J Ornithol (2007) 148:85–90
DOI 10.1007/s10336-006-0105-1

ORIGINAL ARTICLE

Genetically confirmed interbreeding between western Bonelli's warbler (*Phylloscopus bonelli*) and wood warbler (*P. sibilatrix*)

Christian Dietzen · Clemens Hackenberg · Karl-Heinz Heyne · Hedwig Sauer-Gürth · Heidi Staudter · Michael Wink

Received: 26 September 2005 / Revised: 12 June 2006 / Accepted: 15 October 2006 / Published online: 23 November 2006

Abstract During May and June 2003, a mixed breeding pair consisting of a male western Bonelli's warbler (*Phylloscopus bonelli*) and a female wood warbler (*P. sibilatrix*) successfully raised six young in a broad-leafed forest near Jünkerath, western Germany, an area far outside the regular breeding range of *P. bonelli*. The identity of the adult birds was confirmed by song, calls, colouration, and wing measurements. Based on blood samples taken from both parents and three juveniles, the potential interbreeding was analysed by molecular methods. Sequences of the mitochondrial cytochrome *b* gene differed between male and female by 8.7% and confirmed the identification of both parents. Sequences of the mitochondrial cytochrome *b* gene revealed that the male was a western Bonelli's and the female a wood warbler. The hybridisation and the parentage of male and female were corroborated by multilocus DNA fingerprinting. This is the first documented and genetically proven hybridisation event between these two warbler species.

Keywords *Phylloscopus bonelli* · *Phylloscopus sibilatrix* · Hybridisation · DNA fingerprinting · Cytochrome *b*

Communicated by F. Bairlein.

C. Dietzen (✉) · H. Sauer-Gürth · H. Staudter · M. Wink
Institut für Pharmazie und Molekulare Biotechnologie (IPMB), Abt. Biologie, Universität Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany
e-mail: ch.dietzen@urz.uni-hd.de

M. Wink
e-mail: wink@uni-hd.de

C. Hackenberg (✉)
Escher Strasse 10, 54584 Feusdorf, Germany
e-mail: clemens.hackenberg@t-online.de

K.-H. Heyne (✉)
Welschbilliger Strasse 8, 54636 Idesheim, Germany
e-mail: Karl-Heinz.Heyne@wald-rlp.de

Introduction

The breeding distribution of western Bonelli's warbler (*Phylloscopus bonelli*) in Germany is restricted to the Alps and the Black forest, while wood warbler (*P. sibilatrix*) is common throughout Central Europe (Glutz and Bauer 1991; Hölzinger 1999; Gatter 1997). Extra-limit records of over-shooting spring migrants of *P. bonelli* are regularly observed to the north of the breeding range, including the Rhineland-Palatinate (van den Berg and Bosman 1999; Glutz and Bauer 1991; Weitz, in Kunz and Simon 1987; Braun, in Deutsche Seltenheitenkommission (DSK) 1994).

Possible hybridisation between *P. bonelli* and *P. sibilatrix* has been reported several times, but it has never been proven if the male Bonelli's warbler really was the genetic father of the offspring (Schneider 1969; Dewitte et al. 1988; van der Elst 1989).

In this communication we have thoroughly analysed potential interbreeding and hybridisation between a male western Bonelli's warbler and a female wood warbler in the Eifel (West Germany), by using adequate molecular techniques.

Materials and methods

Field studies

On 7 May 2003, one of the authors (C.H.) discovered a singing western Bonelli's warbler in a broad-leafed forest near Jünkerath, Rhineland-Palatinate. The bird was distinguished from wood warbler (*P. sibilatrix*) and eastern Bonelli's warbler (*P. orientalis*) on the basis of measurements (Table 1) and calls. By chance, a female wood warbler was flushed from its nest at the same site on 21 May 2003. It warned extensively while the western Bonelli's warbler was singing and warning nearby, suggesting a close relationship. On 5 June 2003 both birds were seen feeding the young on the nest. Further details of the circumstances of the discovery and identification of this bird were published by Hackenberg et al. (2004).

The two adult birds were captured in a mist-net, measured, and banded with aluminium rings from the Vogelwarte Radolfzell, Germany—ring numbers: BS98758 (female) and BS98759 (male). Shortly before fledging, both adults and the six juveniles were captured again, and a small blood sample was taken from the wing vein for genetic analysis. The male returned to the breeding area in 2004, but none of the 2003 offspring was ever seen again.

