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Source: The Auk, 122(2):684-688. 2005.

Published By: The American Ornithologists' Union

DOI:

URL: <http://www.bioone.org/doi/full/10.1642/0004-8038%282005%29122%5B0684%3AADOMDO%5D2.0.CO%3B2>

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The Auk 122(2):684–688, 2005  
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Printed in USA.

## ADDITIONAL DATA ON MITOCHONDRIAL DNA OF NORTH AMERICAN LARGE GULL TAXA

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**ABSTRACT.**— After publication of the *Larus* phylogeny in Crochet et al. (2002), the taxonomic status of the "*Larus thayeri*" and the "*L. occidentalis*" specimens that were used in that study came into question. For each of those species, we sequenced the same mitochondrial DNA regions in new specimens of known identity. In addition, specimens of *L. glaucescens* were included. Results from using those specimens confirm that *L. occidentalis* was the first to diverge from the large white-headed gulls. *Larus glaucescens*, on the contrary, is part of the Arctic species clade, which also includes *L. hyperboreus*, *L. glaucoides*, *L. thayeri*, and *L. schistisagus*. The three new *L. thayeri* specimens differ substantially in mitochondrial DNA from the previously used sample of *L. thayeri* and share the same haplotype with *L. glaucescens*. The significance of this finding is unclear, because relationships within the "Arctic species" clade are still unresolved; that is attributable to the unusually high incidence of lineage sharing and extremely low divergence of haplotypes in the group. Received 16 April 2004, accepted 13 December 2004.

**Key words:** control region, cytochrome *b*, large white-headed gulls, mitochondrial DNA, phylogeny, recent speciation.

### Phylogénie Mitochondriale des Grands Goélands: De Nouvelles Données en Amérique du Nord

**RÉSUMÉ.**— Cette étude complète la phylogénie des grands goélands publiée dans Crochet et al. (2002), où le statut taxonomique des spécimens "*thayeri*" et "*occidentalis*" était incertain. Les mêmes fragments d'ADN mitochondrial ont été séquencés sur de nouveaux spécimens de chaque espèce, dont l'origine n'est pas ambiguë. Des échantillons de *L. glaucescens* ont également été utilisés. L'ajout de ces spécimens confirme que le taxon *L. occidentalis* a divergé en premier au sein des grands goélands. *L. glaucescens* au contraire est inclus dans le clade des espèces Arctiques, qui regroupe *L. hyperboreus*, *L. glaucoides*, *L. thayeri* et *L. schistisagus*. Les trois nouveaux spécimens de *thayeri* diffèrent substantiellement de l'échantillon utilisé précédemment et partagent le même haplotype avec les spécimens de *L. glaucescens*. La signification de ce résultat ne peut cependant pas être évaluée pour l'instant, les relations au sein du groupe des grands goélands n'étant pas encore résolues du fait de la forte fréquence de polymorphisme partagé et de la faible divergence entre taxons.

LARGE WHITE-HEADED GULLS constitute a group of species whose systematic relationships are poorly understood. Several recent studies have used genetic markers to address parts of

the problem (de Knijff et al. 2001; Liebers et al. 2001; Crochet et al. 2002, 2003; Liebers and Helbig 2002), but none has yet worked with a large sample of all taxa. Extensive sampling within taxa, in particular, is crucial in this group, because of widespread sharing of polymorphisms among species (Crochet et al. 2003).

