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Catching flycatchers: High congruence of field and molecular identifications of *Empidonax* (Passeriformes: Tyrannidae) flycatchers highlights the feasibility of accurate in-hand identification

Amy N. Buxton[1,*], Rodolfo S. Probst[1,2,3,*], Kyle D. Kittelberger[1], Hailey Blair[1,4], and Çağan H. Şekercioğlu[1,5,6]

[1]School of Biological Sciences, University of Utah, Salt Lake City, Utah, USA
[2]Science Research Initiative (SRI), College of Science, University of Utah, Salt Lake City, Utah, USA
[3]Department of Entomology & Nematology, University of California, Davis, California, USA
[4]Utah Department of Natural Resources, Division of Wildlife Resources, Salt Lake City, Utah, USA
[5]Department of Molecular Biology and Genetics, Koç University, Istanbul, Türkiye
[6]KuzeyDoğa Derneği, Istasyon Mahallesi, Ismail Aytemiz Caddesi, Kars, Türkiye

*Corresponding authors: Amy N. Buxton, amy.buxton@utah.edu; Rodolfo S. Probst, rsprobst@ucdavis.edu

ABSTRACT

Recent avian population declines highlight the importance of long-term bird monitoring data such as those collected from bird banding stations. *Empidonax* flycatchers are considered some of the most difficult bird species to identify in North America because of their phenotypic similarity. To determine the congruence of in-hand field and molecular identifications, we collected blood and feather samples from *Empidonax* flycatchers captured during bird banding in southeastern Utah from 2013 to 2017 and generated cytochrome *c* oxidase subunit I DNA barcodes for these individuals. While we expected to see numerous incongruencies due to these species' phenotypic similarity, we found that field and molecular identifications matched for 92.7% of individuals examined. This suggests that *Empidonax* flycatchers can often be accurately identified in the field when banders use carefully collected morphological data in their assessments (including tail length, wing minus tail length, bill size and shape, and calculations based on specific feather measurements). Our findings highlight the importance of using detailed morphometric data while banding *Empidonax* flycatchers, indicating that these kinds of additional measurements can lead to a high degree of accuracy with *Empidonax* in-hand identifications. However, we also show the merit of molecular identifications to supplement field identifications in cases of unknown individuals, rare species, or closely related species with overlapping geographic ranges.

Keywords: bird banding, DNA barcoding, *Empidonax* flycatchers, feathers, morphometrics, ornithology, species identification

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LAY SUMMARY

- Long-term monitoring data when bird banding require accurate species identification.
- *Empidonax* flycatchers are considered one of the most difficult bird lineages to identify in North America.
- We studied how often field and molecular identifications are congruent for North American *Empidonax* flycatchers.
- We collected blood and/or feather samples from flycatchers in southeastern Utah between 2013 and 2017 and generated short DNA barcodes (~400 base pairs) for 103 samples.
- We found that field and molecular identifications matched for 92.7% of the flycatchers included in our study.
- Our findings emphasize that *Empidonax* flycatchers can be accurately identified in the field when banders collect detailed trait information to support the identification process.

Capturando mosqueros: la alta congruencia entre identificaciones de campo y moleculares de *Empidonax* (Passeriformes: Tyrannidae) destaca la viabilidad de una identificación precisa en mano

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RESUMEN

Los recientes declives poblacionales de aves resaltan la importancia de los datos de monitoreo a largo plazo, como los recopilados en las estaciones de anillado de aves. Los mosqueros del género *Empidonax* son considerados algunas de las especies de aves más difíciles de identificar en Norteamérica debido a su similitud fenotípica. Para determinar la congruencia entre las identificaciones en mano de campo y las identificaciones moleculares, recolectamos muestras de sangre y plumas de mosqueros *Empidonax* capturados durante el anillado de aves en el sureste de Utah entre 2013 y 2017, y generamos códigos de barras de ADN COI para estos individuos. Si bien esperábamos observar numerosas incongruencias debido a la similitud fenotípica de estas especies, encontramos que las identificaciones de campo y moleculares coincidieron para el 92.7% de los individuos examinados. Esto sugiere que los mosqueros *Empidonax* a menudo pueden ser identificados con precisión en el campo cuando las personas que anillan utilizan datos morfológicos cuidadosamente recolectados en sus evaluaciones (longitud alar (sin cola), y cálculos basados en mediciones específicas de las plumas). Nuestros resultados destacan la importancia de utilizar datos morfométricos detallados durante el anillado de mosqueros *Empidonax*, indicando que este tipo de mediciones adicionales puede conducir a un alto grado de precisión en las identificaciones en mano de *Empidonax*. Sin embargo, también mostramos el valor de las identificaciones moleculares para complementar las identificaciones de campo en casos de individuos desconocidos, especies raras o especies estrechamente relacionadas con rangos geográficos superpuestos.

Palabras clave: anillado de aves, código de barras de ADN, identificación de especies, morfometría, mosqueros *Empidonax*, ornitología, plumas

INTRODUCTION

Avian extinctions and population declines over recent decades underscore the critical importance of bird research and conservation efforts moving forward (e.g., Rosenberg et al. 2019, Develey and Phalan 2021, Burns et al. 2021, Şekercioğlu et al. 2023, Kittelberger et al. 2024). North America has experienced a net loss of over 2.9 billion birds over the past half-century, including many common species that play key roles in food webs and ecosystem functions (Rosenberg et al. 2019). These declines highlight the need for long-term bird monitoring programs, such as bird banding, to better understand and mitigate future losses (Şekercioğlu et al. 2025), especially in migratory birds (Horns and Şekercioğlu 2018) and those living in climate-sensitive habitats (Neate-Clegg et al. 2022). Bird banding provides a minimally invasive means of tracking populations over time by capturing birds, recording morphological and life history data, and marking individuals with uniquely numbered leg bands (Benson 2017), enabling mark–recapture analyses (Cooch and White 2026). Accurate species identification is crucial for studying avian population dynamics through banding, and when the identification of sister lineages is challenging due to phenotypic similarity, mistakes in identification can undermine efforts to effectively monitor population trends.