DNA isolation and sequencing

Details of sample storage, standardised DNA isolation and sequencing have been described before (Dietzen et al. 2003). The maternally inherited mitochondrial cytochrome *b* gene was amplified by polymerase chain reaction (PCR) from the total genomic DNA using the specific primers *mt-A1* with *mt-Fs-H* (Dietzen et al. 2003). Cycle sequencing was performed with the primers *mt-A1* and *mt-C* Dietzen et al. (2003), producing overlapping sequences. The sequences obtained are deposited at Genbank under accession numbers AY944178–AY944180. For comparative investigations, further *Phylloscopus* sequences from Genbank were used: *P. bonelli* (Z73485, Z73486), *P. orientalis* (Z73490), *P. sibilatrix* (Z73491), *P. collybita* (Z73487) and *P. trochilus* (AY944177). *Sylvia borin* (AJ534549) was used as an outgroup.

Sequence analysis

A 931 bp fragment of the mitochondrial cytochrome *b* gene was sequenced and aligned for a total of six samples (male, female and three juveniles) plus further sequences from Genbank. Pairwise genetic distances between sequences were calculated using MEGA version 2.1 (Kumar et al. 2001). Phylogenetic trees were constructed employing PAUP*4b10 (neighbour-joining and maximum parsimony; Swofford 2001). Neighbour-joining analysis was performed using Kimura's (1980) two-parameter model and bootstrapped 1,000 times (cf. Dietzen et al. 2003, 2006).

Assessment of parentage

From the blood samples collected from three nestlings and the putative parents a multilocus DNA fingerprint was carried out to investigate the genetic relationships among the individuals involved. For each sample, digestion of total genomic DNA by restriction enzymes (*Hae*III), agarose gel electrophoresis, and capillary transfer to a nylon membrane (Biodyne B) followed standard protocols established in our laboratory (Swatschek et al. 1994). Nylon membranes were pre-hybridised in hybridisation mixture (5× SSPE, 0.1% SDS, 1% powdered milk, 5× Denhardt's solution; Sambrook et al. 1989) for 2 h at 39°C. Hybridisation was carried out with a hybridisation mixture containing 10 pmol/ml of the digoxigenated oligonucleotide probe $(GGAT)_4$ (Fresenius) at 39°C overnight. Membranes were washed three times with 6× SSC for 30 min each. DNA/DNA hybrids were detected by an antibody that was raised against digoxigenin (Boehringer). This antibody was coupled to a phosphatase, which, in turn, produced a coloured precipitate at the sites of hybridisation. After colour reactions were completed, nylon membranes were documented and processed by the Bioprofil system (Fröbel, Lindau).

Table 1 Measurements of the male and female of the mixed breeding pair (MBP) compared with measurements of authentic wood warbler (*P. sibilatrix*) and Bonelli's warbler (*P. bonelli, P. orientalis*) following Glutz and Bauer (1991) and Svensson (1992) (*P1*, *P2*, etc. represent primary 1, primary 2, etc., respectively, counted from outwards)

Parameter	Male of MBP	Female of MBP	Wood warbler	Western Bonelli's warbler	Eastern Bonelli's warbler
Wing length	66 mm	74 mm	75.5 ± 1.76 mm	62.9 ± 1.62 mm	66.9 ± 2.30 mm
P1/P2	P1 > P2	P1 < P2	P1 < P2	P1 > P2	P1 > P2
Wing tip	P3 = P4 > P5	P3 > P4 > P2	P3 > P4 > P2	P3 = P4 > P5	P3 = P4 > P5

We did the evaluation considering some fundamental rules outlined by Westneat (1990), by trying to assign all bands of the nestlings to one of the putative parents. The bands of the DNA fingerprint (n = 23) were visually scored into a data matrix as either absent (''0'') or present (''1''), which was than used to calculate the band-sharing coefficients (BSCs) as BSC = $C \times 2/(A + B)$, with C being the number of shared bands, and A and B the number of bands in the profiles of individuals A and B, respectively (Jeffreys et al. 1985; Wetton et al. 1987).

Results

Song, calls, colouration, and wing measurements of the male were all in the range of those of western Bonelli's warbler, and any aberrant wood warbler could be excluded. The female, based on calls, colouration, and measurements, was a typical wood warbler (Table 1).

