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Sex identification in the moorhen (*Gallinula chloropus*) by flow cytometry and morphometric analysis

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ABSTRACT

Sexing individuals in a population is important in many ecological and life-history studies. Since many bird species are monomorphic, non-invasive tools are necessary for sex determination. In this study we utilized flow cytometry to sex individuals in a moorhen population of northern Italy. By improving previous laboratory protocols, we were able to obtain clear and repeatable measures of DNA content from field blood samples. The per cent difference in nuclear content between male and female moorhens was among the highest values reported for birds. We also utilized a discriminant analysis of seven morphological measures to investigate whether birds can be sexed on the basis of biometry. Tarsus and foot lengths were the most influential variables in gender discrimination. However, only 13 females and 10 males (77%) were correctly sexed, while six females and two males were wrongly assigned. When juvenile moorhens were excluded the discriminant analysis correctly sexed 90% of the birds. Since morphometric comparisons with English moorhen populations showed that discriminant biometrical values are geographically different, and thus not useful as universal sexing tools, we recommend the use of the cytometry technique for sex determination.

KEY WORDS: Sex identification - Flow cytometry - Morphometry - Moorhens.

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INTRODUCTION

Behavioural and ecological studies frequently require the sexing of individuals, but this task is difficult in monomorphic species. In the past, ornithologists could only assess sex by the invasive methods of gonadal examination after dissection or karyotype analysis (Hammar, 1970; Belterman & De Boer, 1984; Christidis, 1985). Only recently have other non-invasive techniques been proposed, ranging from morphometric to biochemical analyses. Among the former, univariate body size discrimination is the easiest and in many species the sex has been assigned on the basis of a single morphological measurement. The most discriminating parameters are usually tarsus length, bill length, foot length, wing chord and wing length (Svensson, 1992). However, univariate analyses in general are considered not sufficiently accurate to allow a reliable sexing of all individuals (critical discussions are reported in Pyke, 1995, and Rogers & Rogers, 1995). Bivariate biometric separation of the sexes has also been proposed to improve sex identification, but uncertainty remains for those individuals which have intermediate values (Rogers & Rogers, 1995). Even if multivariate measurements better meet the requirements for sexing birds accurately (van Franeker & ter Braak, 1993), it has been acknowledged that morphometric discriminations fail to sex all individuals accurately and have a very low reliability in the sexing of immature birds (Madsen, 1997).

Recently, non-invasive biochemical methods relying on blood or feather pulp samples have been used (Ellegren & Sheldon, 1997; Redelman *et al.*, 1997). Among them, flow cytometry has been utilized for the accurate estimation of DNA content within cells. It has been successfully applied to a variety of cell types, such as bird erythrocytes and mitotically-active feather pulp cells. In bird studies, this technique was first described by Nakamura *et al.* (1990), who measured DNA content in many species. They observed that in the majority of them the males (the homogametic sex, ZZ) have more DNA content than females (ZW, with W smaller than Z). In cytometric studies (Tiersch *et al.*, 1989; Nakamura *et al.*, 1990) the measurements were usually highly repeatable (low coefficient of variation), even though at times the technique showed some inaccuracy when levels of debris and variability in the fluorescence distributions occurred (Tiersch & Mumme, 1993).

The aim of previous studies was mainly oriented toward evolution and taxonomy. There are only three species in which genome size has been measured in more than 10 individuals (Tiersch *et al.*, 1991), and only one study has been directed toward the sexing of individuals within a population (Tiersch & Mumme, 1993).

In our study, we used the flow cytometric method to sex a population of moorhens *Gallinula chloropus*, a monomorphic bird in which an attempt to sex individuals from morphological measurements was reported by Andersson (1975). This author, studying British popula-

tions, described males as being larger than females on the basis of foot, bill, and wing lengths.

In our study we measured sex differences in DNA content of the moorhen chromosomes. Furthermore, we used multivariate analysis of body measurements to try to discriminate the gender of the moorhens. On the basis of cytofluorimetric analysis we evaluated the ability of multivariate morphometry to discriminate sex. Finally the interest of precise sex identification in an ecologically and behaviourally complex species like the moorhen is discussed.

MATERIALS AND METHODS

The study was carried out along the River Scrivia at Novi Ligure (North Italy, 44°46' N, 8°51' E) during the winter of 1995-96. Moorhens were captured with six funnel traps located in an area of 1 km^2 (Acquarone *et al.*, 1998). Field sessions were performed at least weekly. Standard biometrical parameters were measured: body mass (0.1 g accuracy), wing, tail, and third primary lengths (to the nearest 0.5 mm), tarsus, foot and bill lengths (to the nearest 0.1 mm). According to Baker (1993) the foot length was obtained by measuring the length of the tarsus plus the longest toe, and bill length was obtained from the tip of the bill to the tip of the frontal shield (Andersson, 1975). Age was identified by the different neck and plumage colours, the juveniles having unmolted brown feathers and a dark bill, while the adults have black feathers and a yellow-red bill.

