Conserv Genet (2007) 8:503–507
DOI 10.1007/s10592-006-9179-9

SHORT COMMUNICATION

Molecular evidence for hybridisation between the two living species of South American ratites: potential conservation implications

Frédéric Delsuc · Mariella Superina · Guillermo Ferraris · Marie-Ka Tilak · Emmanuel J. P. Douzery

Received: 6 March 2006 / Accepted: 5 June 2006 / Published online: 22 September 2006

Abstract In a private wildlife research facility and rhea farm of Argentina, artificially incubated eggs of putative hybrid origin between the Greater Rhea (*Rhea americana*) and the Lesser Rhea (*R. pennata*) hatched and gave birth to healthy chicks. Molecular genotyping by the analysis of mitochondrial Cytochrome *b* (Cyt *b*) and nuclear Chromo-Helicase DNA binding (CHD1) gene sequences confirmed the hybrid origin of these chicks which were molecularly sexed as females. The possibility of hybridisation argues for careful management of captive populations of these species, especially if individuals are to be released in the wild for conservation purposes.

Keywords Rhea · Pterocnemia · Hybrid detection · Molecular sexing

Introduction

Ratites (order Struthioniformes) are large flightless birds represented by kiwis, emus, cassowaries, ostriches and rheas (Folch 1992). Among this order, rheas are endemic of South America and are represented by two living species: the common or greater rhea (*Rhea americana*) and Darwin's or lesser rhea (*R. pennata* formerly *Pterocnemia pennata*). Five and three sub-species are recognised within the geographical range of *R. americana* and *R. pennata*, respectively. Among these, the Andean endemic puna rhea (*R. p. tarapacensis/garleppi*) may merit consideration as a full species which is critically endangered since its population is thought to comprise only several hundred individuals at the most (Folch 1992). Both greater and lesser rheas have seen their populations declining dramatically in the recent past as the result of combined effects of high hunting pressure and habitat loss induced by the conversion of plains into farmland and pastures (Bellis et al. 2004). As a consequence, both species are classified as near threatened (NT) in the 2006 IUCN Red List (BirdLife International 2004).

In natural populations of both rhea species, the tasks of incubation and chick rearing are left exclusively to the male, after all females from its harem have laid their eggs in a previously prepared nest (Folch 1992). Some orphan eggs laid outside active nests, long thought to be unfertile, have been shown to produce viable chicks after artificial incubation (Navarro et al. 1998). This property has led to the conservation strategy of using captive reared yearlings for reintroduction in declining natural populations (Bellis et al. 1999). Indeed, both species of rheas are commonly bred in South American farms and zoos. In captivity, some eggs are routinely collected and artificially incubated to increase successful reproduction. After hatching in the incubator, the chicks are brought together with the offspring produced by natural incubation and are reared naturally by

F. Delsuc (✉) · M.-K. Tilak · E. J. P. Douzery
Laboratoire de Paléontologie, Phylogénie et Paléobiologie-CC064, Institut des Sciences de l'Evolution UMR 5554/CNRS, Université Montpellier II, Place Eugène Bataillon, 34 095 Montpellier Cedex 05, France
e-mail: delsuc@isem.univ-montp2.fr

M. Superina · G. Ferraris
Estación de Incubación y Cría de Aves Silvestres (EICAS), Las Palmas 3307, Luján de Cuyo, Mendoza 5507, Argentina

M. Superina
Biological Science Department, University of New Orleans, New Orleans, LA 70148-0001, USA

males. In the first days after the laying, eggs from greater rheas are yellow coloured whereas lesser rheas lay greenish eggs, and thus can be easily distinguished. Consequently, greater and lesser rheas are often bred in the same enclosures.

A private wildlife research facility (Estación de Incubación y Cría de Aves Silvestres-EICAS) in Mendoza (Argentina) maintains about 30 greater rheas together with a single female lesser rhea. Surprisingly, in October 1999, six green lesser rhea-like eggs were found in the enclosure. These eggs were artificially incubated, and two of them hatched 40 days later giving birth to healthy chicks (Fig. 1a). Given the absence of male lesser rheas in the ratite farm, these chicks were suspected to represent viable hybrids of the lesser rhea female and one of the greater rhea males. Hybridisation is actually common in birds as Grant and Grant (1992) estimated that 9.2% of bird species hybridise either in nature or in captivity. However, the authors did not mention this possibility between species of the rhea family (Rheidae). The only mention of such a possibility was briefly noted by Folch (1992, plate 2).

