ORNIS SVECICA 17: 75–80, 2007

Green Warbler *Phylloscopus (trochiloides) nitidus* recorded at Ottenby, Öland: a first record for Scandinavia

Kaukasisk lundsångare Phylloscopus (trochiloides) nitidus *anträffad på Ottenby, Öland: ett förstafynd för Skandinavien*

DARREN E. IRWIN & MAGNUS HELLSTRÖM

Abstract

This paper presents the circumstances surrounding the findings and identification of the first Green Warbler *Phylloscopus (trochiloides) nitidus* for Sweden. The bird was caught and ringed at Ottenby Bird Observatory on May 29, 2003, but was not formally identified to subspecific level until after a DNA analysis of the collected blood-sample was conducted. This constitutes the sixth European record outside the restricted breeding area.

Darren E. Irwin, Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC, Canada, V6T 1Z4. Email: irwin@zoology.ubc.ca
Magnus Hellström, Ottenby Bird Observatory, Pl 1500, SE-380 65, Degerhamn, Sweden.
Email: magnus.hellstrom@netatonce.net

Received 7 February 2007, Accepted 6 March 2007, Editor: S. Svensson

Introduction

Greenish Warblers *Phylloscopus trochiloides* of the subspecies *P. t. viridanus* breed in Eastern Europe, Western Siberia, and central Asia and are regular visitors to Sweden each spring and summer. A related taxon, the Green Warbler *Phylloscopus (trochiloides) nitidus*, which breeds in the Caucasus range in the extreme SE corner of Europe, has never before been recorded in Sweden. This taxon has a breeding distribution that is geographically separated from the rest of the *Phylloscopus trochiloides* group and has diverged to some extent in genetic, behavioural, and morphological traits, leading some authors to treat it as a distinct species *Phylloscopus nitidus* (e.g. del Hoyo et al. 2006). However, other authors treat it as the subspecies *Phylloscopus trochiloides nitidus*, due to its similarity to other taxa within the *Phylloscopus trochiloides* group (e.g. Baker 1997). Helbig et al. (1995) discuss in detail the debate over the taxonomic treatment of *P. (t.) nitidus*, showing that it has reached a level of divergence that is intermediate between subspecies and species.

Findings

During early morning on May 29, 2003, a bird that was initially identified as an unusually yellow Greenish Warbler *Phylloscopus trochiloides* was caught and ringed at Ottenby Bird Observatory, on the Baltic island of Öland, SE Sweden. This bird showed an obvious yellow wash on the supercilium, ear-coverts, throat, breast and, to some extent, also in the wing-bar (Figure 1). The circumstances were rather stressed, and after a quick examination and blood-sampling the bird was released. However, the bird stayed in the light-house garden for the rest of the day, and discussions about the subspecific identity were initiated. In the field it was noted that the bird was seemingly a trifle more green above than most Greenish Warblers seen in Sweden. It also had a longer primary projection, and further the legs appeared notably paler and the bill heavier than usual. During the day the bird proved to be rather silent, but a few calls were heard and were described as more three-syllabic than usually heard from the species, more like the Siberian subspecies *P. t. plumbeitarsus*. All together, the characters seemed to fit the Caucasian form *P. (t.) nitidus*, a taxon never previously recorded in Scandinavia. Because of the hurried situation during the ringing, little extra biometry was available, but the wing was measured to 67 mm which, after consulting literature and the bird observatory's database, was found to be unusually long for the species. Next morning the bird could not be relocated. In order to further investigate whether the bird was a *P. (t.)*

Figure 1. Green Warbler *Phylloscopus (trochiloides) nitidus*, Ottenby, Öland May 29, 2003. (Photo: Daniel Bengtsson).
Kaukasisk lundsångare P. (t.) nitidus*, Ottenby, Öland 29:e maj 2003.*

nitidus or rather an unusual example of *P. t. viridanus*, it was decided to send the blood sample for DNA analysis.

Methods

We analyzed DNA from the putative *P. (t.) nitidus* (sample number B135) as well as a bird that had been caught on May 26 (also at Ottenby) and was identified at that time as a *P. t. viridanus* (sample number B108). This second sample served as a sort of control to ensure the molecular analysis was reliable.