The species identification was confirmed by sequence analysis. Sequences of the mitochondrial cytochrome *b* gene of the male and female revealed that the male was a western Bonelli's and the female a wood warbler (Fig. 1). The pairwise genetic distance between the male and female (8.7%) was of the same magnitude as that between other wood and Bonelli's warblers, 8.8–9.1 and 8.9%, respectively. Sequences of the offspring were identical to that of the mother (Fig. 2), as expected, since mitochondrial DNA is transmitted maternally.

Using the restriction enzyme *Hae*III, we produced an average of 15.0 ± 2.1 bands (n = 5 individuals) by multilocus DNA fingerprinting. The banding patterns of all three nestlings completely matched the banding

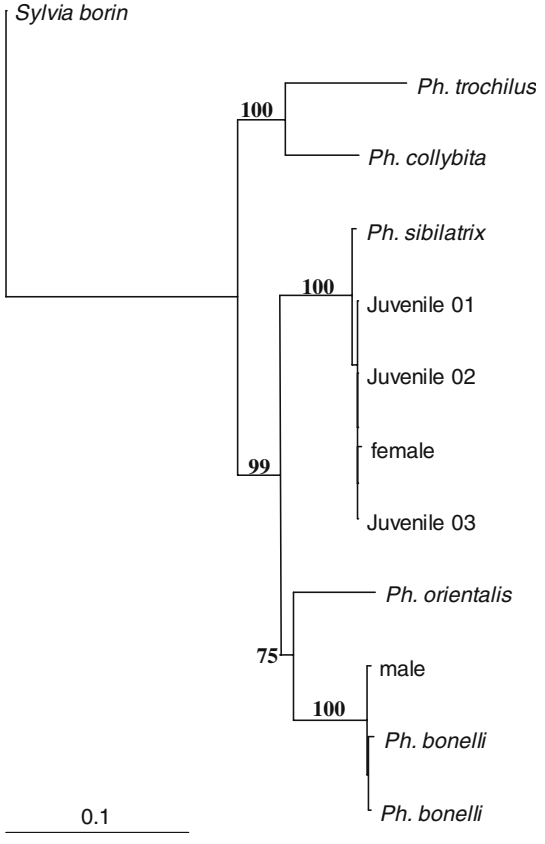

Fig. 1 Neighbour-joining phylogram of the mitochondrial cytochrome *b* gene from the breeding pair and its offspring compared with that of other *Phylloscopus* taxa. *Numbers* indicate bootstrap support values (1,000 replications)

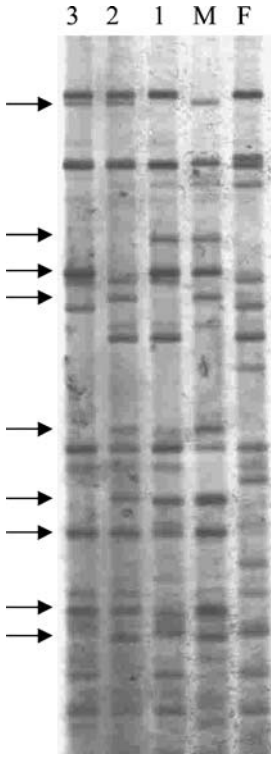

Fig. 2 Multilocus DNA fingerprint of the mixed breeding pair (*M* male, *F* female) and its offspring (*1–3*). Bands verifying the paternity of the father are highlighted by *arrows*

Table 2 Band-sharing coefficients (BSCs) in multilocus DNA fingerprints of the putative parents and their offspring hybridisation. Background BSC (male/female) was 0.23 (*Juv.* juvenile)

Individual	Male	Juv. 1	Juv. 2	Juv. 3
Male	–	–	–	–
Juv. 1	0.6667	–	–	–
Juv. 2	0.6897	0.7500	–	–
Juv. 3	0.4828	0.6875	0.7647	–
Female	0.2308	0.6207	0.6452	0.7742

patterns of their putative parents, with an average BSC of 0.68 ± 0.08 with their mother and 0.61 ± 0.11 with their putative father. The BSC among the nestlings was, on average, 0.73 ± 0.04 (Table 2), indicating that they were full siblings. The juveniles did not reveal any private bands that were not traceable to at least one of the parents. Thus, both adults were unequivocally the genetic parents of the brood.

