FIRST CALIFORNIA RECORDS OF THE LITTLE STINT AND NAZCA BOOBY CONFIRMED THROUGH MOLECULAR ANALYSIS

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ABSTRACT: We used molecular genetic analysis to assess the identification of two bird specimens otherwise difficult to identify with certainty. A *Calidris* sandpiper collected in 1974 had been identified at various times as the Little Stint (*C. minuta*) or as the Red-necked Stint (*C. ruficollis*). The remains of a booby salvaged in 2013 were identified as either the Nazca (*Sula granti*) or the Masked (*S. dactylatra*). We were able to identify the first as *C. minuta* and the second as *S. granti*, providing the first California records of each species.

Figure 1. Nazca Booby carcass in situ, McGrath State Beach, Ventura County, California, 23 July 2013.

*Photo by Alexis Frangis*
Accurate species identification is essential for surveys of wildlife diversity. Over the past few decades, species identification of collected specimens by genetic characters has become commonplace. Genetic identification is particularly useful when the phenotypic characters usually used to distinguish species are difficult to apply or when a salvaged specimen is so degraded that phenotypic characters cannot be used. Here we present results from the genetic identifications of a sandpiper and a booby that constitute first records for California.

McCaskie (1975) described encountering a likely Rufous-necked Sandpiper or Red-necked Stint (*Calidris ruficollis*) at the Salton Sea, California, on 17 August 1974. He collected the bird, in worn first alternate plumage (San Diego Natural History Museum 38887), and identified it as the first example of *C. ruficollis* recorded in California. Subsequently, the possibility was raised that the specimen might be instead an example of *C. minuta*, though the California Bird Records Committee (CBRC) could not reach a consensus on the bird’s identification. Veit (1988), using additional morphological criteria, concluded that the specimen was indeed *C. ruficollis*. However, subsequent morphological comparisons of SDNHM 38887 with over 100 specimens of *C. ruficollis* and *C. minuta* by M. Ralph Browning and Claudia Wilds at the U.S. National Museum of Natural History were inconclusive (Erickson and Hamilton 2001).

On 23 July 2013, Alexis Frangis, a California State Parks biologist, salvaged the remains of a booby (*Sula*) from McGrath State Beach in Oxnard, Ventura County, California. The specimen comprised two feathered wings, a skinless torso and skull, tail feathers, and foot webbing (Figure 1). Nevertheless, at the Western Foundation of Vertebrate Zoology (WFVZ) Searcy and Steven Tucker were able to determine, on the basis of its underwing pattern, that the specimen (WFVZ 56922) was either a Masked Booby (*S. dactylatra*) or a Nazca Booby (*S. granti*). Extensively dark upperparts implied the booby was in its first plumage cycle. Reliable identification criteria for first-cycle Masked/Nazca boobies based on plumage characters have not yet been identified, and mensural characters were not useful because of the degradation of the specimen. At the time California had no specimen records of the Masked Booby, and the Nazca Booby had not been accepted to the main list of California birds. The only Nazca Booby recorded previously in California, a first-cycle individual that had ridden a boat from Mexican waters to San Diego, was identified on the basis of bill coloration and genetic analysis. The CBRC voted to relegate this record to its supplemental list because the bird had traveled to California by boat (Garrett and Wilson 2003).

Though identification of avian specimens to species by genetic techniques has become routine following the publication of the Avian Barcode of Life primers and associated database of sequences for species identification (Hebert et al. 2004), genetic identification of these specimens presented challenges. The *Calidris* was collected over 40 years prior to our genetic analysis, and DNA degradation in the interim did not allow us to use the Barcode of Life primers. Because *S. granti* and *S. dactylatra* are closely related, we also investigated the possibility that the specimen was a hybrid.
METHODS

From each specimen, we sampled a small 5-mm section of foot skin, following the sampling methods of Ellegren (1991) and Mundy et al. (1997). We then extracted DNA from each sample, following the protocol of a standard Qiagen DNEasy Blood & Tissue kit (Valencia, CA), with the addition of a 36-hour digestion with 40 mL (the Sula specimen) or 60 mL (the Calidris specimen) of proteinase K (Crowe et al. 1991). We then amplified DNA regions containing species-diagnostic genetic variation by PCR (the polymerase chain reaction).