Flycatchers in the genus *Empidonax* (Passeriformes: Tyrannidae) are frequently perceived by both birders and bird banders to be one of the most challenging songbird lineages to identify in North America due to interspecific phenotypic similarities (Phillips et al. 1966, Johnson and Cicero 2002, Heller et al. 2016, Lee 2023). This group of insectivorous passerines comprises 14 currently recognized species (Winkler et al. 2020), typically characterized by dull olive, green-yellow, or gray plumage, along with eye-rings and 2 distinct wing bars (Sibley 2014, Lee 2023). *Empidonax traillii* (Willow Flycatchers) and *E. alnorum* (Alder Flycatchers) were formerly both considered to be a single species, *E. traillii* (Traill's Flycatcher), and are very similar in appearance (Sibley 2014). In addition, *E. oberholseri* (Dusky Flycatchers) are phenotypically similar to *E. hammondii* (Hammond's Flycatchers) and *E. minimus* (Least Flycatchers) (Sibley 2014). Although experienced North American birders may distinguish the 10 *Empidonax* species that breed north of Mexico by their vocalizations (Lee 2023), this approach is often not possible during bird banding when the birds are normally silent. Therefore, species-level *Empidonax* identification during banding generally relies on detailed body characteristics and measurements, such as plumage, feather and tail morphologies, and bill color, size, and shape (Pyle 2022). However, identification may not always be precise due to individuals deviating from typical patterns, interspecific overlapping in morphometric measurements, and bander-induced measurement biases/inaccuracies (Pyle 2022, Lee 2023). Furthermore, obtaining additional measurements for species-level identification may not always be feasible during banding due to the extra time required to comprehensively assess each individual (Colorado 2010).

Molecular identification with DNA barcoding offers an alternative source for identifying cryptic taxa such as *Empidonax* flycatchers caught during bird banding (Hebert et al. 2004, Tavares and Baker 2008, Heller et al. 2016). DNA barcoding uses short, standardized gene sequences as references to determine the species identity of organisms (Hebert et al. 2003, 2004). The mitochondrial cytochrome *c* oxidase subunit I (COI) region is a commonly used region for DNA barcoding animal taxa, including birds (Hebert et al. 2003, 2004, Tavares and Baker 2008, Khan et al. 2023). Avian studies focusing on DNA barcoding usually rely on blood (e.g., Bilgin et al. 2016, Pulgarín-R et al. 2021), feather (e.g., de la Hera et al. 2020), or tissue samples (e.g., Colihueque et al. 2021) as molecular sources. While blood samples yield higher amounts of DNA than feathers, they can be time-intensive to collect, require significant training for the bander, and often require additional permits that are increasingly difficult to obtain (Harvey et al. 2006). Given that collecting blood or tissue samples is not always practical, feather samples obtained while banding may present a viable alternative for molecular information, and many studies have found success using feather samples (e.g., Heller et al. 2016, Mendoza et al. 2016, de la Hera et al. 2020, Peters et al. 2020).

As most bird banding stations rely solely on phenotypic and, depending on the species, morphometric information to identify birds in the field, it is important to know how often in-hand vs. molecular identifications align for cryptic and challenging songbird lineages. In this study, we generated short COI DNA barcodes (~400 base pairs [bp]) from blood and feather samples collected from *Empidonax* flycatchers captured during bird banding operations in southeastern Utah from 2013 to 2017 to determine the congruence of field and molecular identifications of these phenotypically similar species. We expected to see numerous incongruencies, especially between *E. oberholseri* and *E. hammondii*, which are similar in appearance and are regularly found in southeastern Utah (Sibley 2014, Lee 2023). Our study provides useful information to help inform future bird banding efforts in North America, where different species of *Empidonax* flycatchers may be captured.

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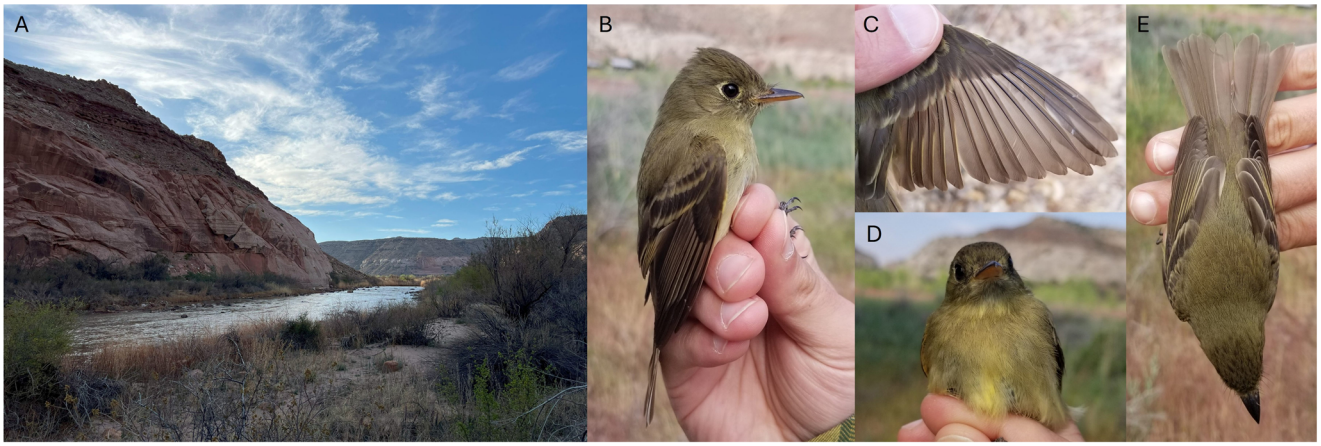


FIGURE 1. (**A**) Dolores River and riparian landscape of our banding site at the Bonderman Field Station at Rio Mesa. Sixteen mist nets are set up along this riparian corridor each spring and fall banding season. Photo by Amy Buxton. (**B–E**) An adult (definitive cycle alternate plumage), unsexed *E. difficilis* individual caught at Rio Mesa. These images represent some of the features that should be considered when determining species identification. Photos by Kyle Kittelberger. (**B**) Profile view depicting important features including overall size and shape, coloration, head shape, eye-ring, and wing bars. The crest and teardrop eye-ring help distinguish this species (Lee 2023). (**C**) Wing view. (**D**) Front view. The orange lower mandible is another characteristic that banders can look for when identifying this species (Lee 2023). (**E**) Top view, including tail.