To test for hybridisation, we studied the two putative hybrid chicks by DNA sequencing of two widely used molecular markers: the nuclear Chromo-Helicase DNA binding (CHD1) (Miyaki et al. 2001) and the mitochondrial Cytochrome *b* (Cyt *b*) genes (Lee et al. 1997). Potential conservation implications of hybridisation between these NT species are discussed in the context of reintroduction plans from captive animals.

Methods

Complete genomic DNA was extracted from blood samples of two putative hybrids (H332 and H055) and a greater rhea *R. americana* (Ram) from the farm, and from muscle tissue of an unrelated lesser rhea *R. pennata* (Rpe) using standard procedures (Sambrook et al. 1989). Because standard molecular sexing protocols based on the study of the CHD genes do not work in ratites which have undifferentiated sex chromosomes (Griffiths et al. 1998; Ellegren 2000), we used the test especially designed by Huynen et al. (2002). The sex-specific sequence (kW1) was PCR amplified in the two putative hybrids using primers w1′ (forward): 5′-ACCAGCCTTTAAACAAGCTATTAA-3′ and k7′ (reverse): 5′-TCTCTTTTGTTTTAGACACCCT-3′ slightly modified from the ones defined in the original publication to specifically target rhea sequences (Huynen et al. 2002). A region of the CHD1 nuclear gene, encompassing approximately equal parts of a complete intron and portions of its flanking exons (García-Moreno and Mindell 2000) was also amplified with the universal sexing primers P8 and P2 (Griffiths et al. 1998) for the two putative hybrids, the greater

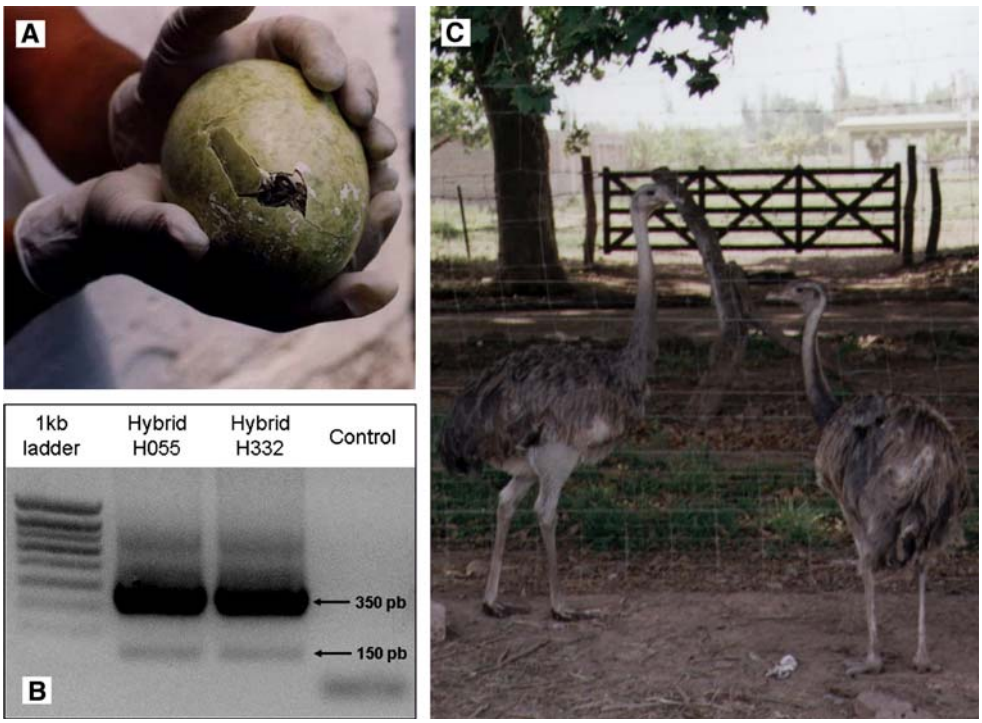

Fig. 1 (**A**) Putative hybrid egg hatching after 40 days of artificial incubation. Note the greenish colour of the shell, characteristic of *R. pennata* eggs, (**B**) molecular sexing of the two putative hybrids. The two PCR amplifications of the kW1 region in the two putative hybrid samples revealed the characteristic fragments of 350 and 150 bp identifying the two individuals as females, and (**C**) juvenile female *R. pennata* × *R. americana* hybrids. Note the presence of mixed characters such as the grey plumage characteristic of *R. americana* and the rounded head typical of *R. pennata*

