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ESTIMATING ISOLATION AND GENETIC DIFFERENTIATION IN TWO BELGIAN POPULATIONS OF MOORHENS *GALLINULA CHLOROPUS* BY USING MINISATELLITE AND MICROSATELLITE DNA MARKERS

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Abstract. Isolation of a population can result in decreased genetic variability as a consequence of inbreeding, random genetic drift and reduced gene flow. This effect is reinforced when it concerns a small population. We used two molecular techniques, multilocus minisatellite DNA fingerprinting and microsatellite analysis, to compare population genetic parameters between a small, possibly isolated natural population of moorhens and a large, presumably non-isolated population at a distance of approximately 30 km. Although sample sizes were still relatively low, both minisatellite and microsatellite analysis indicated that the small population is not genetically impoverished, despite being located at the centre of a large city. Nevertheless, we found significant between-populational genetic differentiation, which suggests that there is little gene flow between the two populations studied.

Key words: *Gallinula chloropus*, DNA-fingerprinting, minisatellites, microsatellites, isolation, inbreeding, population differentiation.

INTRODUCTION

Individuals in small populations are more likely to be inbred because there is a high probability that random pair formation will occur between related individuals (AVISE, 1994; NEIGEL, 1996). Genetically, inbred populations are expected to show increased homozygosity due to the increased probability that individuals carry alleles that are « identical by descent » (AVISE, 1994). Small populations are also more susceptible to loss in genetic polymorphism due to random genetic drift. The main process that counteracts this decrease in genetic variability in small populations is gene flow, largely achieved by migrating individuals (NEIGEL, 1996). Therefore, looking at the genetic variability or the level of inbreeding in a small population can provide information about the degree of isolation, in terms of gene flow, of that population.

Genetic drift, selection and mutation may all result in the divergence of allele and genotype frequencies between isolated populations, such that populations become geneti-

cally different, a process known as population differentiation. This genetic divergence is also reduced by exchange of genetic material (NEIGEL, 1996). As such, the amount of genetic differentiation provides information on the amount of gene flow between populations.

During the course of the last decade, a diverse array of new molecular genetic tools has become available for high-resolution genetic studies of population-level processes (PARKER *et al.*, 1998). In recent years, minisatellite and microsatellite DNA markers have proven their usefulness in numerous applications, including studies on population genetics and relatedness (AVISE, 1994; BRUFORD *et al.*, 1996; BURKE *et al.*, 1996). Minisatellite and microsatellite loci belong to the classes of loci known as variable number tandem repeat (VNTR) loci. Both are usually highly polymorphic, owing to variation in the number of tandem repeats. As a consequence, they can provide information on the genetic structure of individuals (heterozygosity) or populations (allelic diversity) where less sensitive methods such as enzyme electrophoresis might fail (BURKE *et al.*, 1996). Contrary to minisatellites, microsatellite loci can be assayed using the polymerase chain reaction (PCR) combined with electrophoresis (BURKE *et al.*, 1996). This approach allows determination of the exact length in basepairs of alleles, which can be unambiguously assigned to certain loci (QUELLER *et al.*, 1993). As a result, microsatellites are highly suitable to assess genetic variation in terms of heterozygosity levels, genetic polymorphism, and allele frequencies, within and between populations, which makes them powerful tools for estimating inbreeding levels and population differentiation (BRUFORD *et al.*, 1996). Although multilocus minisatellite analysis has also been successfully used in population genetic studies (*e.g.* WAUTERS *et al.*, 1994), this method has more drawbacks than the previous one (QUELLER *et al.*, 1993; AVISE, 1994; BURKE *et al.*, 1996). One of the problems is that multilocus minisatellite analysis reveals complex multiple banding patterns, or «DNA fingerprints», in which it is usually not possible to distinguish every allele and in which alleles generally cannot be assigned to a particular locus (JEFFREYS *et al.*, 1985a,b; BRUFORD *et al.*, 1992; AVISE, 1994; BURKE *et al.*, 1996). Minisatellite fingerprinting is therefore less suitable for studies requiring information on allele frequency distributions, such as needed to estimate the degree of gene flow between populations (BURKE *et al.*, 1996). However, « DNA fingerprints » can be used to elucidate inbreeding because they make it possible to estimate the mean relatedness within populations (AVISE, 1994). BELLAMY *et al.* (1991) have shown in humans that even a moderately inbred population can be recognised by a higher mean band sharing than a known outbred population.