METHODS

Study Site

The blood and feather samples included in this study were collected at the Bonderman Field Station at Rio Mesa (hereafter Rio Mesa), a restricted-access research station located in Grand County, Utah, owned and operated by the University of Utah (Kittelberger et al. 2022, 2025). Situated within the Colorado Plateau, the station is positioned on a canyon floor at an elevation of 1,280 m above sea level, through which the Dolores River flows (Kittelberger et al. 2022, 2025) (Figure 1A). The field station lies within the western or Pacific avian flyway (La Sorte et al. 2014, Rosenberg et al. 2019). Bird banding at Rio Mesa has typically been conducted each spring (generally from early April through early June) and fall (generally from mid-August through early November) since 2011. *Empidonax* species banded at Rio Mesa consist of *E. difficilis* (Western Flycatcher), *E. oberholseri*, *E. wrightii* (Gray Flycatcher), *E. hammondii*, *E. minimus*, and *E. traillii*.

Banding and Sample Collection

Each season, 16 mist nets with 38-mm mesh (approximately 12-m wide × 3-m tall) were erected at consistent locations within the riparian habitat (Kittelberger et al. 2022, 2025). The nets were typically opened 30 min before sunrise and remained open for 6 hr, with checks conducted at approximately 30-min intervals; however, adjustments to the schedule were occasionally made due to weather conditions (Kittelberger et al. 2022, 2025). Captured birds were fitted with a leg band, and 1–2 tail feathers, along with a few breast feathers, were typically collected (for genetic and isotopic analyses). Feather samples were placed in individual envelopes and temporarily stored in a freezer before being moved to a 4°C cold room at the end of each field season. Occasionally, banders also collected blood samples via brachial venipuncture following standard protocols. Twenty to 50 µL of blood (<0.2% of the bird's body weight) was collected from the brachial vein of each bird using a 30-gauge needle. Blood samples were blotted onto blotter paper, which was put into envelopes and stored in the same cold room as the feathers.

For each bird, banders recorded critical information including the presumed species (henceforth "field identification"), age, and sex, as well as morphological measurements such as wing chord and mass (Supplementary Material Table S1). In addition to these standard data, banders generally filled out The Institute for Bird Populations' "Data sheet for *Empidonax* Flycatchers" when banding individuals from this genus (included as Supplementary Material Figure S1 with permission from The Institute for Bird Populations; see also Supplementary Material Table S1). Additional data included morphometric measurements such as tail length and bill width, calculations such as wing minus tail and longest primary feather minus longest secondary feather, and additional morphological observations such as eye-ring shape and wing bar color (Figure 1, Supplementary Material Figure S1). With these data recorded, banders were encouraged to compare their measurements and calculations to those found in Pyle (1997) when determining field identification.

In 2023, 2 *Empidonax* species—*E. occidentalis* (Cordilleran Flycatcher) and *E. difficilis* (Pacific-slope Flycatcher)—were lumped as *E. difficilis* (Western Flycatcher) (Chesser et al. 2023). Our analysis thus treated one individual previously identified in-hand as *E. occidentalis* under *E. difficilis* following this latest classification. While banders were identified by their initials during the banding process, we have replaced bander initials with unique identifiers to ensure anonymity (Table 1 and Supplementary Material Table S1).

DNA Extraction

Calami from the feather samples were cut into ~1-mm pieces and placed in 1.5 mL microcentrifuge tubes. Tail feathers were preferentially used for DNA extraction, while breast feathers were utilized for samples collected in 2013, when no tail feathers were sampled. DNA was extracted from 103 samples of blood ($n = 9$), breast feathers ($n = 10$), or tail feathers ($n = 84$), representing 96 *Empidonax* flycatcher individuals, using a QIAGEN DNeasy Blood and Tissue Kit, following the manufacturer's protocol. To assess extraction success, DNA concentrations were measured for 9 of these extracted samples using a Qubit Fluorometer (Thermo Fisher Scientific) (Supplementary Material Table S2).

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TABLE 1. Unmatched *Empidonax* flycatcher individuals captured at Rio Mesa. Terminals refer to each processed sample of blood, tail feather, or breast feather in Figure 2. Field and genetic identifications use species' alpha codes: DUFL = Dusky Flycatcher (*E. oberholseri*), WIFL = Willow Flycatcher (*E. traillii*), GRFL = Gray Flycatcher (*E. wrightii*), HAFL = Hammond's Flycatcher (*E. hammondii*), WEFL = Western Flycatcher (*E. difficilis*), LEFL = Least Flycatcher (*E. minimus*), and UNEM = unidentified *Empidonax* Flycatcher. Each sample has an anonymous bander ID, season, year, and an indication of whether a "Data sheet for *Empidonax* flycatchers" from the Institute for Bird Populations was filled out for that individual.

Terminal	Field identification	Genetic identification	Tissue type	Bander ID	Season	Year	Empid Sheet
Empidonax_HBEX10	DUFL	GRFL	Tail feather	H	Fall	2016	No
Empidonax_HBEX16	WIFL	GRFL	Blood	C	Fall	2015	Yes
Empidonax_HBEX20[a]	DUFL	GRFL	Tail feather	D	Fall	2015	Yes
Empidonax_HBEX38	GRFL	DUFL	Tail feather	I	Spring	2014	Yes
Empidonax_HBEX42[a]	DUFL	GRFL	Blood	D	Fall	2015	Yes
Empidonax_HBEX70	UNEM	DUFL	Tail feather	G	Fall	2016	Yes
Empidonax_HBEX135	WIFL	HAFL	Tail feather	G	Fall	2016	Yes
Empidonax_HBEX157	DUFL	LEFL	Tail feather	A	Spring	2015	Yes

[a]Empidonax_HBEX20 and Empidonax_HBEX42 represent different samples (tail feather and blood) from the same individual.

DNA Amplification of Barcoding Region

Amplification of the COI marker was performed using polymerase chain reaction (PCR) methods. We followed the primer design of Schäffer et al. (2017), which includes 2 overlapping primer sets: (1) A2f2 + A2r2 and (2) A4f + A4r (A2f2: 5′-GNGCNCCNGAYATRGCNTTYCC-3′ and A2r2: 5′-CNGCNAGRTGNAGNGARAARATNGC-3′ and A4f: 5′-CNTCNATCCTNGGNGCAATYAAC-3′ and A4r: 5′-GCNGGGTCRAAGAANGTNGTGTT-3′). These primers target neighboring regions of the COI marker, each yielding amplicons of ∼150 bp. In our study, we utilized the forward primer of the first set (A2f2) and the reverse primer of the second set (A4r) in combination to generate a single amplicon of approximately 400 bp, under an annealing temperature of 48°C.

