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Delineating fine-scale genetic units in amphibians: Probing the primacy of ponds

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Abstract

The population structure of pond-breeding amphibians is shaped by their distinct breeding foci, but it is unclear to what extent this is reflected in the fine-scale distribution of genetic diversity. We used microsatellite genotypes to investigate the genetic signatures of 24 populations of European newts, *Triturus cristatus* and *T. marmoratus*, inhabiting 21 ponds in a confined study area (7.5 × 3.5 km) in western France. Employing a Bayesian clustering approach based on individual genotypes that minimises departures from Hardy–Weinberg equilibrium and linkage disequilibrium, no evidence was found for within-pond substructuring. Subjecting all sampled ponds simultaneously to this procedure revealed a clear signal of partitioning, with the most likely number of clusters however below the actual number of ponds (seven in *T. cristatus*, three in *T. marmoratus*). A more hierarchical Bayesian approach, with pond as analysis unit, was achieved to separate ponds from genetically more meaningful units, and reduced the *T. cristatus* populations to 11 clusters, and the *T. marmoratus* populations to five clusters. We were unable to specify a minimum nearest-neighbour distance where ponds are separate units, probably due to both historical and current demographic processes. The implications for strategies to manage and conserve endangered amphibians in human-altered landscapes are discussed.

Introduction

The distribution of neutral genetic variation across a species geographical range is, to a large extent, determined by demographic substructuring (Charlesworth et al. 2003). Classical population genetic approaches usually assume non-hierarchical sets of random-mating demes, whereas empirical studies often reveal nested substructuring occurring at different levels (e.g. Sugg et al. 1996; Castric et al. 2001). In conservation science, the accurate delineation of genetic units is vital to manage the evolutionary potential of endangered species (Bowen 1999), and to identify sets of individuals susceptible to genetic threats (Gaggiotti 2003). At a fine study scale, population-diagnostic markers are usually unavailable, and it is *a priori* assumed that the demographic structure apparent in the field will reflect the underlying genetic structure. However, this is not necessarily the case. Important genetic units might occur at different hierarchical levels, and demographic populations, for example defined through habitat patches, will not necessarily be identical to populations defined on genetic grounds (Manel et al. 2003, 2004; Pannell and Obbart 2003).

Habitats play an important role in shaping the spatial distribution of individuals. In pond-breeding amphibians, conservation management is usually based on the assumption that each aquatic site represents a distinct population. However, although this offers a straightforward approach, it remains controversial as to whether populations as defined by ponds usually reflect independent demographic units (Marsh and Trenham 2001). High philopatry to a specific breeding site has been regularly reported from capture–mark–recapture studies, but these results conflict with pond occupancy rates and synchronised population fluctuations which imply the presence of patchy populations characterised by frequent inter-deme migration (Bradford et al. 2003; Petranka et al. 2004). Genetic approaches could shed more light on the question of population structure, but the available information for amphibians remains sketchy (Jehle and Arntzen 2002). In some cases, neighbouring ponds have been shown to differ significantly in allele frequencies (Lampert et al. 2003; Andersen et al. 2004), whereas no clear separation was suggested in other cases (Newman and Squire 2001). The aim of the present paper is to use information drawn from microsatellite genotypes to investigate the fine-scale genetic structure of two amphibian species (the crested newt, *Triturus cristatus*, and the marbled newt, *T. marmoratus*), without *a priori* assuming that genetic units are reflected in ponds. A well-studied close network of breeding sites in western France (Arntzen and Wallis 1991; Jehle et al. 2001) provides a suitable model system. Firstly, we use an individual-based approach to address whether any substructure can be detected within a pond. After we show that this is not the case, we test the hypothesis that ponds are inhabited by groups of individuals which are genetically discernible from neighbours, despite the possibility of interpond migration. We achieve this by applying the individual-based clustering method to all sampled ponds, as well as an alternative approach which treats each pond as an analysis unit.