The Antwerp Zoo, which is located at the centre of the large Belgian city of Antwerp, contains a small population of free-living moorhens. The main goal of our study was to find out whether the surrounding city functions as a barrier that decreases migration in such a way that the moorhens at the Antwerp Zoo form an isolated population. This was tested by estimating the level of inbreeding in the Zoo population compared with that in a large, presumably non-isolated moorhen population at Planckendael, using both multilocus minisatellite and microsatellite DNA markers. The microsatellite analysis also allowed us to determine genetic differentiation between the two populations and to estimate gene flow.

MATERIAL AND METHODS

Study areas and blood sampling

In Belgium, moorhens are common in all kinds of habitats as long as fresh water and low cover are present (WOOD, 1974; STEVENS, 1989). Therefore, moorhens can be abundant in city parks even though they do not inhabit the rest of the city. At the time of its foundation in 1843, the Antwerp Zoo was located at the edge of the town (BAETENS, 1993). Due to increasing urbanisation ever since, the surrounding area of the Zoo has been built over within a radius of five kilometres. The Zoo, with an area of about ten hectares, is now located at the centre of the city and forms, together with a public park nearby the Zoo, an isolated patch of suitable habitat for moorhens in the crowded city (BAETENS, 1993). No records were kept, but moorhens have inhabited the Zoo for as long as can be remembered by long-term employees, which means at least 40 years. Based on thorough capturing and personal observations, the population size was estimated to be about 40 adult moorhens during the sampling period.

The animal park Planckendael, founded in 1956, is situated in a semi-rural setting, near Mechelen, Belgium, at about 30 kilometres distance from the Antwerp Zoo, and has an area of roughly 36 hectares (BAETENS, 1993). Moorhens at Planckendael have been ringed and studied at irregular intervals since 1992, and the turnover population size was estimated to be approximately 300 adult individuals (MERCKX, unpubl.).
At Planckendael, animals were captured between 1993 and 1996; at the Antwerp Zoo all animals were caught in 1996. A wing vein was punctured with a sterile needle (Terumo, 0.55*25 mm) and blood was collected with Na-heparinized haematocrit-capillaries. Within three hours after capture, the blood was stored at -70°C until analyses were done.

Multilocus minisatellite DNA analysis

After a normal phenol-chloroform extraction, DNA was fragmented using the restriction enzyme MboI. DNA fingerprints were generated by «Southern Blotting» following BRUFORD *et al.* (1992). The radio-active (α32dCTP) probe 33.15 was used for hybridisation (JEFFREYS *et al.*, 1985a,b). The mean probability of two individuals sharing a band of apparently similar mobility on the gel, was estimated using the band-sharing coefficient or similarity index $2n_{ab}/(n_a+n_b)$, where n_a and n_b are the numbers of bands present in individuals a and b and n_{ab} is the number of bands shared by a and b (WETTON *et al.*, 1987; WESTNEAT, 1990; LYNCH, 1990; BRUFORD *et al.* 1992; PINXTEN *et al.*, 1994). A band was considered identical in two individuals if it had migrated no more than 0.5 mm from a band in another individual (WESTNEAT, 1990) and if the bands were of approximately the same intensity. A weakly hybridised band in individual «a» was excluded if its possible presence in individual «b» could not be verified because there was a stronger band on the same mobility level or because the entire lane of individual «b» was lighter (WESTNEAT, 1990; BURKE & BRUFORD, 1987). The band-sharing coefficient was calculated for 10 adult pairs from the Antwerp Zoo and 13 from Planckendael. Pairs were ordered in adjacent lanes on the same gel to assure precise comparison of band mobility. The higher the band-sharing coefficient, the more related are the members of the pair (LYNCH, 1990; BRUFORD *et al.*, 1992). A mean

band-sharing coefficient was calculated for each population. These coefficients were arcsine square-root transformed to be normalised, and compared by means of a t-test using the statistical package SPSS/PC (SPSS, 1986). Values given are mean ± SE.