Extraction and amplification of DNA were conducted by D.I. at the Centre for Molecular Biogeography at UBC. Blood samples, which had been collected in SET buffer, were extracted using a standard phenol-chloroform protocol, resulting in high yield of DNA. From both samples we then amplified (using the polymerase chain reaction) a fragment of the mitochondrial cytochrome *b* gene using the primers O-L14851 (5'-CCTAC-CTAGGATCATTCGCCCT-3') and O-H16065 (5'-AGTCTTCAATCTTTGGCTTACAAGAC-3'; Weir & Schluter 2007). The reaction volumes of 50 µl contained 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.03 U/µl of Taq DNA Polymerase (New England BioLabs), and 1X ThermoPol Buffer (New England BioLabs). The reactions were held at 94° C for 2.5 minutes, then cycled 35 times between 94° C for 30 s, 52° C for 30 s, 72° C for 70 s, and then held at 72° C for 5 min. These reactions each resulted in a single clean band when visualized on an agarose checking gel. We sent the PCR products to Macrogen (Seoul, Korea) for sequencing in both forward and reverse directions.

Results

The resulting sequences were aligned and proofread using SE-AL (Rambaut 1996), resulting in 1054 bp of clean sequence from the putative *P. (t). nitidus*, including 461 bp of overlap between clean forward and reverse reads, and 1029 bp from the putative *P. t. viridanus*. The sequences were then aligned and compared with the five cytochrome b sequences from the *P. trochiloides* species available in Genbank. These include a sequence of one *P. (t). nitidus* (accession number Z73489; Helbig et al. 1995), two *P. t. viridanus* (Z73493; Helbig et al. 1995; and L77126; Richman 1996), one *P. t. ludlowi* (L77130; Richman 1996), and one *P. t. trochiloides* (Y10739; Price et al. 1997).

The putative *P. (t.) nitidus* sample showed a clear similarity with the previously published sequence of *P. (t.) nitidus* (Figure 2, Table 1), differing in only a single nucleotide, and statistical significance for this similarity is high (a bootstrap value well above 95%, see caption to Figure 2). These sequences of *P. (t.) nitidus* differ substantially (by 19–37 nucleotides) from sequences of other subspecies in the *P. trochiloides* complex. The putative *P. t. viridanus* (sample number B108) groups as expected with the two published sequences of *P. t. viridanus*, differing from them at only 1 or 2 nucleotides. Given these results, we are quite confident in concluding that sample B135 is indeed from a *P. (t.) nitidus*.

The two new sequences have been deposited in GenBank, under accession numbers EF502039 for sample B135 and EF502040 for sample B108.

Discussion

One should always use caution when inferring species identity from mitochondrial sequence data, for three reasons. First, PCR amplification can be highly sensitive to contamination between samples. This concern, however, cannot be reasonably raised here because there is no actual source of *P. (t.) nitidus* DNA that could have contaminated the sample in question, as the taxon has never previously been known to occur in Sweden and no other *P. (t.) nitidus* samples have been analyzed in our laboratory at UBC. Second, it is conceivable that *P. (t.) nitidus* mitochondrial sequences are sometimes found in other taxa within the *P. trochiloides* complex, raising the possibility that the bird in question is a different taxon, such as *P. t. viridanus*. This also seems highly unlikely, given the fact that sequences of mitochondrial control region sequences from samples throughout the complex show no such sharing of haplotypes between *P. (t.) nitidus* and the other taxa (Irwin et al. 2001). That study showed that 35 samples of *P. t. viridanus* caught between Gotland (in the Baltic Sea) and central Siberia all had extremely similar mitochondrial control region sequences, whereas two samples of *P. (t.) nitidus* were highly divergent. Third, mitochondrial DNA reflects matrilineal inheritance only; there is some small possibility that the individual in question had a *P. (t.) nitidus* mother and a father from another taxon. However, the allopatric breeding range of *P. (t.) nitidus* must be considered, and to our knowledge there is no documented hybridization between

Table 1. Genetic distances between cytochrome *b* sequences of the Ottenby samples (B135 and B108) and five Genbank sequences. Both the number of nucleotide differences (above diagonal) and percent nucleotide differences (below diagonal) are shown.
Genetiskt avstånd mellan cytochrom b-sekvenserna från Ottenbyproven (B135 och B108) och de fem sekvenserna från Genbank. Både antalet nukleotidskillnader (över diagonalen) och procenten nukleotidskillnader (under diagonalen) visas.