Materials and methods

The 15 *T. cristatus* and nine *T. marmoratus* populations across 21 study ponds were located in an area of approximately 7.5 × 3.5 km in the Département Mayenne, western France. Most breeding sites were man-made cattle ponds roughly 5–100 m^2 in size. Between-pond geographical distances were measured to the nearest mm on topographical maps (1:25,000). Tissue samples (772 and 352 individuals in *T. cristatus* and *T. marmoratus* respectively) were collected in 2001 and 2002. Microsatellite genotypes were obtained using methods and loci described elsewhere (Jehle et al. 2001; Krupa et al. 2002). For this study, the seven and four loci in *T. cristatus* (*Tcri*13, *Tcri*27, *Tcri*29, *Tcri*32, *Tcri*35, *Tcri*36, *Tcri*43) and *T. marmoratus* (*Tcri*27, *Tcri*32, *Tcri*35, *Tcri*43), respectively, which were in Hardy–Weinberg equilibrium except for a single population (*T. cristatus* of Pond N11, Jehle et al. in press), were considered. Putative hybrids (about 4% of individuals, Arntzen and Wallis 1991) and individuals with putatively introgressed species-diagnostic alleles bias the calculations and were excluded from the analysis. Sample size per population ranged between eight and 117 individuals, and pairwise genetic differences (F_{st}) ranged between 0.01 and 0.21 in *T. cristatus* and between 0.01 and 0.30 in *T. marmoratus* (Jehle et al. in press). Although there was no evidence that the two sampling years represent distinct gene pools (Jehle et al. in press), we aimed for a careful analysis and treated them separately when testing for within-pond substructure. For the subsequent analysis across ponds, however, years were pooled due to computational constraints.

The potential existence of substructure within ponds was addressed using the Bayesian clustering approach implemented in the software STRUCTURE 2.0 (Pritchard et al. 2000; Falush et al. 2003). Using a Markov chain Monte Carlo (MCMC) algorithm, individual genotypes are assigned to a predefined number of clusters (K) in a given sample (X), in order to achieve Hardy–Weinberg and linkage equilibrium. As recommended in Falush et al. (2003) to obtain the most unbiased model, we firstly inferred the Dirichlet parameter λ for the allele frequency prior used the uncorrelated allele frequency model at $K = 1$. We then fixed λ at the inferred value to calculate *ln* posteriors of $P(X|K)$ using the correlated frequency model (10^6 runs after 100,000 burn-ins), followed by obtaining $P(K|X)$ using Bayes' rule. As potentially inferred subdivisions would further reduce the sample size per cluster, we set a maxi-

mum K at 3, and only analysed pond and year combinations for which >20 genotypes were available.

To investigate whether the genetic structure across the study area is reflected in breeding sites, we subsequently performed a similar analysis considering all sampled ponds simultaneously. We assumed values of K ranging between 1 and the actual number of ponds, the maximum expected number of clusters. As we did not attempt any subdivision below the pond level, we also incorporated sites for which <20 individuals were available (see Table 2). Again, we used the correlated allele frequency model. Due to computational constraints, the allele frequency prior λ was fixed at 1, a value close to the one usually obtained when conducting the within-pond analysis (see above).

For a more hierarchical analysis to assess the spatial structure, we used the Bayesian clustering method described in Corander et al. (2003). This approach enables us to distinguish an enforced substructure (in our case, defined on the basis of ponds) from a potentially more meaningful structure reflected in the data set. The model treats the number of populations as unknown, and, considering all group combinations between subunits to be equally likely, circumvents the problem of *a priori* population labelling. The criteria used to separate populations are based on whether any population pair in the sample can in fact be regarded as being a single population (for details see Corander et al. 2003). Posterior distributions are derived from an enumerative calculation where the number of predefined populations is below 10 (in our case, *T. marmoratus*), or an MCMC algorithm with >10 populations (in our case, *T. cristatus*; we considered 500,000 runs after 100,000 burn-ins). We set a lower probability bound of 0.05 for posterior distribution of partitions to be considered in a final model, and present all models to enable an assessment of alternative scenarios other than the most likely partition.