rhea, and the lesser rhea. Finally, a 337 base pair (bp) fragment of the mitochondrial Cyt *b* gene from the two putative hybrids was directly PCR amplified using the primers L2 (forward): 5′-TACCATGAGGACAAATATC-3′ and H10 (reverse): 5′-CTGGGGTGTAGTTCTCTGGGTC-3′. PCR products for CHD1 and Cyt *b* genes were purified from 1% agarose gels using Amicon Ultrafree-DA columns (Millipore), and directly sequenced with PCR primers on both strands using automatic sequencing (Big Dye Terminator cycle kit) on an ABI 310 (PE Applied Biosystems). Sequences were manually aligned with the ED editor of the MUST package (Philippe 1993). The CHD1 PCR product was 319 bp long for all four individuals analysed and no gaps needed to be introduced in the alignment. Sequences have been deposited in the EMBL data bank under Accession Numbers AJ430514 to AJ430519.

Results and discussion

The two putative hybrids were molecularly sexed as females by the presence of the female specific 150 bp fragment of the kW1 region in the two PCR amplifications (Fig. 1b). Alignment of the mitochondrial Cyt *b* sequences from the two putative hybrids with previously published sequences of *R. americana* and *R. pennata* has shown a perfect identity with *R. pennata* (Fig. 2). Results from this maternally inherited gene confirmed that the artificially incubated lesser rhea-like eggs, giving birth to the putative hybrids, were laid by the lesser rhea female of the breeding facility. No lesser rhea males were kept on the farm and accidental mating with a wild living male was highly unlikely, as the facility is located in a semi-urban area of Mendoza free of wild rhea populations. This result thus strengthened the possibility that the chicks arose from hybridisation with a captive greater rhea male. Definitive confirmation of this suspicion came from the comparison of CHD1 gene sequences (Fig. 3). Only three variable sites on the 319 sequenced sites of the CHD1 alignment were detected, of which two were diagnostic for the hybrids (positions 104 and 109). These variable sites were all situated within the intronic region of the gene. When looking at these sites in chromatograms of the hybrids, we observed two exactly superimposed fluorochrome peaks corresponding to the nucleotides found in the parental species *R. americana* and *R. pennata* (Fig. 3). As CHD1 appeared as non sex-linked in ratites with an autosomal mode of inheritance (Ellegren 2000), these sites indicated that the chicks possessed the two parental alleles: one likely inherited from the lesser rhea female and the other from one of the greater rhea males. The third variable site between the two parental sequences (position 149) was not diagnostic since hybrids unambiguously possessed an Adenine like their lesser rhea mother whereas a Guanine was found in the putative greater rhea father sequence. This observation suggests that the greater rhea male we randomly sampled in the farm is not actually the father of the hybrids. However, our results provide clear molecular evidence that hybridisation in captivity between the two living species of South American ratites led to viable first generation hybrids (F1 hybrids).

```
                    4 4 4 4 4 4 4 5 5 5 5 5 6 6 6 6 6 6 7 7 7 7 7 7
                    2 2 3 4 8 9 9 0 2 3 4 8 1 2 4 9 9 9 1 1 2 3 4 5
                    0 9 8 8 1 2 5 4 2 1 6 5 5 8 8 6 7 9 1 7 3 2 4 1

Ram (AF090339)      A A T C T A T G T C G T G T T A C A T G A C C C
Ram (Y16884)        . . . T . . . . . . . . . . . . . . . . . . . .
Rpe (AF338709)      C G C . C G C A C T A C A C C G T C C A G T T T
Rpe (U76054)        C G C . C G C A C T A C A C C G T C C A G T T T
Hybrid H332         C G C . C G C A C T A C A C C G T C C A G T T T
Hybrid H055         C G C . C G C A C T A C A C C G T C C A G T T T
```

Fig. 2 Alignment of the 24 variable sites over 337 bp from the mitochondrial Cytochrome *b* gene obtained from the two hybrids compared with previously published sequences (accession numbers in brackets) for *R. americana* (Ram) and *R. pennata* (Rpe). Dots (.) indicate identity with the first nucleotide sequence and numbers refer to the position in the complete Cytochrome *b* sequence

As expected, the hybrids showed mixed morphological characters from both maternal and paternal inheritance. Shortly after hatching, the hybrid chicks had greyish brown feathers, resembling offspring of greater rheas. A white line at the tip of the feathers could be observed at 1 year, which made the chicks look more similar to lesser rheas. Approximately 2 years after hatching, the hybrids showed a steel grey plumage that reaches the tarsus. The head is rounded as in lesser rheas, whereas the beak is long and flattened as in greater rheas (Fig. 1c). The hybrids are slightly larger in size than greater rheas of the same age. Very unfortunately, we have been unable to collect any data on the age at which the described hybrids may reach sexual maturity, since they were sold after 2 years by the owner of the farm for commercial purposes. Therefore, we cannot make any prediction about their potential fertility.