Discussion

Natural hybridisation in birds is a well-known phenomenon (Johnsgard 1960; Randler 1999, 2000b; Tubaro and Lijtmaer 2002). Reticulation has received much attention in the recent past, focused on interspecific hybridisation, its reasons and consequences for evolution, species concepts and identification (e.g. Grant and Grant 1992; Sibley 1994; Arnold 1997; Randler 2000a, 2002, 2004). Until very recently, hybridisation was—and still is—regarded as an abnormal phenomenon caused by errors in mating systems. However, new insights into evolutionary processes have confirmed that interspecific hybridisation can play an important role in sympatric speciation, natural selection, introgression, and environmental adaptations, especially if the hybrid offspring equals the parent species in fitness and viability (Grant and Grant 1994; Grant et al. 2004; Sibley 1957).

The detection of possible hybridisation is straightforward in brightly coloured species such as ducks and geese, but this is much more difficult in species with less pronounced interspecific plumage differences, as in many passerines (cf. Randler 2004). Evidence for hybrids in songbirds usually comes from captured birds that show morphological measurements that are intermediate between two similar species. However, whether the differences are really based on hybridisation can only be corroborated by molecular techniques (e.g. Cordero and Summers-Smith 1993; Becker 1995; Panov et al. 2003). Recent examples for genetically proven hybridisations within the Sylviidae family included reed warbler × great reed warbler (*Acrocephalus scirpaceus*, *A. arundinaceus*) (Beier et al. 1997; Hansson et al. 2004), clamorous reed warbler × great reed warbler (*A. stentoreus*, *A. arundinaceus*) (Hansson et al. 2003), and common chiffchaff [*Phylloscopus* [*c.*] *collybita*] × Iberian chiffchaff [*P.* (*c.*) *brehmii*] (Bensch et al. 2002).

The results of the present study unequivocally confirm for the first time the hybridisation between a male *P. bonelli* and a female *P. sibilatrix*. Usually, hybridisation occurs between more or less closely related species in areas of overlap where one of them is rare and hybridisation is encouraged by restricted mate choice (Hubb's principle; Short 1969; Wirtz 1999; Randler 2000, 2002), but, even intergeneric reticulations are not uncommon in birds (Kabus 2002; Parkes 1978; van Balen et al. 2001; Bledsoe 1988; Randler 2004). Speciation rates for birds are similar to those of mammals, but, in birds, the evolution of hybrid inviability requires much more time (Fitzpatrick 2004). With this in mind, hybridisation between species showing relatively large genetic distances (e.g. 8–9% between *bonelli* and *sibilatrix*) is not really surprising.

There are no data available concerning the fate and fertility of the hybrid *bonelli* × *sibilatrix* offspring. Also, the final plumage and morphometric characters are not known, since the nestlings were still growing their feathers when they were last seen. Some detail of juvenile *bonelli* × *sibilatrix* hybrids was given by Schneider (1969). As in many other Sylviidae and their hybrids, it would be difficult to identify these hybrids correctly in the field without capturing them (Hansson et al. 2003, 2004; Beier et al. 1997; Bensch et al. 2002).

Zusammenfassung

Genetischer Nachweis einer Hybridisierung von Berglaubsänger (*Phylloscopus bonelli*) and Waldlaubsänger (*P. sibilatrix*) in Westdeutschland

Im Mai und Juni 2003 zog ein Mischpaar bestehend aus einem männlichen Berglaubsänger (*P. bonelli*) und einem weiblichen Waldlaubsänger (*P. sibilatrix*) in einem Laubwald bei Jünkerath, Rheinland-Pfalz, erfolgreich sechs Jungvögel auf. Der Brutplatz liegt außerhalb des regelmäßigen Brutgebietes des Berglaubsängers. Die Bestimmung der Altvögel basierte auf Gesang, Rufen, Färbung und Flügelmaßen. Anhand von Blutproben beider Alt- und dreier Jungvögel wurde eine molekulargenetische Analyse der Hybridisierung zwischen diesen beiden Arten durchgeführt. Sequenzen des mitochondriellen Cytochrom *b*-Gens (maternale Vererbung) bestätigten die Artzuodnung beider Altvögel und den Waldlaubsänger als Mutter der Jungen. Die Hybridisierung wurde durch Multilocus-DNA-Fingerprinting eindeutig nachgewiesen. Die Bandenverteilung wies die beiden fütternden Altvögel als die genetischen Eltern der Nestlinge aus. Es handelt sich hierbei um die erste dokumentierte und genetisch verifizierte Hybridisierung zwischen diesen beiden Laubsänger-Arten.