Results

Within ponds, the probability that the sample was drawn from a single cluster always exceeded the probability for two or three clusters (Table 1). In other words, all ponds are likely inhabited by

Table 1. Posterior probabilities P for substructuring within *Triturus cristatus* (*c*) and *T. marmoratus* (*m*) populations (following Pritchard et al. 2000; Falush et al. 2003) assuming between one and three genetic units in samples with $n>20$ individuals drawn from one pond in one year

Pond (year)	Species	n	P (1)	P (2)	P (3)
232 (2001)	*c*	48	0.999	0.000	0.000
2E4 (2002)	*c*	29	0.999	0.000	0.000
2H6 (2001)	*c*	60	1.000	0.000	0.000
2N8 (2001)	*c*	51	0.999	0.000	0.000
2N8 (2002)	*c*	27	0.999	0.000	0.000
2P7 (2001)	*c*	33	0.984	0.015	0.001
N3 (2001)	*c*	50	0.550	0.450	0.000
N6 (2001)	*c*	51	0.999	0.000	0.000
N6 (2002)	*c*	27	0.867	0.003	0.130
N7 (2001)	*c*	117	0.999	0.000	0.000
N8 (2001)	*c*	35	0.999	0.000	0.000
N10 (2001)	*c*	28	1.000	0.000	0.000
N11 (2002)	*c*	34	0.999	0.000	0.000
N13 (2002)	*c*	35	0.993	0.005	0.001
278 (2001)	*m*	36	0.995	0.000	0.004
2G9 (2002)	*m*	30	0.989	0.006	0.004
N1 (2002)	*m*	29	0.592	0.012	0.340
222 (2001)	*m*	89	1.000	0.000	0.000
232 (2001)	*m*	50	0.597	0.401	0.000
2D5 (2001)	*m*	78	1.000	0.000	0.000

a single genetic unit, and only two populations (Pond N3 in *T. cristatus* and Pond 232 in *T. marmoratus*) showed considerable ($P > 0.3$) evidence for a bipartition, and one population (Pond N1, *T. marmoratus*) showed similar evidence for a tripartition. Employing this approach across all ponds combined clearly indicated the presence of substructure, however not in agreement with the actual number of sampled ponds. $P(X|K)$ was maximal for $K = 7$ (*T. cristatus*) and $K = 3$ (*T. marmoratus*, Figure 1). For the most likely partition, the proportion of samples from ponds assigned to each cluster was overall uneven, but with only a loose match of clusters with (sets of) ponds (Table 2).

The hierarchical analysis revealed that most ponds represented their own genetic units. In *T. cristatus*, the most likely partition (posterior $P = 0.94$) reduced the 15 populations into 11 clusters, including four pairs of ponds (Figure 2). One pair comprised two neighbouring ponds (N6, N7: 450 m); the other pairs (2A1–2N8, 2C8–2P7, 232–N13) covered distances of 1825, 2150, and 2250 m respectively. The second most likely partition ($P = 0.06$, $n = 10$ clusters) merged pond N10 (situated 450 m from 2N8) to the cluster of 2N8 and 2A1. In *T. marmoratus*, the most likely partition reduced the nine ponds into five clusters ($P = 0.73$, Figure 2). One cluster comprised the three ponds 278, 222, and N12 in the north-east of the study area (at geographical distances not exceeding 625 m), and another cluster combined the adjacent ponds 2C8 and N1 (625 m distance). One genetic unit, however, consisted of ponds 2D5 and 2G9 spaced at 3925 m. The second most likely partition ($P = 0.09$) again consisted of five units, however splitting the three combined ponds and merging pond N12 with pond 2A1; a third partition ($P = 0.08$) merged the four ponds 278, 222, N12 and 2A1, reducing the number of clusters to four.