Microsatellite DNA analysis

Twenty-six and thirty adult moorhens were sampled at the Zoo and at Planckendael, respectively. DNA was obtained from blood by a normal phenol-chloroform extraction. Microsatellite polymorphism was analysed at 9 loci. The primer sets used were originally developed for the Tasmanian native hen *Gallinula mortierii* (unpubl. data). For the exact defining of the primer sets we refer to Jason Buchan (see acknowledgements) by whom they were kindly provided. We adjusted reaction conditions to moorhens by « trial and error ». Amplification was achieved in a 25 µl reaction volume containing 0.2 to 0.4 µM of each primer (one of which was fluorescently labelled with CY5, Pharmacia), 1.5 to 2 mM $MgCl_2$, 200µM dNTP's, about 200 ng of genomic DNA, 1 x buffer (1.5 mM $MgCl_2$ included for the DynaZyme buffer) and 0.5 to 1 U of Pro-HA DNA polymerase (Eurogentec) or DynaZyme polymerase (Life Sciences Int.). The optimal thermal profile for PCR amplification is : 94°C, 3 min. ; 30 cycles of : 94°C, 50 sec. ; locus specific annealing temperature (Table 1), 55 sec. ; 72°C, 45 sec. and a final extension step at 72°C for 5 min. in a Techne Genius. Successful PCR reactions were diluted 1 :10 and exact fragment size was calculated by Allelinks (Pharmacia), after electrophoresis and fluorescent detection on a 6% acrylamide gel in an automated sequencer (A.L.F. express, Pharmacia).

TABLE 1

Volumes (µl) of reagents and annealing temperatures used in the PCR amplification of 9 microsatellite loci in Gallinula chloropus. *The total PCR volume was 25 µl. The length range of the PCR products is given in base pairs (bp). Multiplexing of PCR reactions was feasible for Tm19–Tm20 and Tm18–Tm38, while PCR products were run together for loci Tm31B–Tm105 and Tm36–Tm101.*

	Tm18–38	Tm19–20	Tm27	Tm31B	Tm36	Tm101	Tm105
DNA (200ng/µl)	1	1	1	1	1	1	1
H_2O	14.85	15.85	17.9	19.85	18.85	18.4	18.35
10 X buffer	2.5	2.5	2.5	2.5	2.5	2.5	2.5
$MgCl_2$ (25 mM)	2	2	2	/	0.5	1.5	1.5
DNTPs (10mM)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Forward primer (10 µM)	1–1	1–0.5	0.5	0.5	0.75	0.5	0.5
Reverse primer (10µM)	1–1	1–0.5	0.5	0.5	0.75	0.5	0.5
Taq (5U/µl)	0.15 PRO	0.15 PRO	0.1 PRO	0.15 DYN	0.15 DYN	0.15 PRO	0.15 PRO
Locus specific annealing temperature (°C)	57	57	55	52	57	57	57
Length range (bp)	145–147/ 112–126	149–155/ 132–136	143–173	180–252	146–153	132- 133	111–151

For each population, microsatellite loci were tested for deviations from the Hardy-Weinberg equilibrium and for linkage disequilibrium (following the method of BLACK & KRAFSUR, 1985), using exact probabilities generated in Genepop 3.1b (Markov chain parameters: dememorization:10000; batches:200; iterations per batch (or permutations):10000; RAYMOND & ROUSSET, 1995, 1998). To correct for multiple tests, p values were adjusted (p_a) for comparison to alpha (=0.05) based on the number of tests, Ntest, carried out: the most significant value was multiplied with Ntest, the second most with Ntest-1, etc. (modification of the sequential Bonferroni method of RICE, 1989; see ROS *et al.*, 1997).