As demonstrated by the occurrence of occasional hybridisation, *R. americana* and *R. pennata* seem to be phylogenetically closely related species. At the molecular level, we observed only three differences on 319 nucleotides in the CHD1 gene (0.9% divergence) and 23 differences on 337 nucleotides in the mitochondrial Cyt *b* gene (6.8% divergence). Our results confirm that the genus-level taxonomic distinction introduced with the long use of the name *P. pennata* Grey 1870 for the lesser rhea might not be justified. This gives credit to the recent taxonomic revision coming

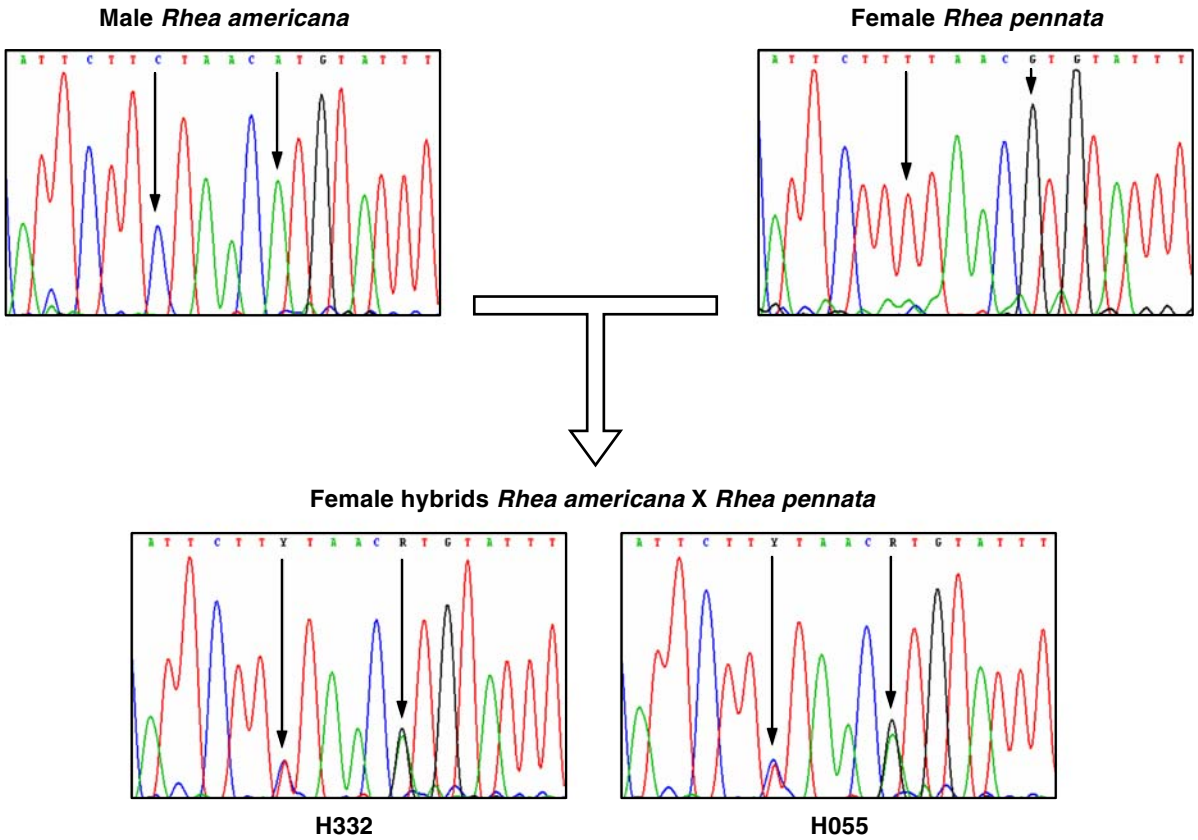

Fig. 3 Chromatograms showing the hybrid diagnostic region of the CHD1 gene. Hybrids possess the two parental alleles in their genomes resulting in perfectly superimposed peaks on the chromatograms at the sites where the *R. americana* and *R. pennata* nucleotide sequences differ. Y (for pYrimidine) and R (for puRine) are the ambiguity codes for cytosine/thymine (C/T) and adenine/guanine (A/G), respectively

back to *R. pennata* as originally proposed by d'Orbigny in 1834. It also provides a good example of the trend for taxonomical splitting observed in birds where species have tended to be placed into separate genera at a lower level of genetic divergence than is often true for other vertebrates (Johns and Avise 1998).

