Ringing & Migration (2010) **25**, 42–46

Can Iberian Water Rail *Rallus aquaticus* be sexed reliably using simple morphometrics?

BENITO FUERTES[1,2]*, JAVIER GARCÍA[2], JUAN FERNÁNDEZ[2], SUSANA SUÁREZ-SEOANE[1] and JUAN JOSÉ ARRANZ[3]

[1]Departamento de Biodiversidad y Gestión Ambiental, Universidad de León, Facultad de Ciencias Biológicas y Ambientales, Campus de Vegazana s/n, 24071 León, Spain [2]Grupo Ibérico de Anillamiento, Daoiz y Velarde, 49, 24006 León, Spain [3]Departamento de Producción Animal I, Universidad de León, Facultad de Veterinaria, Campus de Vegazana s/n, 24071 León, Spain

In this paper we classified the sex of 39 Iberian Water Rails *Rallus aquaticus* using external morphometric measurements and genetic analysis. Logistic regression and classification tree model techniques (CART) were used to test whether simple morphometric measurements alone could classify sex correctly. For most of these measurements the overlap between sexes was too great to be of value. Bill length was the most relevant variable according to all the statistical analyses for the population under study. The applied combination of statistical techniques on biometric and genetic data correctly classify 80% of individuals. However, in view of the apparent variability in morphometric characteristics between populations, morphometric techniques to sex individuals from other populations should be validated using other criteria.

The Water Rail *Rallus aquaticus* is a polytypic, medium-sized bird (102–128 g) with a discontinuous trans-Palearctic distribution (Voous 1960, Cramp & Simmons 1980). In Europe, the subspecies *aquaticus* is widespread (Del Hoyo *et al* 1996) but accounts for less than half of its global breeding population of 140,000–360,000 pairs (BirdLife International 2004). Therefore, it is considered 'Not Threatened'. However, during recent decades it has suffered a continuous decrease in abundance, in common with other species of rails (Collar *et al* 1994, BirdLife International 2004), due to the disappearance and degradation of wetlands (Jenkins *et al* 1995, De Kroon 2004). The subspecies *aquaticus*, which includes Iberian birds, is mainly resident in the west and south of the range, but is partially migratory in the north and east (Cramp & Simmons 1980, De Kroon 1984a).

Methods for determining the sex of Water Rails have scarcely been studied, in spite of the relative ease of capture of the species (Zembal & Massey 1983, De Kroon 1984b, Kearns *et al* 1998, Fuertes *et al* 2002). Biometric differences between the sexes have been described for European Water Rails, with males being significantly larger than females (Flegg & Glue 1973, Cramp & Simmons 1980, Becker 1990, 1995, De Kroon 2000). However, to our knowledge, there is no specific published information on morphometric parameters for sexing Iberian birds (Cramp & Simmons 1980, Baker 1993), even though sex ratio determination is fundamental for understanding both behaviour and population structure and dynamics, and for the design of management strategies and conservation plans (Jones *et al* 1995, Millar *et al* 1997, McGregor & Peake 1998).

We study a Water Rail population in León province (NW of the Iberian Peninsula), which is strategically positioned at the boundary between the Mediterranean and Euro-Siberian biogeographical zones. Due to the lack of knowledge of this population, and since the use of biometric data from Northern and Central Europe to sex Iberian birds is not recommended (Campos *et al* 2005, Zuberogoitia *et al* 2005), we have employed DNA analyses as an alternative and more accurate method for classifying the sex of Iberian Water Rails (Griffiths *et al* 1998). These techniques have recently been applied to ornithological studies because they can provide the basis for exploring biometric variation between the sexes in species without strong sexual dimorphism (Sweeney & Tatner 1996, Brown *et al* 2003, Quintana *et al* 2003, Campos *et al* 2005). We have also explored the potential for simple external morphometric cues to allow sex determination of an Iberian-breeding Water Rail population.

METHODS

Study site

The site consists of four wet areas (2–10 ha), located in the south-eastern quarter of León Province (Spain): the shallow lagoons of Villadangos del Páramo (UTM 30T 271960,

* Correspondence author
Email: benito.fuertes@unileon.es

4711782) and San Andrés (UTM 30T 271416, 4696543) and the streams of Oncina (UTM 30T 286431, 4716895) and Valcavado (UTM 30T 272734, 4681052). All sites were surrounded by meadows, aquatic vegetation (mainly *Juncus* spp, *Carex* spp and *Typha* spp) and scattered willows (*Salix* spp) within a matrix of irrigated agricultural lands.