PCR reactions were conducted in a 25 µL PCR volume, consisting of 2.5 µL of 10X PCR Buffer, 1.25 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.5 µL of each primer (A2f2 and A4r), 0.1 µL of Platinum Taq Polymerase, 12.15 µL of H2O, and 7.5 µL of template DNA. PCR conditions, adapted from Schäffer et al. (2017), were as follows: initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. Gel electrophoreses were employed to verify the success of PCR amplification of the targeted COI region, using 4 µL PCR product, 6 µL TAE Buffer, and 2 µL loading dye for each sample; samples were referenced with a Quick-Load 100-bp ladder. PCR cleanup was performed using ExoSAP-IT PCR Cleanup Reagent, diluted 1:2 with 50 mM Tris HCl, and reactions were incubated at 37°C for 15 min followed by 80°C for 15 min.

DNA Sequencing

DNA sequencing was performed using traditional Sanger sequencing methods and the optimized protocol for the BigDye Terminator v.3 Cycle Sequencing Kit following the manufacturer's instructions. Sequencing reaction cleanup was performed using ethanol precipitation. Sequence reads were obtained with the assistance of the Genomics Core Facility (Health Sciences Cores at the University of Utah). When available, both forward and reverse reads were combined to produce the most comprehensive and accurate sequence for each sample. In cases where only forward or reverse sequences were successfully obtained, the available sequence was used in the absence of its complementary read.

Phylogenetic Analysis

We identified *Empidonax* flycatchers to species level using phylogenetic analyses to estimate relationships among sampled individuals and a set of reference sequences. We employed a variety of strategies to address issues with the quality of some DNA barcodes obtained for our study (see Supplementary Material File S2). To complement our dataset, additional *Empidonax* sequences were downloaded from the Barcode of Life Database (BOLD) (Ratnasingham et al. 2024) and included to assess whether molecular data corroborated the existing taxonomic identifications or revealed potential topological conflicts. We retained the original BOLD taxonomic IDs to evaluate congruence with our sequences. Only a subset of BOLD sequences had associated images or voucher data; these were inspected where available, but the remainder of the dataset was analyzed as provided. Sequences were obtained for *E. traillii*, *E. hammondii*, *E. oberholseri*, *E. wrightii*, *E. minimus*, *E. alnorum*, *E. fulvifrons* (Buff-breasted Flycatcher), *E. flaviventris* (Yellow-bellied Flycatcher), *E. virescens* (Acadian Flycatcher), *E. difficilis*, and *E. occidentalis* (see Supplementary Material File S3). Sequences from *Lathrotriccus euleri* (Euler's Flycatcher) were included as outgroups to provide a reference for tree rooting and comparison (Supplementary Material File S4). This species was previously placed in *Empidonax*, with the genus recently recovered as a sister taxon to a clade containing *Empidonax*, *Contopus*, *Mitrephanes*, and *Sayornis* (Ohlson et al. 2008).

Sequence alignments for all datasets were conducted using MAFFT v7.130b with optimized parameters (Katoh and Standley 2013). All phylogenetic reconstructions were conducted in IQ-Tree v2.1.3 (Minh et al. 2020). Initially, we conducted a phylogenetic analysis using the GTR model of nucleotide evolution on the BOLD dataset alone, with 1,000 ultrafast bootstrap (UFB) replicates (Hoang et al. 2018) to gain preliminary insights into *Empidonax* species relationships. We then merged our *Empidonax* dataset with the BOLD data. To verify species placement, we performed an initial phylogenetic reconstruction with all samples. In doing so, we identified multiple poorly aligned regions and gap-only columns, which were subsequently trimmed using GBlocks v0.91b with reduced stringency parameters (b1:0.5, b2:0.5, b3:12, b4:7) (Talavera

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and Castresana 2007). We then aligned the BOLD data along with our short DNA barcodes, recognizing the need for further end trimming due to length disparities (full COI barcoding amplicons from BOLD sequences compared to our short DNA barcodes of ~400 bp) and removal of potential pseudogenes, a product of some BOLD sequences. Pseudogenization can directly interfere with assigning molecular identifications to closely related taxa, especially in cases of cryptic taxa such as *Empidonax*. An effective approach to recognizing pseudogenes is to assess phylogenetic placement. Therefore, our final alignment, including both the BOLD sequences and our datasets, was meticulously trimmed to improve quality (Supplementary Material File S5). All alignments are available in the supplemental material (Supplementary Material Files S2 and S5). We then conducted phylogenetic inference using the optimized alignment (Supplementary Material File S6). For the complete dataset, ModelFinder2 within IQ-Tree (Kalyaanamoorthy et al. 2017) was used to select the best-fit model for each locus based on AICc. Phylogenetic relationships were inferred and branch support evaluated with 1,000 UFB replicates (Hoang et al. 2018) and 1,000 replicates of the branch-based, Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT) (Guindon et al. 2010).

RESULTS

Field Identification

Initial field identifications for the 96 banded individuals included in our study between 2013 and 2017 were as follows: *E. oberholseri* ($n = 63$), *E. wrightii* ($n = 13$), *E. traillii* ($n = 9$), *E. hammondii* ($n = 8$), and *E. difficilis* ($n = 2$), as well as one unidentified bird left at genus level (Supplementary Material Table S1). Banders captured 37 of these birds in the fall and 59 in the spring (Supplementary Material Table S1). The individuals were aged as "hatching year" ($n = 15$), "after hatching year" ($n = 21$), "second year" ($n = 14$), "after second year" ($n = 41$), and "unknown" ($n = 5$) (Supplementary Material Table S1).

DNA Extraction, Amplification, and Sequencing

We successfully extracted, amplified, and sequenced DNA for 103 samples (9 blood, 84 tail feathers, and 10 breast feathers), representing 96 *Empidonax* individuals (Supplementary Material Table S1). Among the samples included in our study, blood samples yielded 53–132 ng μL^{-1}, tail feather samples yielded 0.228–0.712 ng μL^{-1}, and breast feather DNA concentrations were too low to be measured with a high-sensitivity double-strand DNA (HS dsDNA) Qubit kit (Supplementary Material Table S2). We completed gel electrophoresis for all samples to confirm that PCR reactions successfully amplified the desired region of the COI marker. All sequence data can be accessed on GenBank (Accession Numbers PV876626–PV876728; see also Supplementary Material Table S1).