	B135 EF502039	*P. (t.) nitidus* Z73489	*P. t. ludlowi* L77130	B108 EF502040	*P. t. viridanus* Z73493	*P. t. viridanus* L77126	*P. t. trochiloides* Y10739
B135		1	20	24	26	25	37
nitidus	0.001		19	23	25	24	36
ludlowi	0.029	0.027		8	10	9	33
B108	0.035	0.033	0.012		2	1	35
viridanus	0.038	0.036	0.014	0.003		1	35
viridanus	0.036	0.035	0.013	0.001	0.001		36
trochiloides	0.053	0.052	0.048	0.050	0.050	0.052	

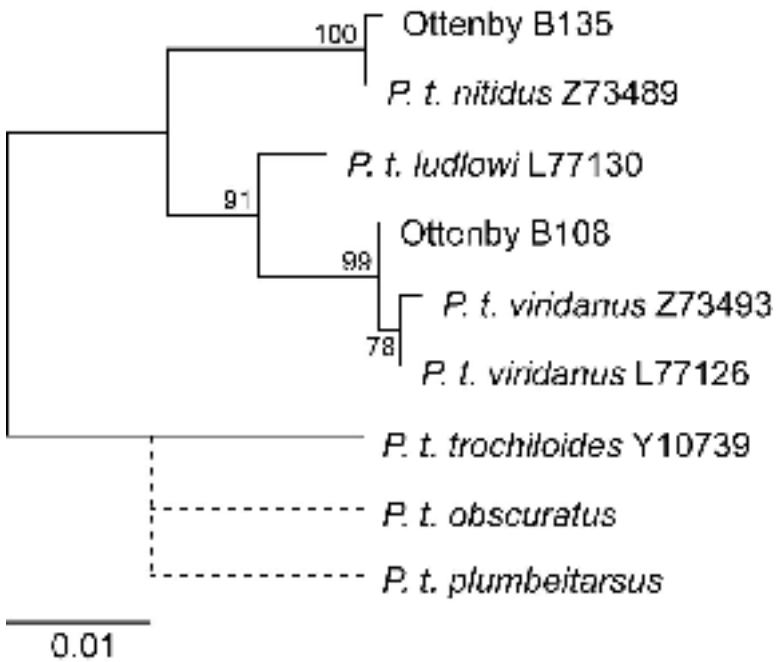

Figure 2. Phylogenetic tree showing estimated relationships between the mitochondrial cytochrome *b* gene of the putative *P. (t.) nitidus* (B135) and a *P. t. viridanus* (B108) ringed at Ottenby as well as other samples from the *P. trochiloides* complex. Sample B135 groups with *P. (t.) nitidus* and sample B108 groups with *P. t. viridanus*. The tree also shows positions of *P. t. ludlowi*, a northwestern Himalayan form, and *P. t. trochiloides*, a central and eastern Himalayan form. Dashed lines show the estimated mitochondrial relationships of two other taxa in the complex, *P. t. obscuratus* in central China and *P. t. plumbeitarsus* in eastern Siberia, as estimated by Irwin et al. (2001) using control region sequences. The position of the root of the tree was chosen to be consistent with the control region phylogeny (Irwin et al. 2001), which was produced using an assumption of a constant molecular clock, thus generating a high confidence level in the position of the root. The scale bar represents substitution rate along a lineage. The phylogeny was produced using a maximum likelihood approach by the program DNAML of the PHYLIP package (Felsenstein 2005) and drawn using TreeView (Page 1996). Numbers above nodes represent bootstrap percentages, based on 100 resampled data sets, calculated using PHYLIP.
Fylogenetiskt träd med de beräknade släktskaperna mellan cytochrom b-gener från Ottenbyproven (B135 och B108) samt fem andra taxa i P. trochiloides-*komplexet.*

P. (t.) nitidus and other taxa. Overall, given that sample B135 was predicted based on visual appearance to be a *P. (t.) nitidus*, the agreement of the DNA analysis is extremely strong confirmatory evidence.

In Sweden the Greenish Warbler is a scarce spring and summer visitor with 25–235 records per year during the period 1990–2005 (SOF 2001–2006, SOF 2002b). Breeding records are made regularly, mainly in the eastern parts of the country. The species is unsurprisingly represented by the northwestern taxon *P. t. viridanus*, but there are also two accepted records of birds from the Siberian population *P. t. plumbeitarsus* (ringed at Ottenby, Öland 5 July 1991; ringed at Utklippan, Blekinge 6 October 1999), which replaces *P. t. viridanus* east of the Jenisey River, Russia.