Water Rail biometrics and blood samples

Birds were captured between 2000 and 2005 using a modified fish net trap (Fuertes *et al* 2002). To ensure the capture of only local individuals, we set the traps between the end of April and August. We aged the birds on plumage traits and removed yearling birds from subsequent analysis.

Six simple morphometric variables were measured: maximum wing chord length (WL; ± 0.5 mm), eighth primary feather length, numbered descendently (F8L; ± 0.5 mm), tarsus length, (TL; ± 0.1 mm) (Svensson 1996), tarsus plus medium toe length (TTL; ± 0.5 mm) (Baker 1993), bill length from the tip to beginning of the feathers (BL; ± 0.1 mm) (Borras *et al* 2000) and, finally, body mass (W; ± 1 g).

By venepuncture, 50 µl of blood was collected from the brachial vein under the wing of each bird using capillary tubes and 100% ethanol was added to preserve the sample. Blood sampling was carried out under Spanish national licences (DGMNPF/120.009, DGMNPF/120.027) and the supervision of the Animal Production Department (University of León).

Molecular sexing

Sex identification was determined by DNA analyses of the extracted blood following a salting-out procedure (Miller *et al* 1988). Briefly, sex-specific regions of DNA were amplified using a polymerase chain reaction (PCR) following procedures used by Griffiths *et al* (1998) with the following adjustment in the protocol. The PCR reaction volume was 10 µl consisting of 30 ng of total DNA, 2.5 mM $MgCl_2$, 0.25 µl of each primer P8 (5'CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3'), 2 mM of each deoxynucleotide triphosphate, 0.5 U Taq DNA polymerase and 1 µl of 10 x PCR Buffer (Ampli Taq Gold, Applied Biosystems, Madrid). Thermocycling parameters included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s and extension at 72°C for 40 s and then, a final extension step at 72°C for 10 min. The resulting PCR products were separated by electrophoresis for 90 min at 7–10 V/cm in a 3% agarose gel stained with ethidium bromide.

Data analysis

Mean values of all morphometric measures collected to discriminate between males and females were firstly compared using the Mann–Whitney U-test. To prevent further collinearity problems among the predictors, we applied Spearman bivariate correlations to explore the associations between all morphometric measures. Wing length, tarsus plus medium toe length and body mass were excluded in the subsequent analyses because they showed values with $r_s > 0.8$. The remaining set of independent variables (eighth primary feather length, tarsus length and bill length) was explored to separate males from females (dependent variable) using two multivariate methods: logistic regression and classification tree models (CART).

A logistic regression with logit link function and binomial error term (Jongman *et al* 1995) was carried out using a backwards stepwise procedure. At each step, each variable and its interactions with other predictors were tested for significance in turn. When any variables were non-significant, the one making the smallest contribution was dropped from the starting null model (including all the variables). The procedure continued until all remaining variables were statistically significant at $P < 0.01$. We selected this level of significance rather than the usual threshold (0.05) to ensure robust conclusions. Finally, an objective cut-off point (Pereira & Itami 1991, Brito *et al* 1999) was defined to convert the probability output (values 0 to 1) into dichotomous data (male–female) and the performance of the model for sexing individuals was evaluated. To avoid prevalence problems in the analysis, equal numbers of males and females were included. This aids interpretation of model performance (Hosmer & Lemeshow 1989, Fielding & Bell 1997, Manel *et al* 2001). Birds not used for the training model were used as an independent data set for testing the predictive power of the model. Model accuracy was assessed using two alternative measures: (1) Nagelkerke's R^2 and (2) the area under the 'Receiver Operating Characteristic' curve (AUC; Beck & Shultz 1986, Fielding & Bell 1997). Analyses were carried out in SPSS 13.0 (SPSS Inc, Chicago).