To estimate within population genetic variability, we looked at the level of polymorphism (P), the mean number of alleles per locus (MNA) and heterozygosity levels (H). WRIGHT's F_{IS} (1951) measures the deviation from Hardy-Weinberg proportions within populations and was calculated as a metric of the level of inbreeding for each population. Significance of F_{IS} values was estimated by resampling over alleles (1000 permutations). F_{ST} represents the amount of genetic differentiation between two populations (WRIGHT, 1951, 1969) and was estimated by means of θ (WEIR & COCKERHAM, 1984). We estimated the overall F_{ST} and the F_{ST} for each locus separately. We also checked whether linkage or deviation of H-W of loci had an effect on the overall F_{ST}. Significance of θ values was estimated by permuting individual alleles across the two areas (Markov chain parameters: dememorization: 10000; batches: 200; iterations per batch (or permutations): 10000). Using θ and assuming an island model, it is possible to estimate Nm or the mean number of individuals that migrate between the studied populations each generation to maintain the population differentiation (WEIR & COCKERHAM, 1984). Genetic diversity (H) was compared between the two populations by means of permutation following VAN DONGEN *et al.* (1998). Parameters H, P, MNA, θ-values and significance of θ-values were calculated with Genepop3.1b (RAYMOND & ROUSSET, 1995b, 1998). Nm, F_{IS} and significance of F_{IS} were calculated using Genetix version 3.0 (BELKHIR *et al.*, 1996). The probability level for significant differences (α) was set at 0.05.

RESULTS

Multilocus minisatellite DNA analysis

In each lane we could score a mean of 21.3 ± 5.5 bands. The difference between the mean band-sharing coefficient of ten adult pairs at the Zoo (0.257 ± 0.038) and that of 13 pairs at Planckendael (0.254 ± 0.036) was not significant (t-test, t=0.15, df=21, p=0.88).

Microsatellite DNA analysis

At Planckendael, we found a significant linkage between loci Tm18-TM19 and loci Tm36-Tm38 (permutation test, sequential Bonferroni correction, p_a=0.042 and p_a=0.0036 respectively), while there was a significant linkage between locus Tm18 and Tm38 at the Zoo (permutation test, sequential Bonferroni correction, p_a=0.027). We found a significant

deviation from Hardy-Weinberg equilibrium at locus Tm36 at the Zoo and at locus Tm38 at Planckendael (after Bonferroni correction respectively: p_a=0.023 and p_a=0.0072); the other loci showed no such deviation (after Bonferroni correction: p_a>0.05)

TABLE 2

Allele frequencies at nine microsatellite loci for 26 adult moorhens at the Zoo and 30 at Planckendael. Names of alleles represent the size in base pairs (or fragment length) of the alleles

Locus	*Allele*	*Antwerp Zoo*	*Planckendael*	*Locus*	*Allele*	*Antwerp Zoo*	*Planckendael*
Tm20	132	0.6346	0.6	Tm36	146	0.1346	0.2
	134	0.2115	0.3833		151	0.6538	0.5833
	136	0.1538	0.0167		153	0.2115	0.2167
Tm27	137	0.0192	0	Tm38	112	0.1346	0.0333
	143	0.0577	0		114	0.0192	0.1667
	144	0	0.0333		116	0.2885	0.1
	145	0.0385	0.1		118	0.2692	0.0667
	147	0.2115	0.1167		120	0.1154	0.1
	149	0	0.0167		122	0.1731	0.5167
	151	0.3462	0.1667		126	0	0.0167
	153	0.0769	0.1	Tm101	132	0.9615	0.9464
	155	0.1538	0.0167		133	0.0385	0.0536
	157	0	0.2333	Tm105	111	0.0192	0.0167
	159	0.0385	0.1833		115	0.0192	0.1
	163	0	0.0333		117	0.0192	0
	173	0.0577	0		119	0.1538	0
Tm31B	180	0	0.0833		131	0.1154	0.0167
	194	0.1154	0.1333		133	0.0192	0
	200	0.0192	0		135	0.0192	0.0667
	202	0.0962	0.0667		137	0.0192	0.05
	206	0.0385	0		139	0.0962	0
	210	0.0192	0.0333		143	0.2692	0.0333
	216	0.0385	0.2		145	0.0769	0.4167
	218	0.0577	0		147	0.0192	0.25
	220	0.0385	0		149	0.0192	0
	224	0.0385	0.05		151	0.1346	0
	228	0.2885	0		153	0	0.05
	234	0	0.1833	Tm18	145	0.4423	0.2333
	236	0.1538	0		147	0.5577	0.7333
	238	0.0385	0		149	0	0.0167
	240	0	0.0167		151	0	0.0167
	246	0.0577	0.2	Tm19	149	0	0.2
	252	0	0.0333		155	1	0.8