The fact that hybridisation can occur in captivity points out that the differentiation between the two species is not complete and raises questions about the possibility of its occurrence in natural populations. The two species have a parapatric distribution range with a sympatric zone North of Patagonia in Argentina (Folch 1992) suggesting that hybridisation could potentially occur in this area. However, it is clear that captive living conditions are far from the natural ones. In the wild, it is likely that a number of pre-zygotic barriers, which are relaxed in captivity, prevent hybridisation between the two species. In particular, the complex courtship behaviour displayed by both species of rheas (Folch 1992) constitutes an ethological barrier to hybridisation. Continuing habitat fragmentation, reduction of population sizes by illegal hunting and isolation of subpopulations of both species could, however, increase the encounters between greater and lesser rheas (Bellis et al. 1999; Bouzat 2001). It cannot be excluded that wild rheas would hybridise with individuals of the other species if conspecifics are rare or absent during the mating season.

Numerous rhea populations are managed by man in farms and zoos and our results argue for a careful management of captive breeding populations in order to avoid hybridisation between greater and lesser rheas. In private husbandries and zoos, the two species must therefore be strictly separated and meaningful captive breeding programs should be followed to conserve the integrity of both species. This latter point is of primary importance in cases where captive bred animals are thought to be released in the wild for reintroduction or population increase purposes (Bellis et al. 1999; Allendorf et al. 2001).

Acknowledgements Mr. Caesar Claude (Zoologisches Museum Zürich) generously provided us with *R. pennata* tissue samples. Jaime García-Moreno kindly shared CHD alignment files and chromatograms. Lura Williamson provided helpful comments on

the manuscript. This work is the contribution ISEM 2006-39 of the Institut des Sciences de l'Evolution de Montpellier (UMR 5554-CNRS).

References

Allendorf FW, Leary RF, Spruell P, Wenburg JK (2001) The problems with hybrids: setting conservation guidelines. Trend Ecol Evol 16:613–622

Bellis LM, Martella MB, Navarro JL, Vignolo PE (1999) Experience of release of yearlings of greater rhea reproduced artificially. In: Proc IV Neotropical Ornithological Congress, Monterrey, Mexico, pp 55–56

Bellis LM, Martella MB, Navarro JL, Vignolo PE (2004) Home range of greater and lesser rhea in Argentina: relevance for conservation. Biodivers Conserv 13:2589–2598

BirdLife International (2004) *Rhea americana* and *Rhea pennata*. In: 2006 IUCN Red List of Threatened Species (http://www.iucnredlist.org). IUCN 2006. Downloaded on 23 May 2006

Bouzat JL (2001) The population genetic structure of the greater rhea (*Rhea americana*) in an agricultural landscape. Biol Conserv 99:277–284

Ellegren H (2000) Evolution of the avian sex chromosomes and their role in sex determination. Trends Ecol Evol 15:188–192

Folch A (1992) Order Struthioniformes. In: Del Hoyo J, Elliott A, Sargatal J (eds) Handbook of the birds of the world, vol 1: ostrich to ducks. Lynx Edicions, Barcelona, pp 84–89

García-Moreno J, Mindell DP (2000) Rooting a phylogeny with homologous genes on opposite sex chromosomes (gametologs): a case study using avian CHD. Mol Biol Evol 17:1826–1832

Grant PR, Grant BR (1992) Hybridization of bird species. Science 256:193–197

Griffiths R, Double MC, Orr K, Dawson RJG (1998) A simple DNA test to sex most birds. Mol Ecol 7:1071–1075

Huynen L, Millard CD, Lambert DM (2002) A DNA test to sex ratite birds. Mol Ecol 11:851–856

Johns GC, Avise JC (1998) A comparative summary of genetic distances in the vertebrates from the mitochondrial Cytochrome *b* gene. Mol Biol Evol 15:1481–1490

Lee K, Feinstein J, Cracraft J (1997) The phylogeny of ratite birds: resolving conflicts between molecular and morphological data sets. In: Mindell DP (ed) Avian molecular evolution and systematics. Academic Press, San Diego, pp 213–247

Miyaki CY, Faria PJ, Griffiths R, Araujo JCC, Barros YM (2001) The last Spix's Macaw and an Illiger's Macaw produced a hybrid. Conserv Genet 2:53–55

Navarro JL, Martella MB, Cabrera MB (1998) Fertility of greater rhea orphan eggs: conservation and management implications. J Field Ornithol 69:117–120

Philippe H (1993) MUST: a computer package of management utilities for sequences and trees. Nucleic Acids Res 21:5264–5272

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York