Classification and regression tree models (CART) (Breiman *et al* 1998, De'ath & Fabricius 2000) provide an interesting alternative to regression. Possible non-linear or discontinuous relationships between independent and dependent variables can be explored using this tool. In this study, we built classification tree models fitted by successively splitting the data into more homogeneous subsets. Each split is based on the independent variable, which upon splitting at some break point (threshold), minimises the error sum of squares for the response variable. The result is a hierarchical tree which forms terminal nodes when the fractional reduction in total error decreases below 0.01. The predicted value at the end of each node is the mean probability of the individuals classified in that node to be sexed as male. For this analysis we used S-plus 6.1 (Insightful Corporation, Seattle, 2002).

RESULTS

During the sampling period, in total 39 adult birds were captured, measured and genetically sexed. The genetic analysis revealed a typical pattern of bands, one band for males and two bands for females, as described by other authors for other bird species (Griffiths *et al* 1998). In summary, 25 birds were males and 14 females, though not all of the sexed individuals could be fully measured. Table 1 shows the variation in morphometric parameters and the results of univariate analyses. These data showed that males differed significantly from females only according to bill length (not considering wing length, eighth primary length, tarsus length, toe–tarsus length and body mass). Bill length was also the least variable measure, while body mass was the most variable, particularly in males.

For statistical multivariate analysis, we used a roughly equal sample of 26 individuals (14 males and 12 females). From the remaining 13 individuals, 11 (males) were used for independent model validation, while two (females) could not be used for this purpose because some relevant body measurements were missing. Moreover, according to the bivariate correlation analysis, only three body measures were retained for those multivariate analyses: eighth primary feather length (F8L), tarsus length (TL) and bill length (BL).

Logistic regression analysis correctly sexed 88% (SD = 6.90) (AUC value) of the 26 individuals included within the analysis. This model had a good fit, as confirmed by a Nagelkerke R^2 value of 0.55. The contribution of each variable to the final model was very similar. The equation below presents the function for calculating the probability (values from 0 to 1) that an individual is male. If this probability is higher than 0.58 (cut-off point), the bird can be included within this category. The application of this equation to the independent validation set correctly classified 80% of birds:

*Male prob. = 1/[(Exp–(8.14*F8L–15.41*TL –0.20*F8L*BL–0.40*BL*TL–24.62)) +1]*

where F8L is the eighth primary feather length; TL the tarsus length and BL bill length.

The sex classification tree model (Fig 1) explained 46% of the data variability, structured around four terminal nodes. The first split, which accounted for 85% of the initial model heterogeneity (deviance explained by each split divided by the total deviance explained by the model), was due to bill length, the most relevant variable also according to this analysis. For a bill length of less than 36.4 mm, the probability that the bird is male is very low: 0.11 (1/9). The second split accounted for only 6% of the explained variability. It divided the remaining individuals (with a bill longer than 36.4 mm) according to eighth feather length (F8L): if this is 87.4 mm or longer, the probability that the individual is a male is 0.60 (3/5). Finally, the third split explained 8% of the variability, again relating to eighth primary length (F8L). If the remaining males have an F8L between 85.5 and 87.3 mm, then the probability of male is 1.00 (5/5). Individuals with an F8L less than 85.5 mm are predicted to be male with a probability of 0.71 (5/7). This model was tested using the independent validation set. Using an arbitrary cut-off point of 0.60, all 11 males were correctly classified with a median probability of 0.71.

DISCUSSION

The preliminary sex-ratio estimation (based only on biometric data from European birds) of the study population showed a female-biased result, which conflicts with our results and the available information about social structure, mating behaviour and territorial distribution of the species (Cramp & Simmons 1980, Brambilla & Rubolini 2004, De Kroon 2004, Jenkins & Ormerod 2004). Even though birds from southern Europe may be expected to be smaller than birds from northern regions (Meiri & Dayan 2003), the use of this rule for other European populations was of no value when applied to the Iberian population under study, as for other bird species (Pepin 1985, Campos *et al* 2005, Zuberogoitia *et al* 2005).

Table 1. Morphometric parameters of 39 Water Rails sexed by molecular techniques (note that not all birds could be fully measured).