Thirty-one moorhens were analysed. For each animal, about 1 ml of blood was collected in Sigma ACD after wing vein puncture. The samples were immediately stored in a bag at 4° C to be transported to the laboratory and there frozen in liquid nitrogen and stored at -80° C.

The flow cytometer was set up daily using a suspension of human lymphocytes (HL) and chicken red blood cells (CRBC). Analyses were performed by mixing every moorhen sample with CRBC. On each day of analysis, a sample from a known female moorhen was analysed together with the undetermined samples. We tested three different protocols: the first one had been used by Dolezel & Göhde (1995) for plant sex determination, with the DNA binding dye DAPI; the second one, proposed by Nakamura *et al.* (1990) for sexing birds, consisted mainly in a modification of the classic method of Krishan (1975); the third one, described hereafter, was our modification of the protocol of Nakamura *et al.* (1990). Blood samples were diluted in PBS (Phosphate saline buffer, 0.1 M, pH 7), and 1 ml of the suspension was centrifuged (2000 rpm, 30 min) on a 1 M sucrose cushion. The pellet was resuspended in 1 ml 0.1% sodium citrate – 0.1% Triton X-100. Two hundred ml of this suspension were diluted in 1800 ml of the same solution and stained with 100 mg of propidium iodide. All the previous operations were carried out on ice. RNase from *Aspergillus clavatus* (35 units) was added to the suspension, which was finally filtered through a 20 µm nylon mesh, kept at room temperature and analysed within 15 min. Hence, the modifications mainly consisted in a strong dilution of the blood sample and in the centrifugation on the sucrose cushion.

The instrument we utilized was a PAS-IIIi flow cytometer (Partec, Münster, Germany), with an excitation light provided by an Argon ion laser (488 nm) and fluorescent light selected through a TK420 filter, a TK560 filter, a TK610 filter and an RG610 filter. Signals were collected and analysed with the Parteclist software package. At least six repetitions were carried out for each sample. The average mode position of the repeated analyses was calculated and used to evaluate the DNA content (Galbraith, 1990):