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The study by Crochet et al. (2002) included most of the taxa, but sampling was often limited to one or a few specimens per taxon. In addition, after the study's publication, the validity of some of its results came into question because the identity of some specimens was challenged (K. G. Smith pers. comm., R. C. Banks pers. comm.). The "*Larus thayeri*" specimen (from the Louisiana State University Museum of Natural Science [LSUMZ]; tissue LSUMZ B-21816, voucher LSUMZ 160609) was collected in an unusual location—unusual in that Louisiana is not part of the normal winter range for *L. thayeri*. Furthermore, after publication of the study, correspondence with staff at the LSUMZ revealed that the specimen is actually labeled "*L. thayeri*?" and should not have been used as a representative of the taxon (S. W. Cardiff pers. com.). The *L. occidentalis* specimen (tissue LSUMZ B-20480) was collected in Grays Harbor, Washington, which is the northern-range limit for that species and lies in the hybrid zone of *L. occidentalis* and *L. glaucescens* (Scott 1971; Bell 1996, 1997; Good et al. 2000). It is thus possible that the specimen had an *L. glaucescens* haplotype, even if the morphology is typical of *L. occidentalis*. Here, we analyzed additional specimens of *L. thayeri* and *L. occidentalis* of known identity, together with several *L. glaucescens* specimens, to resolve ambiguities in the phylogenetic relationships among those taxa of large white-headed gulls.

## METHODS

Specimens of *L. occidentalis* and *L. glaucescens* and all available samples of *L. thayeri* with no identification ambiguity were loaned by the Museum of Vertebrate Zoology, University of California, Berkeley (see Table 1). Localities of the *L. occidentalis* and *L. glaucescens* samples were chosen as far from the hybrid zone as possible. Details about the other specimens used here are described elsewhere (see table 2 in Crochet et al. 2002).

DNA was extracted from tissue samples by complete digestion in 10% Chelex 100 (Biorad, Hercules, California) with 5  $\mu$ L of proteinase K, followed by two periods of 15-min boiling. A 674-base-pair (bp) fragment of the mitochondrial DNA (mtDNA) control region was amplified by polymerase chain reaction (PCR). We used the primers L438: 5'-TCA-CGT-GAA-ATC-AGC-AAC-CC-3' and H1561: 5'-CGG-TTA-ATT-AGG-GTC-TCT-TG-3'. A 307-bp fragment from the cytochrome-*b* gene was amplified from the same specimen, using the following primers: L15008, 5'-AAC-TTC-GGA-TCT-CTA-CTA-GG-3' and H15326, 5'-GAA-TAA-GTT-GGT-GAT-GAC-TG-3'. The PCR amplification was performed in a 50- $\mu$ L reaction volume containing 2  $\mu$ L of DNA solution (variable concentration), 5  $\mu$ L of 10 $\times$  buffer (Tris-HCl 100 mmol + KCl 500 mmol), 6.25  $\mu$ mol of MgCl<sub>2</sub>, 8.32  $\mu$ mol of dNTP, 1.67  $\mu$ mol of each primer, and

TABLE 1. Taxonomic designation, sample size, sampling localities, type of specimen, and voucher specimens for each taxon sequenced.

Taxon	Number of specimens	Locality	Type of specimen	Voucher specimens <sup>a</sup>
<i>Larus glaucescens</i>	4	Middleton Island, Alaska	Three adults and one 4th-year	Wing and partial skeleton: MVZ 172515, MVZ 172518, MVZ 172519, MVZ 172522
<i>L. o. occidentalis</i>	3	San Francisco Bay, California	Adults	Study skin: MVZ 172380, MVZ 172381 Wing and partial skeleton: MVZ 172389
<i>L. o. wymani</i>	3	Anacapa Island, California	Adults	Wing and partial skeleton: MVZ 172465, MVZ 172468, MVZ 172469
<i>L. thayeri</i>	3	Monterey Bay, California	Two adults and one unknown	Skeleton: MVZ 175953, MVZ 175954, MVZ 175955

<sup>a</sup> All voucher specimens were loaned by the Museum of Vertebrate Zoology (MVZ), University of California, Berkeley.

0.5 units of *Taq* DNA polymerase (Eurogentec, Seraing, Belgium). The annealing temperature was 58°C for the control region and 56°C for the cytochrome-*b* gene. Annealing duration was 45 s. Sequencing reactions were conducted with the ABI Prism BigDye terminator kit (Applied BioSystems, Foster City, California), following the standard ABI cycle sequencing protocol, and were electrophoresed on an ABI Prism 310 genetic analyzer following the manufacturer's recommended procedures. Sequencing primers were L438, HHTR (5'-ATC-GCT-GTT-GTT-GAC-ATG-TA-3'), and DL11F (5'-AAA CCC TTC CAG TGC ACC GGG-3') for the control region, and L15008 for the cytochrome-*b* gene. Sequences have been deposited in GenBank (accession numbers: AY615681–AY615706).