	Males			Females			All	
	Mean (SD)	range	n	Mean (SD)	range	n	U-test	n total
WL	117.78 (5.18)	(109.5–128.0)	21	116.96 (4.51)	(110.0–125.0)	12	ns	33
F8L	85.36 (4.00)	(78.5–95.0)	21	84.49 (2.72)	(79.5–89.0)	12	ns	33
TL	40.98 (2.54)	(37.9–45.7)	25	39.61 (2.01)	(36.2–42.5)	14	ns	39
TTL	89.45 (5.40)	(81.7–99.5)	24	87.34 (4.06)	(80.5–94.2)	14	ns	38
BL	39.42 (2.44)	(35.1–43.4)	24	36.92 (1.71)	(34.9–41.2)	14	$P = 0.001$	38
W	112.09 (13.27)	(89.6–139.0)	25	104.11 (10.51)	(87.0–118.5)	14	ns	39

WL = wing length (mm), F8L = eighth primary feather length (mm), TL = tarsus length (mm), TTL = tarsus plus medium toe length (mm), BL = bill length (mm) and W = body mass (g).

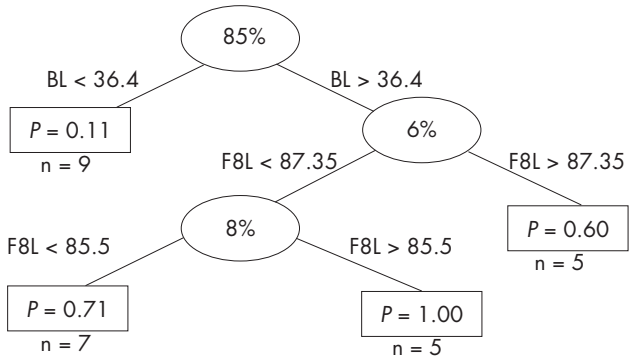

Figure 1. Classification tree model for sexing Iberian Water Rails. Squares represent terminal nodes. Values inside those squares indicate the predicted probability (mean values) of being a male. The variance explained by each split (referred to the total variance retained by the tree) is highlighted with ovals. Critical thresholds for predictors are displayed between node connections. Numbers in bold are the observations included within each branch of the tree. BL = bill length (mm); F8L = eighth primary feather length (mm).

In spite of the small sample size, we detected variation in the morphometric measurements in our study population which, moreover, overlapped between the sexes more than in other European populations (Cramp & Simmons 1980). It is necessary to emphasise the extreme variability in the morphometric measures of males and the need for further research to get more robust conclusions about this variability.

Bill length appears to be the most useful morphometric measure for general sex discrimination, as has been shown by other authors (Flegg & Glue 1973, Cramp & Simmons 1980, Baker 1993). The length of the eighth primary, alone or in combination with the bill length, is the other most relevant variable for sexing birds. The use of this variable is common for sexing passerines (Jenni & Winkler 1989) but, up to now, it has not been used for sexing rails. Body mass, tarsus length and tarsus–toe length did not differ statistically between the sexes, although these measures have been used by other authors to sex rails and other aquatic birds (Baker 1993, Baker *et al* 1999). Perhaps more complicated measurements might prove useful for determining sex in Water Rails, utilising body measurements not taken in this study.

The CART technique has seldom been utilised in ecology and even less in the interpretation of bird biometric data. When, as in our case, CART analyses are applied to small sample sizes, results may be affected by overfitting. In this case, other techniques such as the Breiman–Cutler classification (Lawrence *et al* 2006) may be helpful for further research.

In conclusion, as morphometric features seem to vary strongly across regions and between the sexes, we do not advise the use solely of commonly used measurements to classify the sex of Iberian Water Rails, unless they have been validated using other criteria.

ACKNOWLEDGEMENTS

We want to thank GIA (Grupo Ibérico de Anillamiento) of León (in particular A. Acebes, E. Álvarez, H. Astiárraga, F. de la Calzada, J. Casado, J.M. Colino, J. Falagán, R. González, M. Juan, D. Miguélez, A. Ramos, I. Roa, C. Rodríguez, N. Rodríguez, F. Sandoval, I. San Martín and C. Zumalacárregui) for their dedication with the fieldwork. Susana Pedrosa assisted with genetic analyses and Javier Lozano helped with CART development. Gerard de Kroon, Jenny Gill and three anonymous reviewers are thanked for commenting on the manuscript. We also acknowledge the improvements in English usage made by Hugh Insley.

