Occasionally, even a single breeding event may provide significant biological information, of which the present report of a pairing between a female meadow pipit (*Anthus pratensis*) and a male water pipit (*A. spinolaletta*) may be a good example. Events of mixed-species pairing appear to be relatively common in birds (Arnold 1997). However, only rarely do mixed-species pairings appear to result in the production of hybrid offspring. In a study of hybridization between collared flycatchers (*Ficedula albicollis*) and pied flycatchers (*F. hypoleuca*), Veen et al. (2001) showed that females involved in mixed-species pairs often have extra-pair copulations (EPCs) with conspecific males, and that these extra-pair males sire a significant proportion of the nestlings. Accordingly, in these species the rate of actual hybridization is much lower than the frequency of heterospecific pairs would seem to imply. Here we report, to our knowledge, the first incidence of a mixed species pairing during the study period.

### Materials and Methods

About 25 µl blood from each bird was taken from a brachial vein and stored in Queens lysis buffer (Seutin et al. 1991). DNA was extracted from the blood samples by overnight incubation at 45°C with 50 µl proteinase K solution (10 mg/ml) and 50 µl of 10% SDS. Each sample was extracted twice with 1 ml phenol/chloroform/water solution before DNA precipitation in 1 ml 95% ethanol. The samples were centrifuged at max speed and the resulting DNA pellet rinsed with 70% ethanol, air-dried and resuspended in 500 µl bidistilled H₂O.

We applied BLAST-search (Altschul et al. 1997) of chicken (*Gallus gallus*) sequences to identify potential intron-fragments for sequencing. Primers were designed based on homology alignment with more distantly related taxa (e.g. humans and rodents). Primers were set in conserved exon-regions, according to the alignment results, that would amplify less conserved introns in the pipits. We chose one intron-fragments from each of the following three genes for further analysis:

- TGFB2 (F: 5'-GAAGCGTGCTCTAGATGCTG-3', R: 5'-AGGCAGCAAATATTCTGCAC-3')
- BC-K (F: 5'-GCTTCACCCTGGATGATGTC-3', R: 5'-CGTCGCCAGCTACGCATCCT-3')

The meadow pipit is olive-brown and streaked above, grey-white below and spotted and streaked on chest and flanks. The water pipit has slightly streaked greyish-brown upper parts and dull white under parts with pale buff-pink wash from throat to mid-belly. The first author has studied pipit populations on alpine meadows 1350–1491 m above sea level in the Jeseniky Mountains (Czech Republic, 50°04 N, 17°14 E) since 1986. In total, 84 meadow pipit pairs were caught during 1996–2001. The stated event is the first incidence of a mixed species pairing during the study period.

**Brief report**

**Hybridization and apparent hybridization between meadow pipit (*Anthus pratensis*) and water pipit (*A. spinolaletta*)**

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Table 1. Genotype of water pipits (Anthus spinoletta), meadow pipits (A. pratensis) and an apparent hybrid at seven nucleotide positions (Pos.) exhibiting inter- or intraspecific variation

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</thead>
<tbody>
<tr>
<td>A. spinoletta (control)</td>
<td>G/G</td>
<td>T/T</td>
<td>C/C</td>
<td>C/C</td>
<td>A/A</td>
<td>C/C</td>
<td>G/G</td>
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<tr>
<td>A. spinoletta (putative father)</td>
<td>G/G</td>
<td>T/T</td>
<td>C/C</td>
<td>C/C</td>
<td>A/A</td>
<td>C/C</td>
<td>G/A</td>
<td></td>
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</tr>
<tr>
<td>Nestling 1 (hybrid)</td>
<td>G/G</td>
<td>T/A</td>
<td>C/G</td>
<td>C/G</td>
<td>A/G</td>
<td>C/A</td>
<td>G/A</td>
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<tr>
<td>Nestling 2</td>
<td>G/A</td>
<td>A/A</td>
<td>G/G</td>
<td>G/G</td>
<td>G/G</td>
<td>A/A</td>
<td>G/G</td>
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<tr>
<td>Nestling 3</td>
<td>G/A</td>
<td>A/A</td>
<td>G/G</td>
<td>G/G</td>
<td>G/G</td>
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<tr>
<td>A. pratensis (putative mother)</td>
<td>G/G</td>
<td>A/A</td>
<td>G/G</td>
<td>G/G</td>
<td>G/G</td>
<td>A/A</td>
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<tr>
<td>A. pratensis (control)</td>
<td>G/G</td>
<td>A/A</td>
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LaminA (F: 5'-CCAGGACAGCTGCAGGATGAGATGC-3', R: 5'-CTGCGGCCCCGTGTGCTCAGTCTCACCAG-3').

PCR amplification was performed in 10 μl reactions containing 2 μl of template DNA (~10 ng/μl concentration), 0.5 U AmpliTaq (Perkin Elmer), 200 μM dNTPs, 10 mM Tris-HCl, 1.75 Mm MgCl2 and 4 pmol of each primer. A thermal cycler (Perkin Elmer 9600) with the following PCR profile was used: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55–67°C for 30 s and 72°C for 40 s. After the final cycle a prolonged extension step of 7 min was added. Annealing temperature for each fragment was optimised to 65°C for TGFB2, 55°C for BC-K and 67°C for Lamin A. Sequencing of Qiaquick (Qiagen) purified PCR-products was performed on an ABI 377 automated sequencer (Perkin Elmer) using the same primers as in amplification.

Sequences were base-called and aligned using Sequence Navigator, SeqPup (Gilbert 1996) and manual inspection. High quality sequence alignments were obtained from the TGFB2 (581 bp) and BC-K (527 bp) genes. However, a polymorphic insertion-deletion in the LaminA fragment rendered most of the sequence unreadable in some individuals. Accordingly, the latter fragment was excluded from the analysis. In addition to the mixed-species family (a female meadow pipit, a male water pipit and three nestlings) one additional male from each of the species was sequenced for comparison. The sequences are available at the EMBL database (Accession nos. AF527050-AF527053).

RESULTS AND DISCUSSION

The two adult meadow pipits and the two adult water pipits were homozygous for respectively different nucleotides at 5 sites (Table 1). In addition, intraspecific polymorphisms at two sites were observed. Assuming Mendelian inheritance, one of the three nestlings had a genotype consistent with being sired by the heterospecific pair members. However, the latter two nestling genotypes were not consistent with that of their putative father. At the five (apparently) species-specific sites these nestlings were homozygous for “meadow-pipit alleles”. In short, our results suggest that the mixed-species pairing event resulted in one hybrid offspring, whereas two of the nestlings resulted from extra-pair paternity of a male conspecific with the female pair member.

In molecular studies of hybridization it is a prerequisite to find appropriate markers to identify hybrid genotypes. Since hybridizing taxa usually are closely related a general problem is to find nuclear markers with sufficiently different allele frequencies. Allozymes may evolve too slowly for this purpose (Tegelström and Gelter 1990). The peculiar mutation pattern of microsatellites results in considerable homoplasy even though the mutation rate is high at such loci (Angers et al. 2000). Accordingly, even rather distantly related species may still have overlapping allele frequencies at microsatellite loci (Sætre et al. 2001). The present study suggests that sequence analysis of intron fragments may provide a good alternative to these two methods. Intron-sequences would evolve considerably faster than allozymes. Moreover, since back-mutations are much less likely to occur at a specific nucleotide site of a sequence compared to those resulting from addition or subtraction of microsatellite repeat units, homoplasy is much less of a problem at the former loci.

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