A composite segment, including the control region and cytochrome-*b* segments, was aligned manually and subjected to phylogenetic analysis. *Larus crassirostris* and *L. canus* were selected as outgroup taxa, because they are both closely related to the large white-headed gulls (Crochet et al. 2000, 2002). We used MODELTEST, version 3.06 (Posada and Crandall 1998) to determine the appropriate model of substitution for the combined fragment of control region and cytochrome-*b* sequences (we reiterated the analysis for each fragment separately with similar results). The selected model was Tamura-Nei, with a proportion of invariable sites ( $P = 0.8555$ ). We used PAUP\*, version 4.0b10 (Swofford 1998) to generate a phylogenetic tree of the mtDNA haplotypes by the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods. All three methods gave near-identical topologies (see below). We evaluated the robustness of the trees by bootstrap (1,000 replications) based on the ML method. We also estimated branch support using the Bayesian methodology as implemented in MRBAYES, version 3.0b4 (Huelsenbeck and Ronquist 2001). MRBAYES estimates posterior probabilities of the best set of trees for a given model of sequence evolution. The GTR model with eight classes of substitution rates and gamma distribution (GTR + I + gamma) was used; model parameters were estimated by MRBAYES. Analyses were initiated with random starting trees, and three Markov chains of 1 million generations were simulated, with trees sampled every 100 generations. *Larus canus* was selected as the outgroup taxon.

## RESULTS AND DISCUSSION

Because NJ, MP, and Bayesian trees have a similar topology to the ML tree, only the ML tree is presented in Figure 1. The six additional *L. occidentalis* samples cluster together with the *L. occidentalis* sample used in Crochet et al. (2002), which indicates that despite the frequent hybridization with *L. glaucescens*, that specimen had a typical *L. occidentalis* haplotype. The addition of those six *L. occidentalis* samples confirms that *L. occidentalis* and *L. livens* have diverged first. The "fuscus clade," as defined in Crochet et al. 2002, is thus recovered in the analysis. As expected from their subspecific status, the taxa *L. o. occidentalis* and *L. o. wymani* are very closely

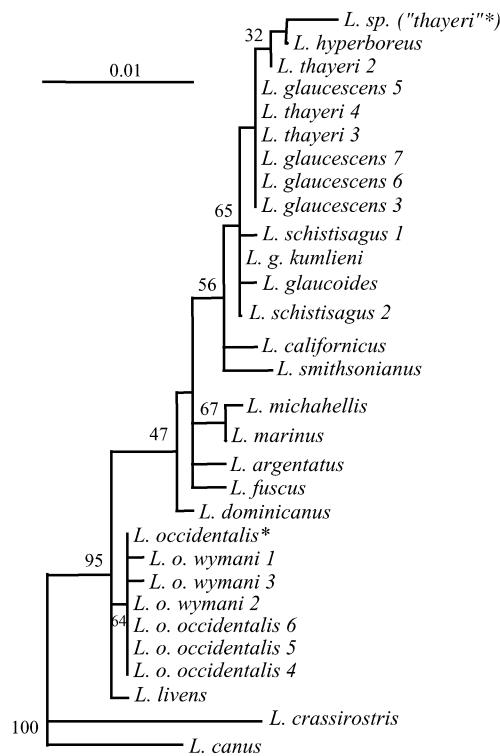


FIG. 1. Maximum-likelihood (ML) tree of the large white-headed gulls (genus *Larus*) based on mtDNA control region and cytochrome-*b* sequences. Bootstrap values are based on 1,000 permutations under the ML method and are displayed at nodes supported by a value >40. An asterisk (\*) indicates specimens whose taxonomic status was uncertain in Crochet et al. (2002).

related, with one *wymani* individual sharing the same haplotype as *occidentalis* and two differing by one mutation each in the control region segment (Table 2).