REFERENCES

Baker, K. (1993) *Identification Guide to European Non-Passerines*. BTO Guide **24**. British Trust for Ornithology, Thetford.

Baker, A.J., Piersma, T. & Greenslade, A.B. (1999) Molecular vs. phenotypic sexing in red knots. *Condor* **101**, 887–893.

Beck, J.B. & Shultz, E.K. (1986) The use of relative operating characteristic (ROC) curves in test performance evaluation. *Archives of Pathology and Laboratory Medicine* **110**, 13–20.

Becker, P. (1990) Kennzeichen und Kleider der europäischen kleinen Rallen und Sumpfhühner *Rallus* und *Porzana*. *Limicola* **4**, 93–144.

Becker, P. (1995) Identification of Water Rail and *Porzana* crakes in Europe. *Dutch Birding* **17**, 181–211.

BirdLife International (2004) *Birds in Europe: population estimates, trends and conservation status*. BirdLife Conservation Series 12. BirdLife International, Cambridge.

Borras, A., Pascual, J. & Senar, J.C. (2000) What do different bill measures measure and what is the best method to use in granivore birds? *Journal of Field Ornithology* **71**, 606–611.

Brambilla, M. & Rubolini, D. (2004) Water Rail *Rallus aquaticus* breeding density and habitat preferences in northern Italy. *Ardea* **92**, 11–17.

Breiman, L., Friedman, J.H., Olsen, R.A. & Stone, C.J. (1998) *Classification and regression trees*. Chapman and Hall, Boca Ratón.

Brito, J.C., Crespo, E.G. & Paulo, O.S. (1999) Modelling wildlife distributions: logistic multiple regression vs. overlap analysis. *Ecography* **22**, 251–260.

Brown, A.W., Brown, L.M. & Stevick, P.T. (2003) Sexing Mute Swans *Cygnus olor* by discriminant analysis. *Ringing & Migration* **21**,174–180.

Campos, F., Hernández, M., Arizaga, J., Miranda, R. & Amezcua, A. (2005) Sex differentiation of Corn Buntings *Miliaria calandra* wintering in northern Spain. *Ringing & Migration* **22**, 159–162.

Collar, N.J., Crosby, M. & Stattersfield, A.J. (1994) *Birds to watch 2: the World List of threatened birds*. BirdLife Conservation Series 4. BirdLife International, Cambridge.

Cramp, S. & Simmons, K.E.L. (1980) *The Birds of the Western Palearctic*. Volume 2. Oxford University Press, Oxford.

De'ath, G. & Fabricius, K. (2000) Classification and regression trees: a powerful yet simple technique for ecological data analysis. *Ecology* **81**, 3178–3192.

De Kroon, G.H.J. (1984a) Zug und Wintervorkommen der Wasserralle. *Beiträge zur Vogelkunde* **36**, 97–110.

De Kroon, G.H.J. (1984b) Method and provisional results of trapping Water Rails in the Netherlands. *Ringing & Migration* **2**, 132–136.

De Kroon, G.H.J. (1986) Moult and feather growth in the Water Rail *Rallus aquaticus* on the Waddensea Island of Vlieland. *Ringing & Migration* **7**, 57–59.

De Kroon, G.H.J. (2000) Are rails of the putatively subspecies *Rallus aquaticus korejewi* (Zarudny, 1905) proportionally bigger than rails of the nominate *Rallus aquaticus aquaticus* (Linnaeus, 1758)? *Selevinia* **2000**, 59–66.

De Kroon, G.H.J. (2004) A comparison of two European breeding habitats of the Water Rail *Rallus aquaticus*. *Acta Ornithologica* **39**, 21–27.

Del Hoyo, J., Elliott, A. & Sargatal, J. (eds) (1996) *Handbook of the Birds of the World*. Volume 3. Lynx Edicions, Barcelona.

Fielding, A.H. & Bell, J.F. (1997) A review of methods for the assessment of prediction errors in conservation presence/absence models. *Environmental Conservation* **24**, 38–49.

Flegg, J.J.M. & Glue, D.E. (1973) A Water Rail study. *Bird Study* **20**, 69–79.

Fuertes, B., García, J. & Colino, J.M. (2002) Use of fish nets as a method to capture small rails. *Journal of Field Ornithology* **73**, 220–223.