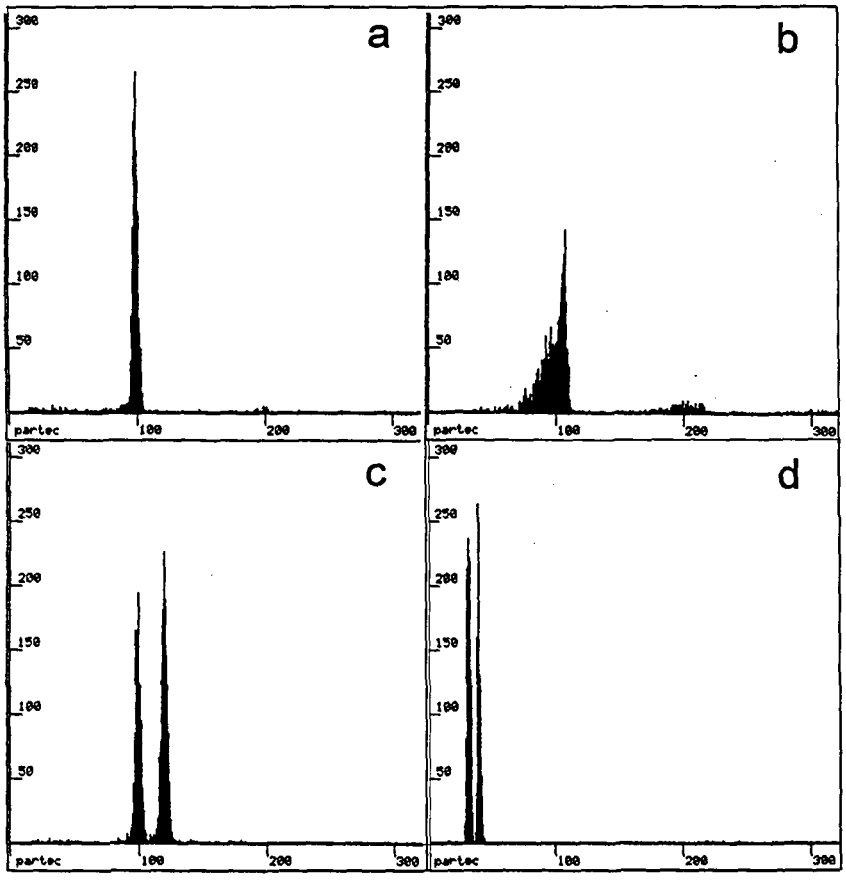

Fig. 1 - Flow cytometry histograms obtained with three different protocols: (a) chicken red blood cells prepared according to Dolezel & Göhde (1995), using the fluorescent dye DAPI; (b) mixed chicken and moorhen red blood cells, prepared with the previous protocol, the two species cannot be distinguished; (c) mixed chicken (peak on the right) and male moorhen (peak on the left) red blood cells, prepared according to Nakamura *et al.* (1990), stained with propidium iodide; (d) mixed chicken (peak on the right) and male moorhen (peak on the left) red blood cells, prepared according to the modified Nakamura *et al.* protocol that we propose.

DNA (pg) = 2.33 × Average mode position of moorhen / Average mode position of chicken, where 2.33 pg is the genomic DNA content of CRBC.

Three individuals died after the collection of blood sample and they were sexed by examination of gonads. This allowed us to verify that also in moorhen, as reported for birds in Nakamura *et al.* (1990), the females have a lower value of DNA content. Hence, all individuals with low DNA content (left peak of Figure 2 in the Results section) were sexed as females, and birds with a high DNA content as males. Furthermore, all individuals classified as males had a significantly different DNA content in comparison with the known females (t-test), while no significant difference in the DNA content was observed for all individuals classified as females.

Statistical analysis

The t-test was employed in univariate comparisons of male and female body measurements, with the exception of foot length, whose variance was not homogeneous (Bartlett's test). In this case the non-parametric Mann-Whitney U test substituted the t-test. We also utilized the three functions provided by Andersson (1975) to sex moorhens and, with Yates' corrected chi-square, we tested whether or not they were able to assign sex correctly in our population. These three functions are based, respectively, on foot and bill, foot and wing, and foot and weight measurements.

The multivariate discriminant analyses were computed by SYSTAT (Wilkinson, 1992).

RESULTS

Cytofluorimetric analysis

The flow cytometry histograms obtained with the DAPI protocol (Dolezel & Göhde, 1995) are shown in Figure 1a, b. The quality of the peaks was always excellent when individual samples were analysed (CVs ranged from 1.2 to 1.4%, Fig. 1a), but when two samples were mixed (i.e., moorhen and chicken, or two moorhens) the quality of the peaks dramatically decreased, which prevented the resolution of the two different samples (Fig. 1b).

By analysing the blood samples with Nakamura's protocol, we could recognize the presence of two mixed samples (Fig. 1c), but the CVs were always higher than 2% ($\bar{x}$ = 3.36, range 2.0-4.5).

With the modified Nakamura protocol that we propose, the CVs were as low as 1.0% ($\bar{x}$ = 2.05, range 1.0-3.3), and different samples always had the best resolution (Fig. 1d).

The mean DNA content of the 19 females was 2.802 ± 0.028, while that of the 13 males was 2.920 ± 0.024 (Fig. 2, Table I). The difference between the two genders was 4.1%.

Biometric analysis

The results of univariate morphometric analysis are reported in Table II. The length of the foot was the only statistically different variable between males and females.

The three functions provided by Andersson (1975) did not assign correctly the sex of birds in our population. Since the Italian moorhens were smaller than the English ones, all individuals were wrongly predicted to be females from Andersson's foot and bill (χ^2 = 19.6, P < 0.01) and foot and weight (χ^2 = 19.6, P < 0.01) functions, while Andersson's foot and wing function correctly assigned only one male individual (χ^2 = 16.4, P < 0.01).

The multivariate analysis of the body measurements from all individuals (adults and juveniles) extracted one discriminant factor. The canonical correlations between the parameters and the extracted factor are reported in Table III. Tarsus and foot lengths were the most influential variables in gender discrimination. On the basis of the body measurements the discriminant analysis was able to assign correctly a significant number (χ^2 = 6.32, P < 0.05) of males and females. However, the success in classification was not complete, since only 13 females and 10 males (77% of individuals) were correctly predicted, while six females and two males were wrongly assigned.

The discriminant analysis performed on adults (with juvenile moorhens excluded) enhanced the rate of correctly assigned individuals (Table IV): nine out of 10 adults (90%, χ^2 = 4.9, P < 0.05) were correctly sexed.