P. (t.) nitidus breeds in mountain forests in the Caucasus range, extending NW into the lowlands of the Russian region of Krasnodar, and the wintering areas are mainly located in the southern parts of the Indian sub-continent (Cramp 1992). Outside the restricted breeding range the European occurrence of the taxon is rather limited with the following records accepted: Greece (1 cy ringed at Antikythera 18 September 1998 [to be re-assessed by the Hellenic Rarities Commitee]); Faroe islands (ringed at Nólsoy Bygd 8 June 1997); France (1 cy Audinghen, Pas-de-Calais 20–21 September 2003); England (St. Mary's, Isles of Scilly 26 September

to 4 October 1983); Germany (shot on Helgoland 11 October 1867 [skin preserved at Inst. of Avian Research, Wilhelmshaven]). Two further records from Helgoland, Germany are still pending: 8 June 1997 (ringed) and 1 June 1998 (Jochen Dierschke, Tim Melling, Pierre-André Crochet, Nikos Probonas, Kasper Thorup in litt.). Hence, the Ottenby record constitutes the sixth accepted for Europe outside the breeding range, and to our knowledge the first to be documented with DNA. The record was accepted by the Swedish Rarities Committee on their meeting in December 2006.

Concerning the taxonomical status of *P. (t). nitidus* the AERC TAC (Taxonomic Advisory Committee of the Association of European Records and Rarities Committees) stated that the taxon, although positioned outside the distribution ring consisting of the other five taxa within *P. trochiloides* (*viridanus-ludlowi-trochiloides-obscuratus-plumbeitarsus*), is best treated as conspecific (AERC TAC 2003). It was acknowledged that its allopatric range, genetic differentiation and apparently distinct morphology could defend a treatment as a separate species (*P. nitidus*), but based on uncertainties concerning the full morphological variation it was decided that the taxon, at present, does not fulfil the diagnosability requirements presented in Helbig et al. (2002), and therefore is best regarded as a subspecies within *P. trochiloides*.

Acknowledgements

Thanks to the ringing staff at Ottenby Bird Observatory for taking the first steps towards the identification of the bird. We also thank Jason Weir for recommending primer sequences, and Alexander Hellquist and Mats Wærn for valuable comments on the manuscript. This is contribution no. 219 from Ottenby Bird Observatory. For funding we thank the Natural Sciences and Engineering Research Council of Canada, the Canadian Foundation for Innovation, and the British Columbia Knowledge Development Fund.

References

AERC TAC. 2003. *AERC TAC's Taxonomic Recommendations*, 1 Dec 2003. Online version: http://www.aerc.eu.

Baker, K. 1997. *Warblers of Europe, Asia, and North Africa*. Princeton University Press, Princeton, New Jersey.

Cramp, S. (ed). 1992. *The Birds of the Western Palearctic*. Vol VI. Oxford University Press, Oxford.

del Hoyo, J., Elliott, A. & Christie, D. A. (eds.) 2006. *Handbook of the Birds of the World*. Vol. 11. Old World Flycatchers to Old World Warblers. Lynx Edicions, Barcelona.

Felsenstein, J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Helbig, A. J., Knox, A. K., Parkin, D. T., Sangster, G. and Collinson, M. 2002. Guidelines for assigning species rank. *Ibis* 144.

Helbig, A. J., Seibold, I., Martens, J. & Wink, M. 1995. Genetic differentiation and phylogenetic relationships of Bonelli's Warbler *Phylloscopus bonelli* and Green Warbler *P. nitidus. J. Avian Biol*. 26: 139–153.

Irwin, D. E., Bensch, S. & Price, T. D. 2001. Speciation in a ring. *Nature* 409: 333–337.

Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357–358.

Price, T. D., Helbig, A. J. & Richman, A. D. 1997. Evolution of breeding distributions in the Old World leaf warblers (genus *Phylloscopus*). *Evolution* 51: 552–561.

Rambaut, A. 1996. Se–Al: Sequence Alignment Editor. Available at http://evolve.zoo.ox.ac.uk/.