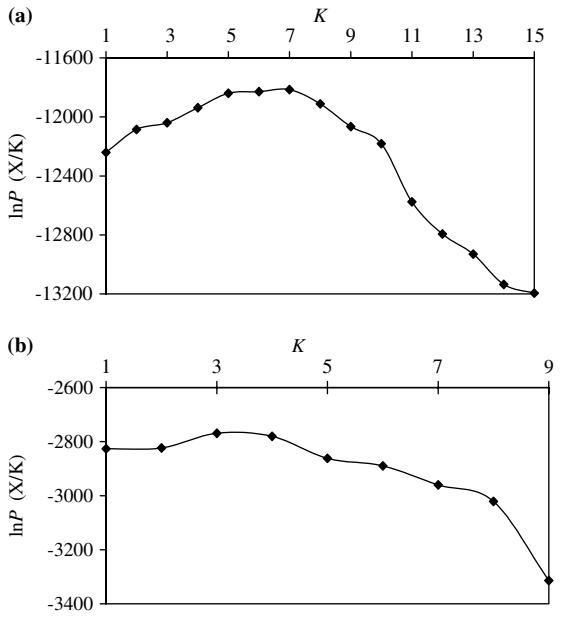

Figure 1. Bayesian posterior probabilities estimated following Pritchard et al. (2001) and Falush et al. (2003), assuming between 1 and *n* clusters (*n*, the number of sampled ponds). Higher values represent a higher probability of the assumed partition; for more details see text. (a) *Triturus cristatus*, (b) *T. marmoratus*.

Discussion

The traditional view of the amphibian conservation biologist that breeding ponds represent distinct populations requires significant demographic uncoupling (for example so that variation in population size will be independent, Petranka et al. 2004), an assumption which is not consistent with the fact that newly created habitats are often rapidly colonised (Bradford et al. 2003). In contrast to previous studies on genetic structure (e.g. Scribner et al. 2001), we refrain from the *a priori* assumption that populations are reflected in ponds. We show that all individuals inhabiting a breeding site can indeed be regarded as a genetically panmictic unit. Without considering potential demographic causes (colonisation: Arntzen and Wallis 1991; current gene flow and drift: Jehle et al. in press), we furthermore reveal that in our study system the majority of ponds can be distinguished genetically from close neighbours.

The within-pond analysis confirmed that breeding sites represent unstructured groups of individuals. The approach we have employed tends to suggest partitioning despite its actual absence, for example due to mild departures from linkage or Hardy–Weinberg equilibrium (Falush et al. 2003). Nevertheless, evidence ($P > 0.3$) for more than one cluster was only present in three populations. The *T. cristatus* population comprises around eight individuals, implying potential assortative mating in a small group, whereas one of the *T. marmoratus* populations (pond 232)

Table 2. Proportion of membership for each pond assigned to each cluster for the most likely partition (following the approach described in Pritchard et al. 2000 and Falush et al. 2003). (a) *Triturus cristatus* ($K = 7$), (b) *T. marmoratus* ($K = 3$)

Pond	*n*	1	2	3	4	5	6	7
(a)								
233	27	0.066	0.099	0.034	0.139	0.072	0.075	0.516
232	48	0.076	0.189	0.122	0.349	0.050	0.132	0.083
2A1	17	0.236	0.151	0.057	0.194	0.161	0.059	0.141
2C8	11	0.166	0.148	0.132	0.159	0.135	0.155	0.105
2E4	35	0.072	0.105	0.126	0.120	0.043	0.116	0.419
2H6	60	0.111	0.037	0.043	0.039	0.652	0.039	0.080
2N8	78	0.162	0.322	0.053	0.058	0.111	0.127	0.166
2P7	45	0.651	0.050	0.100	0.075	0.055	0.031	0.038
N10	45	0.103	0.300	0.155	0.145	0.080	0.130	0.087
N11	66	0.182	0.201	0.082	0.241	0.139	0.055	0.100
N13	36	0.065	0.114	0.045	0.395	0.132	0.115	0.133
N3	60	0.047	0.076	0.081	0.251	0.072	0.392	0.080
N6	78	0.109	0.129	0.205	0.080	0.096	0.220	0.160
N7	117	0.068	0.089	0.268	0.101	0.050	0.227	0.197
N8	48	0.127	0.076	0.533	0.068	0.025	0.110	0.060
(b)								
278	89	0.205	0.429	0.366				
2G9	36	0.275	0.399	0.326				
N1	50	0.734	0.118	0.148				
222	78	0.249	0.342	0.409				
2A1	18	0.175	0.505	0.320				
232	33	0.284	0.359	0.357				
2D5	29	0.272	0.398	0.330				
N12	8	0.183	0.553	0.263				
2C8	10	0.396	0.215	0.389				