Table 2 shows the allele frequencies that were determined at the nine microsatellite loci for 26 adult moorhens at the Zoo and 30 at Planckendael. Some loci are highly variable (e.g. Tm31B) while locus Tm101 is uniform. Locus Tm19 seems to be fixed in the Zoo population. Table 3 presents the genetic diversity in both populations. No significant

differences in genetic variability between the two populations were found (permutation tests, all p>0.05). The inbreeding coefficient F_{IS} equalled 0.061 for the Zoo population and 0.033 for the Planckendael population; both values were not significantly different from 0 (permutation tests, p=0.1 and p=0.18 respectively). As shown in table 4, there was a significant overall genetic population differentiation with an estimated θ value of 0.082 (permutation test, p<0.05). This value corresponds to 2.79 migrants per generation (Nm). Table 4 also lists F_{ST}-estimates for separate loci. Omitting linked loci or the loci that deviated from Hardy-Weinberg equilibrium did not alter the magnitude order nor significance of the overall F_{ST}-estimate (values not given).

TABLE 3

Microsatellite variability for two populations of moorhens: expected heterozygosity (Hexp), observed heterozygosity (Hobs), proportion polymorphic loci (P) and mean number of alleles per locus (MNA). SD = Standard Deviation

	Hexp (SD)	*Hobs (SD)*	*P*	*MNA*
Antwerp Zoo	0.54 (0.32)	0.52 (0.34)	0.9	5.89
Planckendael	0.56 (0.25)	0.55 (0.27)	1.0	5.56

TABLE 4

Estimates of genetic differentiation between the Antwerp Zoo and Planckendael moorhen population for each locus separately and for all loci combined. Significance levels are defined as follows: p<0.05 :; p<0.01 :**; p<0.001 :****

Microsatellite locus	*F_{ST}–estimate*	*significance*
Tm18	0.062	*
Tm19	0.178	***
Tm20	0.032	**
Tm27	0.066	***
Tm31B	0.091	***
Tm36	–0.016	NS
Tm38	0.118	***
Tm101	–0.032	NS
Tm105	0.142	***
Total	0.082	***

DISCUSSION

Although sample sizes were rather low, both genetic analyses provided indications that the small moorhen population inhabiting the Antwerp Zoo is not genetically impoverished compared to the ten times larger population at Planckendael. Firstly, the study of microsatellite variability at nine loci revealed that the Zoo population does not suffer a decreased genetic variability. There were no significant differences in heterozygosity

levels nor in the proportion of polymorphic loci nor in mean number of different alleles between the two populations studied. For both populations, inbreeding coefficients (F_{IS}) were too small to regard the populations as being inbred. This also suggests that there is no unusually high level of relatedness among moorhens in either of the populations. In addition, mean band-sharing coefficients, calculated from the DNA-fingerprints, did not differ significantly between the Zoo and Planckendael populations. This indicates that moorhens at the Zoo are not more related to each other than at Planckendael. We found similar mean band-sharing coefficients to those reported for unrelated animals in other studies (European starling *Sturnus vulgaris*: 0.21 (PINXTEN *et al.*, 1994); indigo bunting *Passerina cyanea*: 0.23 (WESTNEAT 1990); blue tit *Parus caeruleus*: ± 0.20 (KEMPENAERS *et al.*, 1992); moorhen: 0.29 (MCRAE & BURKE, 1996)), whereas close relatives have mean coefficients of around 0.60 in the indigo bunting (WESTNEAT 1990) and the blue tit (KEMPENAERS *et al.*, 1992). Thus our data indicate that the small moorhen population inhabiting the Antwerp Zoo is probably not isolated from other moorhen populations, as this would have resulted in a higher band-sharing and inbreeding coefficient.