Molecular Identification

Our phylogenetic reconstructions reveal a generally well-supported topology within the genus *Empidonax*, with nearly all nodes exhibiting high statistical support (UFBoot ≥90, SH-aLRT ≥95) (Figure 2). Our outgroup, *L. euleri*, was distinctly recovered from the *Empidonax* ingroup, further reaffirming its placement as a distinct lineage.

We recovered *Empidonax* as strongly monophyletic (99.9/100), with a key finding being the recovery of sister relationships to all *Empidonax* taxa included in our study. Sister to all remaining *Empidonax* taxa was a clade composed of a BOLD sequence identified as *Empidonax traillii extimus* (Southwestern Willow Flycatcher, an endangered subspecies) and a set of BOLD sequences identified either as *E. alnorum* or *E. flaviventris*. Internally, we recovered a BOLD sequence identified as *E. virescens* sister to, with low support, a moderately supported clade of BOLD sequences identified as *E. flaviventris*, and shallower, a clade of *E. difficilis* (mix of newly generated plus BOLD sequences) sister to *E. occidentalis* (represented by 2 BOLD sequences) with very short branches. Another clade includes sequences attributed to *E. traillii* and *E. alnorum*, which, despite being considered separate species in modern taxonomy, were grouped together in our analysis. In our tree, these sequences cluster closely together in a well-supported clade (94/98) (Figure 2).

Following this clade, the next major group includes 3 BOLD sequences identified as *E. fulvifrons*, sister (with considerable support, 87.3/91) to the remaining *Empidonax* included in our analyses (*E. wrightii*, *E. minimus*, *E. hammondii*, *E. affinis*, and *E. oberholseri*). *Empidonax wrightii* was recovered as a well-supported clade (99.3/100), with DNA barcodes from both our collections and BOLD clustered, reinforcing *E. wrightii* as a monophyletic assemblage. Still within that major clade, *E. minimus* (100/100), *E. hammondii* (99.6/100), and *E. oberholseri* (90.6/97) are all supported by strong node values (Figure 2). Within the clade of *E. oberholseri*, we recovered one BOLD sequence originally identified as *Empidonax affinis* (Pine Flycatcher), probably a product of field misidentification (GBMIN134025-17_E_affinis_KT806384).

The relationships within the clade containing *E. minimus*, *E. hammondii*, *E. affinis*, and *E. oberholseri*, especially regarding the placement of *E. minimus* as the sister of *E. hammondii*, E. *affinis*, and *E. oberholseri*, are supported by lower internal support, suggesting some uncertainty about the exact placement of *E. minimus* (Figure 2).

Congruency Between Field and Genetic Identification

Of the 96 individuals successfully sequenced, 89 individuals (92.7%) had matching field and genetic identifications (hereafter "matched individuals"), while 6 individuals had unmatched field and genetic identifications and 1 individual that was left unidentified in the field was identified genetically (all hereafter "unmatched individuals") (Tables 1 and 2 and Supplementary Material Table S1). The 7 unmatched individuals were banded by 6 different banders, meaning there was only one instance of a bander banding 2 unmatched individuals (Table 1). One individual originally identified in the field as Cordilleran Flycatcher (*E. occidentalis)* was identified as Pacific-Slope Flycatcher (*E. difficilis)* using DNA barcoding information (Figure 2). Since Cordilleran and Pacific-Slope Flycatchers have been combined under *E. difficilis* (Chesser et al. 2023), we gave this specimen a "matched" designation for the purpose of our analysis.

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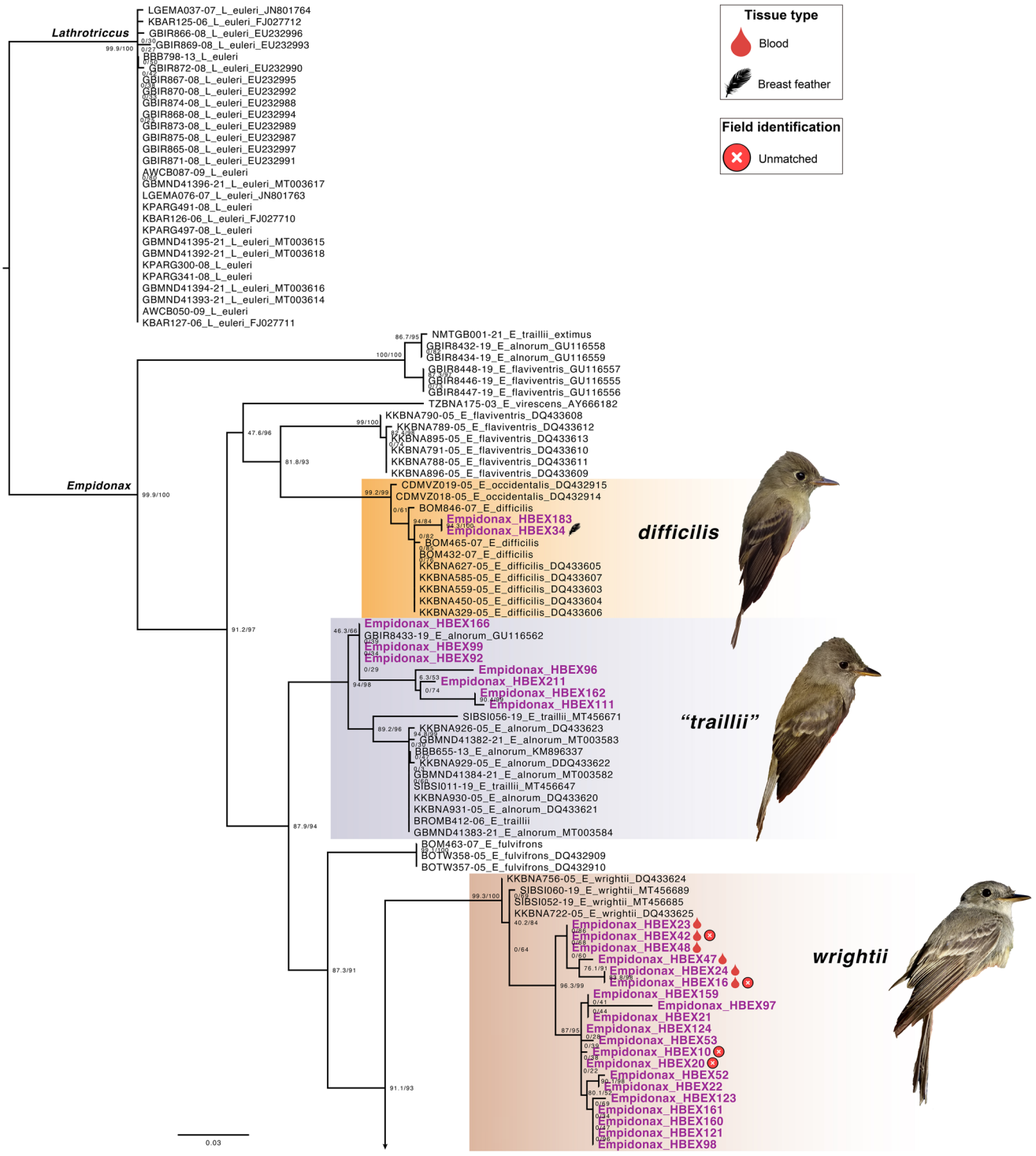