DISCUSSION

Sexing individuals in populations of monomorphic species is important in behavioural and ecological studies. For example in our study species, the moorhen, the presence of a complex mating system, involving intraspecific egg parasitism (McRae, 1996) and female competition for fat males (Petrie, 1983), requires the precise sex identification of each individual for behavioural studies. Moreover, social or ecological circumstances can alter the fitness benefits of producing daughters or sons (Emlen, 1997): moorhen families can have helpers at the

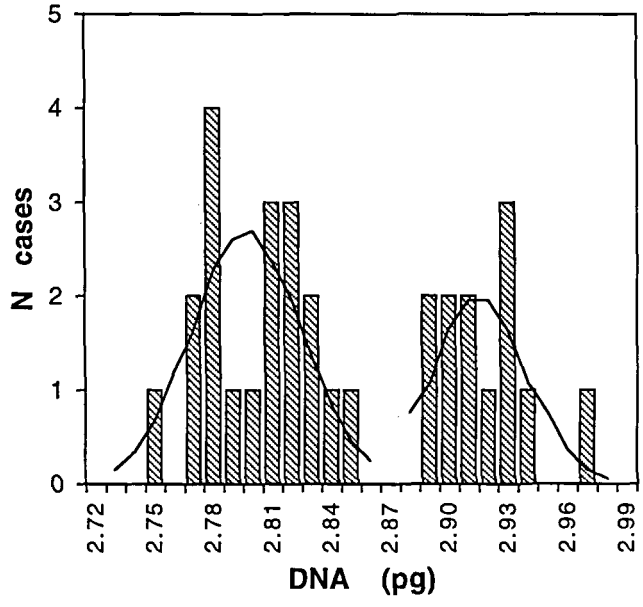

Fig. 2 - Distribution of the DNA content in the 31 moorhens.

TABLE I - *Mean and dispersion (SD) of DNA (pg) values for individual moorhens.*

Individuals	DNA ($\bar{x}$ ± SD)	Repeated samples (*n*)
Female 1	2.753±0.019	6
Female 2	2.767±0.025	6
Female 3	2.769±0.018	6
Female 4	2.777±0.010	6
Female 5	2.780±0.031	6
Female 6	2.781±0.018	6
Female 7	2.782±0.042	9
Female 8	2.790±0.040	6
Female 9	2.798±0.032	7
Female 10	2.809±0.016	6
Female 11	2.810±0.013	6
Female 12	2.812±0.022	6
Female 13	2.818±0.032	6
Female 14	2.822±0.027	6
Female 15	2.822±0.040	11
Female 16	2.828±0.027	6
Female 17	2.834±0.030	7
Female 18	2.837±0.029	6
Female 19	2.853±0.030	10
Male 1	2.887±0.039	6
Male 2	2.892±0.022	5
Male 3	2.902±0.036	12
Male 4	2.904±0.040	13
Male 5	2.911±0.020	9
Male 6	2.914±0.045	9
Male 7	2.920±0.026	7
Male 8	2.931±0.030	12
Male 9	2.933±0.028	8
Male 10	2.934±0.034	8
Male 11	2.940±0.035	9
Male 12	2.974±0.040	7

TABLE II - *Morphometric and DNA values ($\bar{x}$ ± SD) in female* (n = *19) and male* (n = *12) moorhens.*

Variables	Females	Males	t	P
Wing (mm)	177 ± 8.1	175 ± 9.8	0.45	ns
Third primary (mm)	123 ± 6.1	122.4 ± 5.7	0.46	ns
Tail (mm)	71.4 ± 4.2	71.1 ± 5.3	0.17	ns
Tarsus (mm)	56.24 ± 4.1	58.65 ± 4.2	1.62	ns
Foot (mm)	109.7 ± 4.1	122.4 ± 10.5	60.0*	0.02
Bill (mm)	33.1 ± 2.3	33.8 ± 1.5	0.94	ns
Weight (g)	302.4 ± 60.7	281.8 ± 49.5	1.01	ns
DNA (pg)	2.805 ± 0.034	2.908 ± 0.041	10.90	0.01

* Mann-Whitney U test (see Methods).

TABLE III - *Discriminant analysis of 31 different moorhens (adults and juveniles combined).*

Variables	First factor loadings
Wing	0.026
Third primary	0.026
Tail	0.035
Tarsus	0.603
Foot	0.650
Bill	0.306
Mass	-0.181

Success of classification

Predicted		Observed	
Females	15	Females	13
		Males	2
Males	16	Females	6
		Males	10
Total	31		31

TABLE IV - *Discriminant analysis of 10 adult moorhens.*

Variables	First factor loadings
Wing	0.285
Third primary	0.315
Tail	0.349
Tarsus	0.421
Foot	0.472
Bill	0.231
Mass	-0.052

Success of classification

Predicted		Observed	
Females	6	Females	6
		Males	0
Males	4	Females	1
		Males	3
Total	10		10

nest (Gibbons, 1987), a situation where altered sex-ratios in favour of the helping sex have been predicted (Emlen *et al.*, 1986).