We found a significant overall genetic population differentiation, which suggests that there is little gene flow between the Zoo and Planckendael populations. When considering the loci separately, the population differentiation was significant for seven of the nine loci; only Tm36 and Tm101 would have concealed differentiation. Nm equalled 2.71, which indicates that the observed genetic differentiation can be maintained when two to three individuals migrate between the study areas each generation. However, one should be aware of the fact that Nm is only an indirect assessment of the degree of gene flow, and should assume that an equilibrium exists, that the alleles considered are neutral in terms of selection, and that the migration is equal in both directions (NEIGEL, 1996). Because DNA was collected at Planckendael up to three years earlier than at the Zoo, we cannot exclude completely that the differentiation we found is in fact a temporal differentiation and that there was no genetic differentiation at the time we sampled moorhens at the Zoo. This would imply that the genetic composition of both populations altered during the time interval of our study, for instance as a consequence of a bottle-neck due to bad weather conditions. This is an unlikely scenario, however, because moorhen density at Planckendael remained high at all times. Moreover, we only used adults in our analysis, and at least some of the adults ringed in 1992 were still alive in 1997.

The fact that we found no signs of isolation even though the populations were genetically differentiated is probably due to an « isolation by distance » effect where the Zoo and Planckendael are reproductively isolated from each other due to geographical distance (WRIGHT, 1943), while migration of moorhens occurs between the Antwerp Zoo and the immediate surroundings. The distance between the Zoo and Planckendael is about 30 kilometres, which is rather large, given that the moorhens in both populations are considered to be mainly resident (MERCKX, 1993; VAN DUYSE, 1997).

A disadvantage of microsatellites is that identifying appropriate regions from a genomic library for a new species can be time-consuming (PARKER *et al.*, 1998). Known primers are not expected to amplify the same locus across related taxa unless the microsatellite region is flanked by highly conserved sequences where priming sites are located (ELLEGREN, 1992). Recent work, however, suggests that this may occur more often

than originally thought (PRIMMER *et al.*, 1996; PARKER *et al.*, 1998). Therefore, we began our study on moorhens by trying primers that had been developed for a closely related *Gallinula* species, namely the Tasmanian native hen. In agreement with several recent studies (DEKA *et al.*, 1994; PRIMMER *et al.*, 1996), the primers originally developed for Tasmanian native hens also worked for moorhens. As far as we know, our study reports the first application of microsatellite analysis in moorhens.

It has been recommended that one should be careful in interpreting data obtained from molecular markers (AVISE, 1994). Sometimes the results depend on the markers used, for example when different parts of the genome experience different selection pressures. Nevertheless, we believe that our results are reliable. Firstly, we combined two different molecular techniques, both revealing the same main result, i.e. that the Zoo population does not seem to be genetically impoverished. HAIG (1998) recently emphasised that studies that use several molecular techniques are likely to provide a more definitive assessment than those that use only one technique. Secondly, because minisatellite and microsatellite regions are non-coding regions of DNA, they are generally considered to be good, neutral mendelian markers (JEFFREYS *et al.*, 1985a,b; JARNE & LAGODA, 1996). Thirdly, our microsatellite analysis revealed significant linkage in only one pair of loci at the Zoo and in two pairwise combinations at Planckendael. These three combinations represent only 4.2% of the total of 72 combinations. Therefore, it is likely that we looked at loci that are representative of the entire genome. However, we cannot exclude that the Zoo population of moorhens is isolated to some extent. It could for instance be that the reference population at Planckendael is isolated and that the levels of genetic diversity we found may be typical of « isolated » moorhen populations in both populations. Though we have no proof, we presume, however, that the Planckendael population is not isolated because of its situation in a semi-rural setting, surrounded with plenty of suitable habitat for moorhens. It is also possible that the Zoo has become isolated only recently and that the effects are not yet noticeable. Nevertheless, this seems unlikely because urbanisation has been going on for over a century. Although more data are needed, we tentatively conclude that the surrounding city probably does not serve as a significant barrier for moorhens.

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