Griffiths, R., Double, M.C., Orr, K. & Dawson, R.J.G. (1998) A DNA test to sex most birds. *Molecular Ecology* **7**, 1071–1075.

Hosmer, D.W. & Lemeshow, S. (1989) *Applied Logistic Regression*. Wiley, New York.

Jenkins, R.K.B. (1999) The breeding biology of the Water Rail *Rallus aquaticus* in Britain and Ireland. *Bird Study* **46**, 305–308.

Jenkins, R.K.B., Buckton, S.T. & Ormerod, S.J. (1995) Local movements and population density of Water Rails *Rallus aquaticus* in a small inland reedbed. *Bird Study* **42**, 82–87.

Jenkins, R.K.B. & Ormerod, S.J. (2002) Habitat preferences of breeding Water Rail *Rallus aquaticus*. *Bird Study* **49**, 2–10.

Jenni, L. & Winkler, R. (1989) The feather-length of small passerines: a measurement for wing-length in live birds and museum skins. *Bird Study* **36**, 1–15.

Jones, P., Schubel, S., Jolly, J., Brooke, M. & Vickery, J. (1995) Behaviour, natural history and annual cycle of the Henderson Island rail *Porzana atra* (Aves: *Rallidae*). *Biological Journal of the Linnean Society* **56**, 167–183.

Jongman, R.H.G., Ter Braak, C.J.F. & Van Tongeren, O.F.R. (1995) *Data Analysis in Community and Landscape Ecology*. Cambridge University Press, Wageningen.

Kearns, G.D., Kwartin, N.B., Brinker, D.F. & Haramis, G.M. (1998) Digital playback and improved trap design enhances capture of migrant soras and Virginia rails. *Journal of Field Ornithology* **69**, 466–473.

Lawrence, R.L., Wood, S. & Sheley, R. (2006) Mapping invasive plants using hyperspectral imagery and Breiman Cutler classifications (RandomForest). *Remote Sensing of Environment* **100**, 356–362.

Manel, S., Williams, H.C. & Ormerod, S.J. (2001) Evaluating presence–absence models in ecology: the need to account for prevalence. *Journal of Applied Ecology* **38**, 921–931.

McGregor, P. & Peake, T. (1998) The role of individual identification in conservation biology. In *Behavioral Ecology and Conservation Biology* (ed Caro, T.M.), pp 31–55. Oxford University Press, New York.

Meiri, S. & Dayan, T. (2003) On the validity of Bergmann's rule. *Journal of Biogeography* **30**, 331–351.

Millar, C.D., Reed, C.E.M., Halverson, J.L. & Lambert, D.M. (1997) Captive management and molecular sexing of endangered avian species: an application to the black stilt *Himantopus novaezelandiae* and hybrids. *Biological Conservation* **82**, 81–86.

Miller, S.A., Dykes, D.D. & Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* **16**, 1215.

Pepin, D. (1985) Morphological characteristics and sex classification of Red-legged Partridge. *Journal of Wildlife Management* **49**, 228–237.

Pereira, J.M.C. & Itami, R.M. (1991) GIS-based habitat modeling using logistic multiple regression: a study of the Mt Graham red squirrel. *Photogrammetric Engineering and Remote Sensing* **57**,1475–1486.

Quintana, F., Somoza, G., Uhart, M., Cassará, C., Gandini, P. & Frere, E. (2003) Sex determination of adult Rock Shags by molecular sexing and morphometric parameters. *Journal of Field Ornithology* **74**, 370–375.

Sweeney, J.J. & Tatner, P. (1996) Sexing Wrens *Troglodytes troglodytes indigenus* using morphological measurements and discriminant analysis. *Bird Study* **43**, 342–350.

Voous, K.H. (1960) *Atlas of European Birds*. Nelson, Edinburgh.

Zembal, R. & Massey, B.W. (1983) To catch a Clapper Rail – twice. *North American Bird Bander* **8**, 144–148.

Zuberogoitia, I., Martínez, J.A.B., Zabala, J., Martínez, J.E., Castillo, I., Azkona, A. & Hidalgo, S. (2005) Sexing, ageing and moult of Buzzards *Buteo buteo* in a southern European area. *Ringing & Migration* **22**, 153–158.

(MS received 26 March 2009; accepted 20 October 2009)