wood frogs were shown to recognize and preferentially associate with their siblings or half-siblings and thus it is likely that kin recognition led to aggregation in one pond and repulsion in the other.

If kin recognition was a factor in the spatial distribution, then other questions arise. Because the tadpoles were not mature and were not competing with their parents, explanations most commonly used to explain repulsed kin

distributions such as inbreeding avoidance (Bateson 1983) and parent-offspring competition (e.g. Hurst & Barnard 1991) do not apply. Instead, it is possible that other costs of aggregation were higher than the benefits Quarry and less in LT pond. It is impossible to determine why this difference should exist based on the information from this study. However, there were distinct differences between the two ponds.

First, there were important biotic and abiotic differences between the two ponds. Quarry appears to have more vertebrate and invertebrate aquatic predators than LT (Freidenburg, unpublished). The egg mass survey indicates that the tadpole density was, at least initially, higher in Quarry than in LT pond. However, the density of the tadpoles in the pipe samples was higher in LT pond than in Quarry. This apparent change in relative densities may indicate there was higher mortality in Quarry between the oviposition date and May 8, when the tadpole samples were taken. However, it may also be an artefact of the sampling regime and the change in size of the ponds due to precipitation and evapotranspiration. In addition, although the hydroperiod is about the same, Quarry is larger, deeper, darker and colder on average than LT pond. No data on food resources are available, but this and other factors affecting competition probably also differed between the two ponds. As a result of one or all of these factors, the tadpoles were, on average, smaller and less developed in Quarry than they were in LT pond. Second, there were more kin groups in Quarry than in LT pond, which might have influenced behaviour as individuals are less likely to encounter a sibling or half-sibling in Quarry than in LT pond. Finally, because of the different size of the ponds and the sampling regime, the average distance between samples was different in the two ponds. This raises important questions about the scale at which kin distribution occurs. While this study is not suited to resolve such questions, the techniques described herein may be useful for future studies.

In short, the spatial distribution of wood frog kin groups differed between the two ponds, as did the physical and biotic factors affecting them. If the spatial distribution of the kin groups was context dependent, it would be consistent with the findings of other researchers who showed that the presence of predators, more even food distribution, and lower temperature variability diminished the tendency for anuran larvae to form aggregations (Hokit & Blaustein 1997). Such a distribution is also consistent with the hypothesis that inclusive fitness can be increased by avoiding competition with kin (Hamilton & May 1977), by spreading the risk that lineage will be destroyed by a single predator or catastrophic event (Stearns 1992), and by increasing genetic diversity and thereby reducing the spread of disease (Shykoff & Schmid-Hempel 1991; Garrett & Mundt 1999).

Although kin selection and kin recognition have been more extensively studied in anuran larvae than in any

other vertebrate, this is the first study of these phenomena to be conducted with these animals in the wild. While the limited data presented here make it impossible to assess mechanism with any confidence, and indeed leave more questions than answers, the results do show that spatial distribution of kin may be context dependent and that laboratory experiments are not necessarily indicative of patterns in natural settings. That this is the only study to date to find nonrandom dispersal of nonbreeding animal kin groups in the wild and the first to show that a species may have different nonrandom kin distributions in different circumstances may reflect the actual prevalence of these phenomena (for examples in seminatural conditions see Jasienski 1988; Kasuya 2000; Griffiths *et al.* 2003). More likely, though, they reflect the need to further study kin selection with this and other species under natural settings.

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