FIGURE 2. Maximum-likelihood phylogenetic tree of *Empidonax* species, inferred from COI short barcoding sequences (~400 bp) using IQ-Tree2. This tree was rooted using sequences from the closely related genus *Lathrotriccus* serving as an outgroup. Terminals in purple represent newly generated sequences (HBEX codes refer to extraction codes), integrated with previously published data obtained from BOLD. Node support values are displayed as UFB/SH-aLRT, with values near 100/100 indicating strong support. Symbols at terminal nodes denote the tissue type used for DNA extraction, including blood and/or breast feathers. For terminals without symbols, tail feathers were used as the source for DNA extractions. Unmatched symbols indicate disagreement between DNA barcoding placement and field identifications. See Supplementary Material for specimen information and DNA matrices. *Empidonax traillii* is in quotation marks due to conflict between *E. alnorum* and *E. traillii* sequences (see text for additional information). Bottom portion of the tree is depicted on the following page. Photos by Kyle Kittelberger and Liz Allocca.

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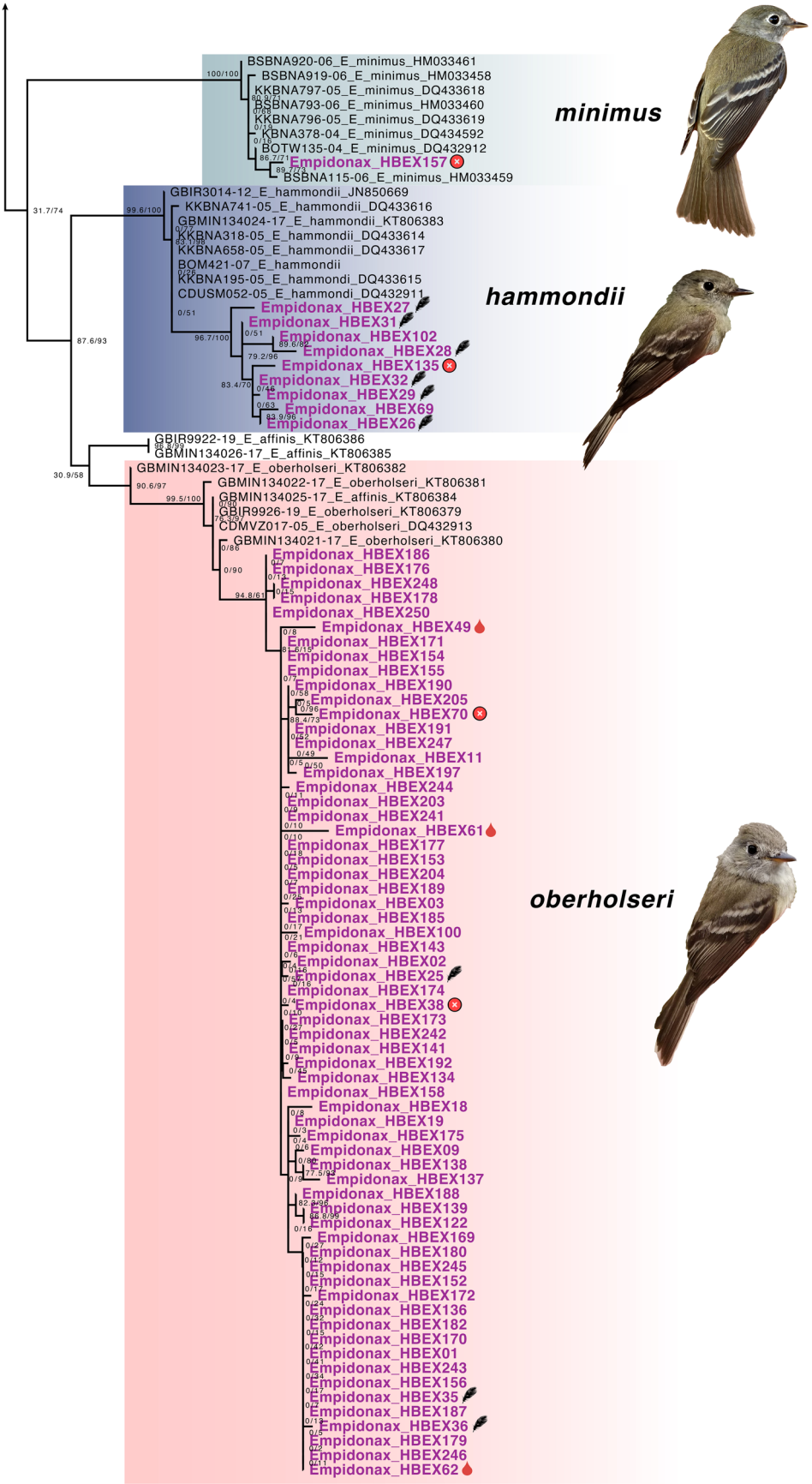


FIGURE 2. (Continued). Bottom portion of the maximum-likelihood phylogenetic tree of *Empidonax* species, inferred from COI short barcoding sequences (~400 bp) using IQ-Tree2.

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TABLE 2. Number of matched and unmatched *Empidonax* flycatchers with each field identification. For unmatched individuals, genetic identification is also given (in parentheses). Identifications use species' alpha codes: DUFL = Dusky Flycatcher (*E. oberholseri*), WIFL = Willow Flycatcher (*E. traillii*), GRFL = Gray Flycatcher (*E. wrightii*), HAFL = Hammond's Flycatcher (*E. hammondii*), WEFL = Western Flycatcher (*E. difficilis*), LEFL = Least Flycatcher (*E. minimus*), and UNEM = unidentified *Empidonax* Flycatcher.