Acknowledgements Cordial thanks to Jochen Martens (University of Mainz) and Fernando Jubete Tazo (La Nava Ringing Station, Spain), who provided some samples of *P. bonelli* for the comparative analysis. Rudolf Zenan, Regierung Oberbayern, generously gave permission for us to catch western Bonelli's warblers in Bavaria. Hans-Joachim Fünfstück, Vogelschutzwarte Bayern, was very helpful in locating western Bonelli's warbler territories around Garmisch-Partenkirchen, Bavaria. Javier Gonzalez assisted in catching birds and taking samples. Darrin O'Brien and Julie Craves kindly provided some literature not available to us and improved the English. Marion Steinbüchel assisted in preparing some figures. All experiments complied with the current laws of Germany.

References

Arnold ML (1997) Natural hybridization and evolution. Oxford University, Oxford

Becker J (1995) Sympatrisches Vorkommen und Hybridisierung von Sprosser *Luscinia luscinia* und Nachtigall *L. megarhynchos* bei Frankfurt (Oder), Brandenburg. Vogelwelt 116:109–118

Beier J, Leisler B, Wink M (1997) Ein Drossel- × Teichrohrsänger-Hybride *Acrocephalus arundinaceus* × *A. scirpaceus* und der Nachweis seiner Elternschaft. J Ornithol 138:51–60

Bensch S, Helbig AJ, Salomon M, Seibold I (2002) Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. Mol Ecol 11:473–481

Bledsoe AH (1988) A hybrid *Oporornis Philadelphia* × *Geothlypis trichas*, with comments on the taxonomic interpretation and evolutionary significance of intergeneric hybridization. Wilson Bull 100:1–8

Cordero PJ, Summers-Smith JD (1993) Hybridization between House and Tree Sparrow (*Passer domesticus*, *P. montanus*). J Ornithol 134:69–77

Deutsche Seltenheitenkommission (DSK) (1994) Seltene Vogelarten in Deutschland 1991 und 1992. Limicola 8:153–209

Dewitte T, Hofmans K, Clesse B (1988) Observation of a Bonelli's warbler *Phylloscopus bonelli* at Treignes during the reproductive period and description of his habitat in Belgium. Aves 25:21–32

Dietzen C, Witt H-H, Wink M (2003) The phylogeographic differentiation of the European robin *Erithacus rubecula* on the Canary Islands revealed by mitochondrial DNA sequence data and morphometrics: evidence for a new robin taxon on Gran Canaria? Avian Sci 3:115–131

Dietzen C, Voigt C, Wink M, Gahr M, Leitner S (2006) Phylogeography of island canary (*Serinus canaria*) populations. J Ornithol 147:485–494

Fitzpatrick BM (2004) Rates of evolution of hybrid inviability in birds and mammals. Evolution 58:1865–1870

Gatter W (1997) Waldgeschichte, Buchenprachtkäfer und Rückgang des Berglaubsängers *Phylloscopus b. bonelli*. Vogelwelt 118:41–47

Glutz V. Blotzheim UN, Bauer KM (1991) Handbuch der Vögel Mitteleuropas, vol 12, Passeriformes 3. Aula Verlag, Wiesbaden, Germany

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

Grant PR, Grant BR (1994) Phenotypic and genetic effects of hybridization in Darwin's finches. Evolution 48:297–316

Grant PR, Grant BR, Markert JA, Keller LF, Petren K (2004) Convergent evolution of Darwin's finches caused by introgressive hybridization and selection. Evolution 58:1588–1599

Hackenberg C, Heyne K-H, Dietzen C (2004) Bestimmung eines Berglaubsängers (*Phylloscopus bonelli*) und Nachweis einer Mischbrut zwischen Berg- und Waldlaubsänger (*P. bonelli* × *P. sibilatrix*) in der Eifel. Dendrocopos 31:31–40