Attempts to amplify the Calidris sample with the Barcode of Life primers (BirdF1 and Bird R1; Hebert et al. 2004) were unsuccessful, likely because of the large size of the fragment (750 base pairs) targeted by these primers. Additionally, despite the availability of multiple reference sequences of cytochrome oxidase I for each Calidris species, we were unable to design short (<150 base pairs) primers for this region that encompassed species-diagnostic variation and also amplified successfully. Instead, we acquired sequence data for both C. ruficollis and C. minuta from the mitochondrial DNA (mtDNA) ND2 region from Genbank (www.ncbi.nlm.nih.gov/genbank/) and designed primers to amplify a fragment of 115 base pairs that encompassed DNA polymorphisms distinguishing each species (Calidris ND2-F: 5′-CCC ACC AAA CTC CAC AAA CC-3′; Calidris ND2-R: 5′-GTG ATT ATG GGG GAG AGT GGT-3′). For the Sula specimen, we used the b3 and b4 primers designed by Morris-Pocock et al. (2010) to amplify a 450-base-pair fragment of the mtDNA cytochrome-b region capable of distinguishing S. granti from S. dactylatra. Additionally, to investigate the possibility that the specimen was a hybrid, we also amplified a nuclear DNA region capable of distinguishing S. granti from S. dactylatra (intron 8 of the nuclear gene for α-enolase) by using primers (F: 5′-TGGACCTTCAAATCCCCCGATGATCCCCAGC-3′, R: 5′-CCAGGCACCCCCAGTCTACCTGGTCAAA-3′) described by Patterson et al. (2011).

Our PCR reaction mixes and thermocycling regime varied by primer set. For the Calidris sample we amplified 3 µL of DNA extract in a total volume of 25 µL with 2.5 µL 10X JumpStart REDTaq buffer (Sigma-Aldrich), 1 mM MgCl₂, 200 µM of each dNTP, 0.3 µM primer concentration, and 0.5 units of Jumpstart RED Taq (Sigma-Aldrich). Thermocycling consisted of an initial denaturation at 94°C for 4 minutes, then 40 cycles of 94°C for 15 seconds, 56°C for 1 minute, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. For the Sula sample we amplified 1 µL of DNA extract in a total volume of 20 µL with 2 µL 10X Jumpstart REDTaq buffer, 200 mM of each dNTP, 0.2 µM primer concentration, and 0.4 units of Jumpstart RED Taq. In this case thermocycling comprised an initial denaturation at 94°C for 5 minutes, then 35 cycles of 94°C for 15 seconds, 48°C (65°C for α-enolase) for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. We tested the PCR for success in amplifying the DNA by agarose gel electrophoresis, then sent successful amplifications to Sequetech Corp. (Mountain View, CA) for EXO-SAP PCR cleanup, Sanger sequencing in the forward and reverse directions, sequencing cleanup, and capillary electrophoresis to generate
sequence data. We trimmed and aligned the forward and reverse sequence data to generate consensus sequences for the Calidris specimen and each gene analyzed for the Sula specimen in Geneious (Biomatters Ltd.).

We aligned the Calidris consensus sequence alongside reference sequences of C. minuta and C. ruficollis from Gibson and Baker (2012) acquired from Genbank (accessions KC 969101 and 969105, respectively). To place our results in a larger taxonomic context, we also aligned the Calidris sequence with reference sequences of 19 species of Calidris (including C. minuta and C. ruficollis) also from Gibson and Baker (2012; Genbank accessions KC969093–KC969108). We then visually inspected these alignments for characters diagnostic of species within Calidris. For each of these two Calidris alignments, we also used Geneious to generate a neighbor-joining phylogenetic tree with a Jukes–Cantor model of substitution (Jukes and Cantor 1969) to assign the Calidris specimen to its most closely related species. We used 1000 bootstrap replicates of each data set to assess statistical confidence in the relationships inferred within each phylogenetic tree.