*Larus glaucescens* is included in the "fuscus clade" and is therefore not closely related to *L. occidentalis*, despite the fact that the two species widely hybridize where they breed in sympatry (Bell 1996, 1997; Good et al. 2000). *Larus glaucescens* is grouped with the "Arctic species" clade (see Crochet et al. 2002), which also includes *L. hyperboreus*, *L. glaucooides*, *L. thayeri*, and *L. schistisagus*. That clade is supported by a moderate bootstrap value (ML: 65) and posterior probability (66). Either a longer fragment or a more variable fragment (or both) could improve resolution of this part of the tree, though the hypervariable HV1 fragment of the mitochondrial control region sequenced in Liebers et al. (2004) failed to provide a better support for this particular node.

All three *L. thayeri* samples cluster together in the "Arctic species" clade, which is in agreement with the supposedly close relationship between *L. glaucooides* and *L. thayeri*, as favored by some recent authors (e.g. Sibley and Monroe 1990, American Ornithologists' Union 1998). However, all *glaucescens* specimens sequenced here share the same haplotype with two *thayeri* specimens and differ by one mutation on the control region from the haplotype of our specimen *thayeri* 2 (Table 2). That result certainly underlines the very recent origin of those taxa, but we cannot decide whether extensive lineage-sharing explains this observation or whether *L. thayeri* and *L. glaucescens* are reciprocally monophyletic and the lack of variation in the sequence that we analyzed prevented us from separating their haplotypes. To solve this question, it will be necessary to sequence either a longer segment or a more variable segment (or both), such as the hypervariable HV1 fragment of the mitochondrial control region (see Liebers et al. 2004) that has not been sequenced yet for *thayeri*.

The "thayeri" specimen included in the phylogeny of Crochet et al. (2002) largely differs from all three specimens analyzed here (five mutations on the control region; Table 2) and seems to be more closely related to *hyperboreus* than to the three *thayeri* specimens. That could be attributed either to horizontal gene transfer if the specimen was really *L. thayeri*, which we

TABLE 2. Variable sites in the control region and observed cytochrome-*b* haplotypes.

	Control region	Cytochrome <i>b</i>
<i>L. michahellis</i>	ACGTTTTTTCAC	GTAT
<i>L. occidentalis</i>	GTAG.C....	AC..
<i>L. o. occidentalis</i> 4	GTAG.C....	AC..
<i>L. o. occidentalis</i> 5	GTAG.C....	AC..
<i>L. o. occidentalis</i> 6	GTAG.C....	AC..
<i>L. o. wymani</i> 2	GTAG.C....	AC..
<i>L. o. wymani</i> 3	GTAG.C...T	AC..
<i>L. o. wymani</i> 1	GTAG.C..C.	AC..
<i>L. glaucescens</i> 5	...C.CT..	ACGC
<i>L. glaucescens</i> 3	...C.CT..	ACGC
<i>L. glaucescens</i> 6	...C.CT..	ACGC
<i>L. glaucescens</i> 7	...C.CT..	ACGC
<i>L. thayeri</i> 3	...C.CT..	ACGC
<i>L. thayeri</i> 4	...C.CT..	ACGC
<i>L. thayeri</i> 2	G...C.CT..	ACGC

cannot know for sure, or to misidentification of the specimen. Whatever the reason, the haplotype identified in Crochet et al. (2002) was most likely not a genuine *thayeri* haplotype. The results of Crochet et al. (2002) about the position of *thayeri* are thus partly invalidated by these additional data.

#### ACKNOWLEDGMENTS

We thank C. Cicero from the Museum of Vertebrate Zoology, University of California, Berkeley, who sent us the samples used here. We are also grateful to P. Sourrouille and C. Debain for technical assistance with the molecular work, to C. de Franceschi who participated in the molecular work, and to P. Chevet who provided help with the PAUP analyses. We thank two anonymous reviewers and A. E. Burger for their helpful comments on the manuscript.

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Associate Editor: A. E. Burger