consists of around 147 individuals (Jehle et al. 2001), implying potential subgrouping in a large population. However, the proportion of a given genotype which is assigned to several clusters is mostly symmetric (data not shown), generally suggesting that the calculated di- and tri-partitions are not biologically meaningful.

When incorporating all ponds into the clustering model, a clear signal of substructuring was detected, in agreement with the fact that the sampled individuals stem from different breeding sites. However, any other result should not be over-interpreted. The approach to estimating the probability of a specific K requires caution. When the putative number of subpopulations is large (as in our case, 15 and nine), this approach reveals a partition with the most likely K below the actual value of subgroups (Pritchard et al. 2000), even when subgroups are clearly separated and a large number of loci is available (domestic chicken breeds, Rosenberg et al. 2001). An analysis as described in Dawson and Belkhir (2001) might have been a useful alternative, but was not conducted as the associated software (PARTITION) does not allow for missing data (6.3% and 3.3% of data across all loci were missing in *T. cristatus* and *T. marmoratus*, respectively).

As the analysis implemented in STRUCTURE 2.0 revealed only a loose match between genetic partitions and actual ponds (Table 2), the hierarchical Bayesian clustering method of Corander et al. (2003) seems more appropriate to relate genetic with spatial structure. Most ponds were identified as significant genetic entities, and the clustering was often related to the spatial location of ponds (such as ponds N6 and N7 in *T. cristatus*, or ponds 222, 278 and N12 in *T. marmoratus*; Figure 2). In four cases, however, two populations at geographic distances above 2 km were pooled. For *T. cristatus*, one population pair included

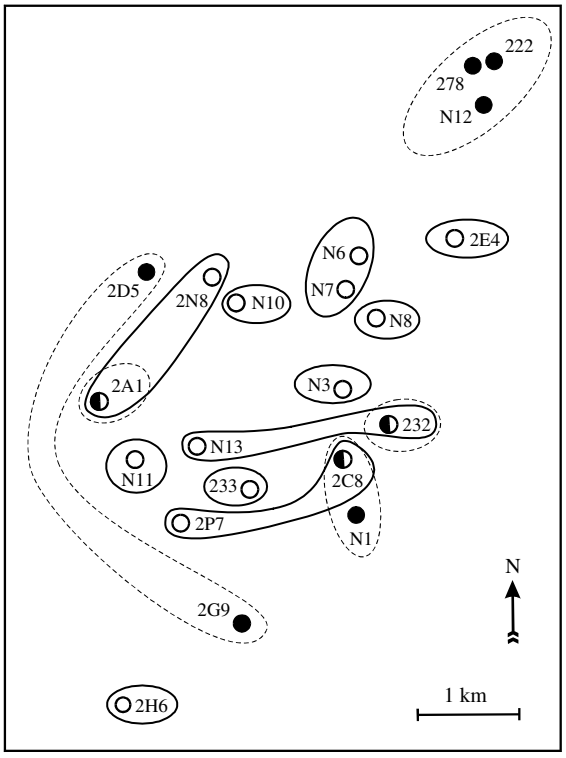

Figure 2. Study ponds (open circles: *Triturus cristatus;* filled circles: *T. marmoratus*; both species considered in three ponds) and the most likely partition of genetic units (solid lines: *T. cristatus*, posterior $P = 0.94$; dashed lines: *T. marmoratus,* posterior $P = 0.73$) identified using the Bayesian approach from Corander et al. (2003).