Hansson B, Gavrilov E, Gavrilov A (2003) Hybridisation between great reed warblers *Acrocephalus arundinaceus* and clamorous reed warblers *A. stentoreus*: morphological and molecular evidence. Avian Sci 3:145–151

Hansson B, Roggeman W, De Smet G (2004) Molecular evidence of a reed warbler × great reed warbler hybrid (*Acrocephalus scirpaceus* × *A. arundinaceus*) in Belgium. J Ornithol 145:159–160

Hölzinger J (1999) Die Vögel Baden-Württembergs, vol 3.1. Eugen Ulmer, Stuttgart, Germany

Jeffreys AJ, Wilson V, Thein SL (1985)Individual-specific ''fingerprints'' of human DNA. Nature 316: 76–79

Johnsgard PA (1960) Hybridization in the Anatidae and its taxonomic implications. Condor 62:25–33

Kabus A (2002) Hybriden zwischen Rauchschwalbe *Hirundo rustica* und Mehlschwalbe *Delichon urbica*. Limicola 16:276–285

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kumar S, Tamura K, Jacobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17:1244–1245

Kunz A, Simon L (1987) Die Vögel in Rheinland-Pfalz—Eine Übersicht. Naturschutz Ornithol Rheinl Pfalz 4:353–657

Panov EN, Roubtsov AS, Monzikov DG (2003) Hybridisation between Yellowhammer and Pine Bunting in Russia. Dutch Birding 25:17–31

Parkes KC (1978) Still another Parulid intergeneric hybrid (*Mniotilta* × *Dendroica*) and its taxonomic and evolutionary implications. Auk 95:682–690

Randler C (1999) Zum Auftreten von Hybriden zwischen Tafel- und Reiherente *Aythya ferina* × *A. fuligula* in Deutschland, Österreich und der Schweiz. Vogelwelt 120:211–220

Randler C (2000a) Wasservogelhybriden (Anseriformes) im westlichen Mitteleuropa—Verbreitung, Auftreten und Ursachen. Ökol Vögel 22:1–106

Randler C (2000b) Die Bestimmung von Tauchentenhybriden der Gattung *Aythya*. Limicola 14:1–35

Randler C (2002) Avian hybridization, mixed pairing and female choice. Anim Behav 63:103–119

Randler C (2004) Frequency of hybrids: does detectability make all the difference? J Ornithol 145:123–128

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

Schneider PA (1969) Mischbrut zwischen Berglaubsänger (*Phylloscopus bonelli*) und Waldlaubsänger (*Phylloscopus sibilatrix*) [im Wurzacher Ried, Kreis Wangen im Allgäu]. J Ornithol 110:101–102

Short LL (1969) Taxonomic aspects of avian hybridization. Auk 86:84–105

Sibley CG (1957) The evolutionary and taxonomic significance of sexual dimorphism and hybridization in birds. Condor 59:166–191

Sibley D (1994) Finding and identifying hybrid birds. Birding 26:162–177

Svensson L (1992) Identification guide to European passerines. Lars Svensson, Stockholm

Swatschek I, Ristow D, Wink M (1994) Mate fidelity and parentage in Cory's shearwater *Calonectris diomedea*. Field studies and DNA fingerprinting. Mol Ecol 3:259–262

Swofford DL (2001) PAUP*, phylogenetic analysis using parsimony (*and other methods), version 4.0b10a. Sinauer, Sunderland, Massachusetts

Tubaro PL, Lijtmaer DA (2002) Hybridisation patterns and the evolution of reproductive isolation in ducks. Biol J Linn Soc 77:193–200

Van Balen JH, Perdeck AC, van Diek H (2001) Hybridisatie tussen Waterhoen en Meerkoet. Dutch Birding 23:196–203

Van den Berg AB, Bosman C (1999) Rare birds of the Netherlands. East Sussex, UK

Van der Elst D (1989) Observation of a warbler considered a probable hybrid between Bonelli's warbler and wood warbler *Phylloscopus bonelli* × *P. sibilatrix*. Aves 26:62–63

Westneat DF (1990) Genetic parentage in the indigo bunting: a study using DNA fingerprinting. Behav Ecol Sociobiol 27:67–76

Wetton JH, Carter RE, Parkin DT, Walters D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327:147–149

Wirtz P (1999) Mother species—father species: unidirectional hybridization in animals with female choice. Anim Behav 58:1–12