Field identification	Number of matched individuals	Number of unmatched individuals	Total number of individuals
DUFL	60	3 (GRFL, GRFL, LEFL)	63
GRFL	12	1 (DUFL)	13
HAFL	8	0	8
UNEM	0	1 (DUFL)	1
WEFL	2	0	2
WIFL	7	2 (GRFL, HAFL)	9
Total	89	7	96

DISCUSSION

We investigated how often in-hand field identifications are congruent with molecular identifications for *Empidonax* flycatchers, which represent some of the most challenging songbird species to identify in North America (Phillips et al. 1966, Johnson and Cicero 2002, Heller et al. 2016, Lee 2023). Based on their phenotypic similarity, we expected to see many incongruencies between the 2 identification techniques for these birds. However, we found that identifications based on the 2 methods agreed for 89 out of 96 individuals examined (92.7%). Recent studies have found DNA barcoding to be a highly effective tool for identifying birds across wide geographic regions, with notable identification rates in areas including North America (94%), the Japanese Archipelago (97%), Argentina (98%), Chile (95%), Colombia (86%-92%), and the Neotropics as a whole (93%) (Kerr et al. 2007, 2009, Tavares et al. 2011, Saitoh et al. 2015, Mendoza et al. 2016, Colihueque et al. 2021). While these studies compare DNA barcodes of a wide range of species within the same region, DNA barcoding techniques can also help identify phenotypically similar species that may be difficult to identify morphologically (e.g., Heller et al. 2016, de la Hera et al. 2020). For example, one study used DNA barcoding to molecularly identify and differentiate *Phylloscopus ibericus* (Iberian Chiffchaffs, Phylloscopidae) from the phenotypically similar *P. collybita* (Common Chiffchaffs), from which they were taxonomically split 2 decades prior (de la Hera et al. 2020). The authors found that 78% of individuals molecularly identified as *P. ibericus* and 72% of individuals molecularly identified as *P. collybita* were congruently identified in the field (de la Hera et al. 2020). Our study of phenotypically similar *Empidonax* species yielded a much higher congruence (92.7%), suggesting that, despite their similarities, many North American *Empidonax* species can be identified successfully in the field with careful consideration of multiple morphometric and phenotypic characteristics (Phillips et al. 1966, Pyle 2022).

This high level of congruence, which differed from our initial prediction, is likely due largely to the banders at Rio Mesa taking careful, detailed morphometric measurements when banding *Empidonax* flycatchers. Accurate phenotypic identification of *Empidonax* flycatchers depends on multiple field marks collectively, so taking additional measurements greatly increases accuracy (Lee 2023, Pyle 2022). Collecting information on bill size, shape, and lower mandible color in conjunction with other morphological data for initial species identification, followed by interpretation of individual species accounts, has been suggested as an important approach for field identification (Pyle 1997, 2022). Key morphological data to record include wing and tail lengths, bill length and width, whether p6 (primary 6) is emarginated, as well as several calculated measurements: wing minus tail length and the differences in length between certain primary and/or secondary feathers in the wing (Pyle 2022). Our study shows that banders who collect this data are likely to make correct in-hand identifications of individuals from this otherwise challenging-to-identify genus. Carefully collecting, recording, and considering these traits to aid in field identification should be standard practice when banding *Empidonax* flycatchers, and we recommend that banders use the "Data sheet for *Empidonax* Flycatchers" published by the Institute for Bird Populations (included as Supplementary Material Figure S1 with permission from The Institute for Bird Populations).

Despite the overall high level of congruence, there remained several incongruencies between field and molecular identification in our study. Two individuals identified in the field as *E. oberholseri* were identified genetically as *E. wrightii*, and one individual identified in the field as an *E. wrightii* was identified genetically as an *E. oberholseri* (Figure 2, Tables 1 and 2). Banders did not fill out an *Empidonax* data sheet for one of these individuals, which could help explain this mismatch (Table 1, Supplementary Material Table S1). The other 2 individuals each had associated *Empidonax* data sheets, but the morphometric measurements/calculations did not perfectly align with the expected ranges predicted in Pyle (2022) for either *E. oberholseri* or *E. wrightii*. While this is not unexpected—Pyle (2022) suggests that measurements may not always align—this does make identification more difficult for certain individuals. We recommend that banders are trained to take careful and accurate measurements, as human error is one possible cause of these discrepancies (Pyle 2022). We also suggest that banders look for other diagnostic features when making their field identification. For example, for one of the individuals identified in the field as *E. oberholseri* and identified genetically as *E. wrightii*, the banders left a note that this individual had a black-tipped bill. This dark tip can help distinguish *E. wrightii* from other *Empidonax* species (Sibley 2014, Pyle 2022) and likely should have played a larger role in the field identification of this individual. Another distinguishing characteristic of *E. wrightii* is the distinct white edging on its outer tail feathers (Sibley 2014, Pyle 2022, Lee 2023). While this trait is not featured on the *Empidonax* data sheet, banders should be aware of its potential to aid in field identification (Sibley 2014, Pyle 2022, Lee 2023). Another individual in our study was originally identified as an *E. oberholseri* but determined to be an *E. minimus* through molecular analysis (Figure 2, Tables 1 and 2). While *E. minimus* has shorter tails than *E. oberholseri* on average (Sibley 2014, Pyle 2022, Lee 2023), this individual's tail length was in the upper part of the expected range for *E. minimus* and in the lower part of the expected range for *E. oberholseri*, making them particularly difficult to identify (Pyle 2022). This highlights the importance of using multiple field marks in combination when identifying *Empidonax* flycatchers (Pyle 2022, Lee 2023); for example, another trait that can help distinguish *E. minimus* from *E. oberholseri* is their convex bill (Pyle 2022). Finally, 2

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individuals identified as *E. traillii* in-hand were molecularly identified as *E. hammondii* and *E. wrightii*, respectively (Figure 2, Tables 1 and 2). In both cases, eye-ring may have been an important and overlooked clue for identification. *Empidonax traillii* tend to have a weak or absent eye-ring (Sibley 2014, Pyle 2022, Lee 2023), while Pyle (2022) notes that *E. hammondii* have a complete white, somewhat almond-shaped eye-ring, and *E. wrightii* have a complete white, round eye-ring. For these 2 individuals, the banders did not select either "lacking" or "indistinct" for the eye-ring shape on the *Empidonax* data sheet, instead selecting "teardrop" for the individual genetically identified as *E. hammondii*, and "round" for the individual genetically identified as *E. wrightii* (Supplementary Material Figure S1). Both banders additionally selected "white" for eye-ring color (Supplementary Material Figure S1). Eye-ring shape and color therefore provided important information that could perhaps have been considered more fully in the identification of these individuals.