Richman, A. D. 1996. Ecological diversification and community structure in the Old World leaf warblers (genus *Phylloscopus*): a phylogenetic perspective. *Evolution* 50: 2461–2470.

SOF. 2001–2006. *Fågelåret* 2000–2005. Vår Fågelvärld. Supplement 35, 37, 40, 42, 44 & 45. Stockholm.

SOF. 2002 b. Sveriges fåglar. 3:rd edition. Stockholm.

Weir, J. T. & Schluter, D. 2007. The latitudinal gradient in recent speciation and extinction rates in birds and mammals. *Science* 315: 1574–1576.

Sammanfattning

Under morgonen den 29 maj 2003 fångades en avvikande lundsångare på Ottenby fågelstation, Öland. Fågeln hade ett påtagligt gulstick över ögonbrynsstreck, örontäckare, strupe, bröst och i viss utsträckning även i vingbandet (Figur 1). Fågeln var långvingad (67 mm) och uppfattades grovnäbbad samt yttrade vid några tillfällen ett lockläte som uppfattades som mer uttalat trestavigt än vad som är brukligt för lundsångare. Ett blodprov togs vid ringmärkningstillfället, och i syfte att utreda fågelns taxonomiska ursprung beslutades att detta skulle skickas till DNA-analys.

Blodet (prov nr B135) analyserades samtidigt med ett prov (nr B108) som togs från en normalfärgad lundsångare (förmodad *P. t. viridanus*) som hade fångats på samma plats ett par dagar tidigare. Detta som en kontrollåtgärd för att försäkra att analysen var tillförlitlig. Resultatet från analysen presenteras i Tabell 1. Prov nr B135 var nära identiskt med sekvensen för *P. (t.) nitidus* som finns att tillgå i Genbank, och prov nr B108 grupperade sig som förväntat med de publicerade sekvenserna från *P. t. viridanus*. Risken för att resultatet påverkats av kontaminering är mycket låg eftersom *P. (t.) nitidus* aldrig tidigare hanterats i vare sig ringmärknings-

laboratoriet på Ottenby eller i analyslaboratoriet i Vancouver, Kanada. Man kan också anföra risken att den aktuella sekvensen teoretiskt skulle kunna återfinnas även hos andra taxa inom *P. trochiloides*-komplexet, men även detta kan betraktas som högst osannolikt eftersom tidigare analyser utförda inom hela komplexet inte uppvisar någon tendens att haplotyper delas mellan *P. (t.) nitidus* och de övriga populationerna. Dessa analyser visade samtidigt att 35 prov av *P. t. viridanus*, insamlade från Gotland till centrala Sibirien, alla hade extremt lika sekvenser, samtidigt som två prov från *P. (t.) nitidus* tydligt avvek från dessa. Vidare finns en teoretisk felkälla i att nedärvning av mtDNA sker på mödernet vilket i praktiken innebär att analysen inte säger något om faderns genetiska ursprung. Det bör dock beaktas att *P. (t.) nitidus* har ett allopatriskt häckningsområde och vad vi känner till finns ingen dokumenterad hybridisering mellan *P. (t.) nitidus* och andra taxa. Om man till ovanstående lägger att prov B135 misstänktes för att vara *P. (t.) nitidus* redan vid den visuella bedömningen av fågeln utgör överensstämmelsen med DNA-analysen en mycket kraftfull bevisföring.

P. (t.) nitidus häckar i bergsskogar i Kaukasus och övervintrar främst i de södra delarna av Indien. Sedan tidigare finns (utanför det begränsade häckningsområdet) fem godkända fynd från Europa: Grekland, Färöarna, Frankrike, England och Tyskland. Ottenbyfyndet var således det sjätte i ordningen. Vad gäller den taxonomiska statusen för *P. (t). nitidus* har AERC TAC (den internationella europeiska taxonomiska kommittén) beslutat att för närvarande behålla *P. (t). nitidus* inom *P. trochiloides*, samtidigt som man erkänner att utbredningsområdet är allopatriskt, en genetisk differentiering finns närvarande och att formen sannolikt är morfologiskt distinkt. Beslutet grundar sig på oklarheter rörande den utseendemässiga variationen, och för närvarande anser man således inte att *P. (t). nitidus* uppfyller de diagnostiska krav som regelmässigt ställs vid denna typ av bedömningar. Fyndet godkändes av SOF:s Raritetskommitté på dess möte i december 2006.