We aligned the cytochrome-b sequence of the booby alongside five reference sequences of S. dactylatra and five of S. granti (Genbank accessions AY156695-156699 and JX569178-569182, respectively). To place our results in a larger taxonomic context we also aligned this dataset alongside reference sequences of S. leucogaster, S. nebouxii, S. sula, and S. variegata (Genbank accessions JX 416755, KC 577254, JX 416799, and KC 577252, respectively). We then visually inspected these alignments to identify species-specific variation and analyzed it phylogenetically as described for the Calidris dataset.

Bootstrap resampling assesses the likelihood of observing a given relationship in a new dataset generated by resampling the dataset with replacement, a “nodal support” value that is not directly analogous to statistical support based on deviation from a normal distribution of variation. For example, our Calidris data set was composed of 115 DNA base pairs (“sites”) that can be numbered 1, 2, 3, etc., to 115. To create a bootstrap replicate, we randomly selected 114 of these sites with replacement (e.g. 1, 7, 7, 12, 23, 23, 50, 72, 72, 103, etc.) and analyzed this resampled data set in the same way. The bootstrap nodal support indicates the percentage of time across bootstrap replicates that two taxa (or groups of taxa) were found to be most closely related to one another. In this way, we could evaluate whether a phylogenetic relationship observed in the original data set was driven by few weakly informative points of variation, which are unlikely to be resampled consistently across bootstrap replicates, or multiple strongly informative points of variation, which should be resampled (at least in part) across the majority of bootstrap replicates. While bootstrap resampling of datasets is frequently used in phylogenetic analyses, the mathematics and methods of the approach are complex, as described by Felsenstein (1985).

RESULTS

The Calidris specimen (SDNHM 38887) possessed the variation characteristic of C. minuta at eight of eight diagnostic sites across the 115
base pairs surveyed (Table 1). The phylogenetic analyses also supported its identification as *C. minuta*, with 99% nodal support in a direct comparison with *C. minuta* and *C. ruficollis* (Figure 2) and 92% nodal support in a comparison of 19 species of *Calidris* combined (Figure 3). The 115-base-pair sequence is available via Genbank as accession KU310674.

The *Sula* specimen (WFVZ 56922) possessed variation characteristic of *S. granti* at four of four diagnostic sites across the 450 base pairs of mitochondrial DNA (mtDNA) surveyed (Table 2). The phylogenetic analysis of mtDNA also supported its identification as *S. granti* with 99% nodal support (Figure 4). Additionally, the nuclear gene-sequence data also placed WFVZ 56922 as *S. granti* (rather than as a hybrid of *S. granti* and *S. dactylatra*) at the single diagnostic site. The mitochondrial cytochrome b sequence for this specimen is available via Genbank as accession KU310675, and the nuclear α-enolase sequence is available as accession KU364145.

**DISCUSSION**

Despite the ever-increasing array of genetic technology available to study wild populations, poor quality and insufficient quantity of template DNA is a fundamental obstacle to genetic analysis. For SDNHM 38887, we initially attempted to acquire sequence data from the region that had been sequenced for the Barcode of Life (Hebert et al. 2004). By design, however, this region is relatively large in order to capture some genetic distinction between

![Figure 2](image-url)
every avian species. The DNA we extracted from SDNHM 38887 was too
degraded to allow amplification of the 750-base-pair segment sequenced for
the Barcode of Life, so we focused on smaller regions of <200 base pairs
in order to amplify DNA successfully. Data analysis with the Barcode of Life
region would have been extremely straightforward, with a simple analysis
matching the sequence of SDNHM 38887 to the existing database. Only
one reference sequence of the mitochondrial gene ND2 was available for

Figure 3. Unrooted neighbor-joining tree with 1000 bootstrap replicates of 122 base
pairs of mitochondrial ND2 data comparing SDNHM 38887 to reference sequences
from 19 species of Calidris. Bootstrap support <90% not shown.
the two candidate species of *Calidris*, so we cannot assess the placement of SDNHM 38887 with respect to the full range of genetic variation within each species. Nevertheless, SDNHM 38887 matched *C. minuta* at all eight diagnostic sites, a pattern unlikely to arise in an individual of *C. ruficollis* by chance alone. Thus SDNHM 38887 represents the earliest record of *C. minuta* in California, by over nine years, preceding one photographed at Bolinas, Marin County, 14–22 September 1983 (Roberson 1986).