Until now, the sexing of moorhens has relied on morphometry (Andersson, 1975), but this method appears to be quite inaccurate. For example, Petrie (1983) observed after autopsy that only 88% of adult moorhens were correctly sexed with morphometric measurements, and she had to verify the sex of each individual by post-mortem autopsy. McRae (1996) also relied on morphometry, but

sex identification had to be validated by behavioural observations during reproduction. In our population, the functions given by Andersson did not work, and our own discriminant analysis correctly assigned only 77% of the birds.

Ambiguous sexing from biometrical data has been reported for other species. For example, in Denmark only 49% of captured robins, *Erithacus rubecula*, met a wing-length criterion used to sex males and females, and a discriminant function using several body measures only increased the success rate to 80% (Madsen, 1997). In conclusion, even if multivariate analyses can carefully sex some monomorphic bird species (van Franeker & ter Braak, 1993), many species are not fully sexable by this method, or the analysis must be restricted to adults or to a single population (Madsen, 1997).

Some recent studies on bird ecology and evolution have used flow cytometry to determine sex. Evolutionists have utilized flow sorting mainly for comparative descriptions of male/female differences in DNA content and genome evolution within the bird class (30 species; Nakamura *et al.*, 1990) or among restricted taxa (Falconiformes and Sphenisciformes, De Vita *et al.*, 1994). Behavioural ecologists have employed this technique in only one study, aimed at identifying gender in a population of the monomorphic Florida scrub jay *Aphelocoma coerulescens* (Tiersch & Mumme, 1993).

In our study on moorhens, the cytometric procedure was successfully applied through the introduction of some improvements to the method of Nakamura *et al.* (1990). When frozen cells are thawed, they are subjected to lysis. The centrifugation step on a sucrose cushion helps to purify the free nuclei both from the ghosts of red blood cells and from other small particles producing background. Our results are highly repeatable and show very low coefficients of variation. This leads to unambiguous results and overcomes the difficulties reported for existing protocols (Tiersch & Mumme, 1993).

The per cent difference in nuclear content between male and female moorhens (4.1%) is in the range reported for birds (0.4-7%) and greater than that reported for another family of the same order Gruiformes (the cranes) which has only 1.8% of male-female difference (Nakamura *et al.*, 1990). Also the DNA content of moorhen cells (2.8-2.9 pg) is in the range reported for birds (2.4-3.4 pg), but in this case the value is similar to those of cranes (3.02-3.05 pg).

In addition to multivariate biometry, field ornithologists now have two molecular methods, flow cytometry and DNA binding (Ellegren & Sheldon, 1997), to ascertain the sex in each bird category (chicks, fledglings, juveniles, and adults). These are non-invasive techniques, requiring only a small quantity of blood (obtainable by vein puncture) or a feather pulp (feathers can be pulled out of live birds without apparent damage). Besides flow cytometry, the identification via PCR of highly repetititve DNA linked to the W chromosome is a highly reliable technique. The chromodomain-helicase-DNA-binding W-linked gene identified by Griffiths & Tiwari (1993) is an example of this technique successfully applied to birds. For many species, sex identification is probably more reliable by molecular than by morphometric analysis. Both molecular methods (Ellegren & Sheldon, 1997; Redelman *et al.*, 1997) fit the needs of field ornithologists, who must sex birds without error to determine the sex-ratio in population studies, and must know the sex of a single specimen in ecological and behavioural studies. In agreement with Redelman *et al.* (1997), we recommend the use of flow cytometry for sexing birds when the cytometer is suitable, a condition met within numerous medical or veterinary schools, universities, and hospital laboratories. The alternative PCR-based method should be recommended when sexual chromosomes are of similar size, or if a flow cytometer is not available (Ellegren & Sheldon, 1997).

In conclusion, in the moorhen the biometrical data can not be utilized to sex individuals, not even with the help of a multivariate statistical analysis. Furthermore, biometrical criteria to sex birds must be largely corrected from one population to another, as demonstrated by our unsuccessful utilization of Andersson's (1975) functions. We recommend the use of other methods, particularly flow cytometry, for the successful sexing of moorhens.

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