pond 2A1, whose genotypic data are not fully interpretable due to an introduction in 1994 (J. W. Arntzen, unpublished data). The other cases involved two out of the three ponds for which only inconclusive results were obtained when estimating dispersal rates (Jehle et al. in press), potentially caused by undetected introgression of non-diagnostic alleles in frequent hybridisation events. *Triturus marmoratus* populations tended to cluster more than *T. cristatus*, such as in the case for the two distant and large populations 2G9 and 2D5. *Triturus marmoratus* is seen as the more terrestrial species (Arntzen and Wallis 1991), but the data structure is insufficient for a firm conclusion that it is a better disperser than *T. cristatus*, leading to more homogenised gene pools.

How do our results integrate into other amphibian field studies such as based on capture–mark–recapture? For example, Trenham et al. (2001) investigated between-pond migration rates in California tiger salamanders (*Ambystoma californiense*) at a spatial scale very similar to ours. On the assumption that one disperser per generation leads to a homogenisation of gene pools, they concluded that a weak genetic differentiation should exist between their ponds. Our empirical data agree with such a finding, but also reveal some degree of variance in spatial differentiation, as a minimum geographical distance where an association between two adjacent ponds breaks into two distinct units was impossible to specify. This is not surprising, as for example different numbers and origins of founders result in varying genetic compositions which might be still apparent in an established population. In addition, different between-pond terrestrial habitats can lead to corridors or barriers to dispersal (Scribner et al. 2001), and gene flow rates can be dependent on population size (Jehle et al. in press). Petranka et al. (2004) argued for wood frogs (*Rana sylvatica*) that emigration and habitat shifts associated with pond perturbation precluded some local ponds to be independent demographic units. However, the conclusion that habitat disturbance might have caused some of our ponds not to bear their own genetic signature is hampered by the fact that the two study species do not show identical patterns of clustering.

Our results also conform to recently documented fine-scale differences in life-history traits and thermal preferences between neighbouring *R. sylvatica* populations (Relyea 2002; Skelly 2004). The (usually) pond-specific signatures of neutral variation revealed by us suggest that additive genetic variance could indeed account for phenotypic differences between amphibian ponds, even when they are potentially connected by migration. On the other hand, when looking at larger geographical scales, non-genetic maternal and dominance effects outweigh the importance of inherited variation with regard to adaptation to different environments (Merilä et al. 2004).

What do these inferences tell us about conservation strategies for pond-breeding amphibians? The consequences of genetic structuring for fitness-related traits, an as yet unresolved issue in amphibians (Rowe et al. 1999; Rowe and Beebee 2001), is beyond the scope of the present study. Moreover, ponds do not carry diagnostic neutral variation, and thus cannot be seen as evolutionary significant units (cf. Crandall et al. 2000). However, there is a need for future studies to investi-

gate whether the observed small-scale differences in life history and neutral genetic variation are reflected in quantitative genetic differences. Our data suggest this is possible, and care should be taken to preserve ponds with different ecological regimes in order to maintain the full adaptive potential of a species. Our study also has consequences for the overall maintenance of neutral genetic variation. When the degree of subdivision is high, the variance in reproductive success across demes becomes reduced, and the retention of genetic diversity can be enhanced (Ray 2001). Natural population turnover in amphibian populations is often deterministically related to pond age, rather than being stochastic (Marsh and Trenham 2001). It should therefore be ensured that fine-scale units are maintained over long periods of time, however without necessarily disrupting the natural process of pond succession, which ultimately leads to the disappearance of a pond, usually after several decades. For the management of existing populations, the distinctiveness of ponds implies that as many ponds as possible should be preserved, rather than only a fraction of seemingly more important (e.g. larger) demes. And finally, although there is rising evidence that pond-breeding amphibians usually do not follow classical metapopulations, it also has to be ensured that habitat connectivity enables the colonisation of newly emerging sites.

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