One drawback of species identification by mtDNA is that mtDNA is inherited exclusively through the maternal line. The lack of paternal contribution to mtDNA variation can lead to difficulties in identifying patterns of gene flow (Yang and Kenagy 2009). Therefore, mtDNA data are not capable of identifying offspring resulting from interspecific hybridization. Nevertheless, we believe that SDNHM 38887 is unlikely to be a hybrid *C. minuta × C. ruficollis* origin, given that *C. ruficollis* is not known to hybridize (Pyle 2008).

In the case of *S. granti* and *S. dactylatra*, however, mixed pairs and apparent hybrid offspring have been observed (Howell and Webb 1990, Pitman and Jehl 1998). Thus we could not rule out the possibility of a hybrid origin for WFVZ 56922 by the mtDNA analysis alone. We addressed this uncertainty by sequencing the nuclear gene for α-enolase, in which *S. granti* and *S. dactylatra* differ (Patterson et al. 2011). In this marker, WFVZ 56922 possessed the sequence characteristic of *S. granti* with no indication of heterozygosity. Patterson et al. (2011) examined a total of five nuclear genes, but they found that among these only intron 8 of α-enolase consistently distinguished *S. dactylatra* from *S. granti*. They generated their reference sequences from two individuals of each species, sampled from opposite ends of each species’ geographic range, increasing their ability to detect intraspecific polymorphism and thereby also increasing the likelihood that any genetic differences reflect interspecific rather than intraspecific differentiation. The low level of genetic differentiation between these two species can be attributed to their relatively recent evolutionary divergence (Friesen et al. 2002, Patterson et al. 2011).

As both mitochondrial and nuclear DNA results support the identification of WFVZ 56922 as *S. granti*, on this basis the CBRC added the Nazca Booby to the main list of California birds, the record from Ventura County being the northernmost of the species.

Our results reflect the utility of genetic analysis for species identification. For this purpose, the discrete nature of genetic data can serve as a valuable supplement to the more continuous nature of morphometric data traditionally used for species identification. In the vast majority of cases, morphometric

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**Table 2** Variation at Four sites within the Mitochondrial Gene ND2 Distinguishing *Sula granti* from *S. dactylatra*

<table>
<thead>
<tr>
<th>Position(^a)</th>
<th>75</th>
<th>192</th>
<th>405</th>
<th>429</th>
</tr>
</thead>
<tbody>
<tr>
<td>WFVZ 56922</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td><em>S. granti</em></td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td><em>S. dactylatra</em></td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

\(^a\)In alignment with reference sequences of *S. dactylatra* (Genbank accessions AY156695–156699).
data are more than sufficient for unambiguous species identification. In the examples we have addressed here, however, where diagnostic morphological characters were degraded, genetic analysis was invaluable. Additionally, in cases where species are distinguished mainly by absolute size rather than morphological ratios or meristic characters, genetic analysis provides a valuable method to ensure that exceptionally large or small individuals are identified accurately.

Grinnell (1910) emphasized that the value of museum specimens may not be realized until the lapse of many years. In the case of the specimen of the Little Stint, McCaskie’s (1975) foresight allowed us the opportunity to revisit this specimen with biological techniques not available at the time it was collected. As shown here, archived natural history collections have been and will continue to be a valuable resource for biologists, often in unexpected ways (e.g., Mundy et al. 1997, Yang et al. 2011).

Figure 4. Neighbor-joining phylogenetic tree comparing sequences of the mitochondrial gene for cytochrome b from WFVZ 56922 with those reference sequences of the Masked, Nazca, Blue-footed (Sula nebouxii), Peruvian (S. variegata), Brown (S. leucogaster), and Red-footed (S. sula) boobies. The data strongly support the placement of WFVZ 56922 with S. granti. Bootstrap support <90% not shown.
LITERATURE CITED


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