While our findings indicate that careful in-hand identification during bird banding is generally sufficient for *Empidonax* flycatchers, collecting blood or feather samples for molecular analysis can further support the identification of undetermined individuals or rarely captured species. Molecular identification techniques have been used to help identify taxonomically challenging passerines (Pulgarín-R et al. 2021), including undetermined and rarely captured *Empidonax* species (Novitch et al. 2015, Heller et al. 2016, Goldberg and Mason 2017). Heller et al. (2016) molecularly identified an undetermined *Empidonax* flycatcher in Virginia as an *E. oberholseri*, far outside of its expected normal range. Likewise, Goldberg and Mason (2017) used molecular techniques to identify 2 vagrant flycatchers in northeastern North America as Pacific-slope Flycatchers (now Western Flycatchers, *E. difficilis*). In our study, we used DNA barcoding to identify an *E. minimus*, a species rarely banded at Rio Mesa between 2011 and 2024 ($n = 5$), that had been identified in the field as an *E. oberholseri*, a species much more commonly banded there ($n > 275$) (Table 1). We also used DNA barcoding to identify an *E. oberholseri* that banders had been unable to identify to species level in the field (Table 1). From 2011 to 2024, banders at Rio Mesa banded 44 unidentified *Empidonax* flycatchers and 7 additional unidentified flycatchers. These 51 individuals represent important candidates for molecular identification and highlight that banding stations should consider implementing protocols to take samples from unknown individuals to identify them through DNA barcoding.

Finally, molecular identification can be useful for distinguishing closely related species with overlapping geographical ranges. For example, *E. traillii* and *E. alnorum* are both members of the "Traill's Flycatcher-complex" and were considered conspecific until 1973 (Eisenmann 1973, Lee 2023). Although there are vocal and some subtle phenotypic differences, experts advise careful exploration of phenotypic and morphometric traits when distinguishing between non-vocal individuals of these 2 very similar species (Pyle 2022, Lee 2023). Novitch et al. (2015) used molecular analysis to determine whether museum specimens labeled as "Traill's Flycatcher" belonged to *E. traillii* or *E. alnorum*, unexpectedly matching several specimens to a third taxon, *E. virescens*. Novitch et al. (2015) also checked the identification of several specimens that had been previously labeled as *E. traillii* or *E. alnorum*, highlighting multiple misidentifications. In our phylogenetic analyses, *E. traillii* and *E. alnorum* clustered together (Figure 2), which raises questions about their reciprocal monophyly at commonly used diagnostic markers; perhaps these are lineages currently undergoing incomplete lineage sorting, but additional markers beyond COI sequences should be analyzed for congruency. Alternatively, our results could suggest possible misidentification of *E. traillii* and *E. alnorum* BOLD sequences. Likewise, *E. oberholseri* and *E. affinis* BOLD sequences showed some overlap in our phylogeny, which could again point to potential misidentification (Figure 2). Our findings also recovered sister relationships between *E. difficilis* and *E. occidentalis* (Figure 2), similar to what has been found in other phylogenetic reconstructions (e.g., Fjeldså et al. 2018, Johnson and Cicero 2002). These previously separate taxa were recently synonymized under *E. difficilis* (Western Flycatcher) (Chesser et al. 2023), and our recovery of a clade of *E. difficilis* as sister to *E. occidentalis* with very short branches supports this recent taxonomic update.

Conclusion

Long-term bird monitoring data, such as those collected at bird banding stations, are particularly important in light of recent population declines (Rosenberg et al. 2019), and accurate species identification is essential for obtaining valid banding data. Despite the phenotypic similarity of *Empidonax* flycatchers (Phillips et al. 1966, Johnson and Cicero 2002, Sibley 2014, Pyle 2022, Lee 2023), 92.7% of individuals in our study were congruently identified using field and molecular identification techniques. This high percentage of congruence importantly suggests that correctly identifying *Empidonax* flycatchers in the field is highly feasible and should perhaps be viewed as less of a daunting task than currently perceived by bird banders (Pyle 2022). To obtain accurate identifications, banding stations should implement rigorous field identification protocols for all *Empidonax* flycatchers, including carefully collecting morphometric data as described by Pyle (2022). In addition, stations should consider complementing field identifications with molecular identification techniques in cases of unidentified individuals, rarely caught species, or closely related species (especially ones that banders may not be familiar with), ideally applying a standard protocol for collecting feathers from all banded birds (Smith et al. 2003, Ruegg et al. 2014). As identifications derived from both the field and DNA barcoding can be subject to biases, a combination of both approaches provides additional taxonomic accuracy, which can enhance our understanding of avian population trends over time. Finally, a cautionary note is warranted when utilizing well-curated mitochondrial COI databases (e.g., BOLD, NCBI RefSeq): although sequences may derive from well-established pipelines, taxonomic identifications can nonetheless show substantial mismatches with morphology, particularly in phenotypically uniform taxa.

Supplementary material

Supplementary material is available at *Ornithology* online.

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Ethics statement

All required permitting was obtained for bird banding and data collection for this study.

Conflict of interest statement

The authors declare that the research in this study was conducted in the absence of any commercial or financial relationships that could be construed as a conflict of interest.

Author contributions

A.N.B. and R.S.P. wrote the manuscript with help from K.D.K. H.B. wrote the original version of this study. Ç.H.Ş. and H.B. conceived the original idea for the project, which was improved upon by A.N.B., R.S.P., and K.D.K. H.B. performed the molecular analysis. R.S.P. performed the phylogenetic analysis. A.N.B., R.S.P., and K.D.K. created the figures. All co-authors contributed to and gave final approval of the manuscript.

Data availability

Sequence data used in this study are available in GenBank (Accession Numbers PV876626–PV876728; see Supplementary Material Table S1). All additional data used in this study are available in the Supplementary Materials.

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