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EVIDENCE FOR CRYPTIC SPECIATION OF *LEUCOCYTOZOON* SPP. (HAEMOSPORIDA, LEUCOCYTOZOIDAE) IN DIURNAL RAPTORS

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ABSTRACT: Species of *Leucocytozoon* (Haemosporida, Leucocytozoidae) traditionally have been described based on morphological characters of their blood stages and host cells, with limited information on their avian host specificity. Based on the current taxonomy, *Leucocytozoon toddi* is the sole valid species of leucocytozoids parasitizing falconiform birds. Using a nested polymerase chain reaction protocol, we determined the prevalence of *Leucocytozoon* infection in 5 species of diurnal raptors from California. Of 591 birds tested, 177 (29.9%) were infected with *Leucocytozoon toddi*. Subsequent phylogenetic analysis of the cytochrome *b* gene revealed that distinct haplotypes are present in hawks of these genera. Haplotypes present in *Buteo* spp. are not found in *Accipiter* spp., and there is a 10.9% sequence divergence between the 2 lineage clades. In addition, *Leucocytozoon* sp. from *Accipiter* spp. from Europe group more closely with parasites found in *Accipiter* spp. from California than the same California *Accipiter* species do with their sympatric *Buteo* spp. Similarly, a *Leucocytozoon* haplotype from a Common Buzzard (*Buteo buteo*) from Kazakhstan forms a monophyletic lineage with a parasite from *B. jamaicensis* from California. These results suggest that *Leucocytozoon toddi* is most likely a group of cryptic species, with 1 species infecting *Buteo* spp. and 1 or more species, or subspecies, infecting *Accipiter* spp.

Avian blood parasites increasingly are being studied as a model system for host-parasite interactions with relevance to the fields of ecology, evolution, and conservation biology. Recently, DNA sequencing has added new information concerning host-parasite relationships among the parasites. The traditional view that the natural host range is a valid taxonomic criterion is in contention. Several groups have provided evidence for extensive host switching of the parasites belonging to the genera *Plasmodium* and *Haemoproteus* (Bensch et al., 2000; Ricklefs and Fallon, 2002; Waldenström et al., 2002; Fallon et al., 2003; Szymanski and Lovette, 2005). Moreover, there are examples of cryptic speciation in blood parasites that infect humans and lizards where several lineages of morphologically similar species constitute reproductively isolated species (Qari et al., 1993; Perkins, 2000). There is also recent evidence for cryptic speciation in avian hemosporidians (Bensch et al., 2004). In that work, parasite mitochondrial DNA haplotypes associated with unique copies of the nuclear gene dihydrofolate reductase thymidylate synthase (DHFR-TS). However, little was shown regarding how these lineages are related to traditional morphologically described species. Here we study 1 morphologically distinct species of avian blood parasite, *Leucocytozoon toddi*, and its phylogenetic relationships within its hosts, diurnal raptors of the family Accipitridae.

Haemosporidian blood parasites (Sporozoa, Haemosporida) of the family Leucocytozoidae are widespread in birds in which they undergo exoerythocytic merogony in numerous organs and tissues and can infect both red and white blood cells (Valkiūnas, 2005). Species of *Leucocytozoon* are transmitted by blackflies (Simuliidae), and pathogenicity mainly has been reported in domestic poultry (Bennett et al., 1993; Valkiūnas, 2005). First discovered by Danilewsky (1884), and subject to extensive investigation for over 100 yr, the leucocytozoids continue to present many problems of taxonomy and host specificity. As with other avian haemosporidian parasites, the concept of host-family/host-subfamily specificity for *Leucocytozoon* spp. was widely accepted in taxonomic studies in the 20th century (Desser and Bennett, 1993) but has been questioned by current studies (Valkiūnas and Ashford, 2002). The recent development of PCR methods for amplifying DNA from *Leucocytozoon* spp. has provided new information on the phylogeny of these parasites. Based on studies using the mitochondrial cytochrome *b* gene, the parasite lies basal to *Plasmodium* and *Haemoproteus* spp. (Perkins and Schall, 2002), and many lineages found in Sweden correspond to several morphological species of *Leucocytozoon* (Hellgren et al., 2004).

In the order Falconiformes, only *Leucocytozoon toddi* is deemed valid based on morphology (Greiner and Kocan, 1977; Valkiūnas, 2005). *Leucocytozoon toddi*, discovered by Sambon (1908), was redescribed by Greiner and Kocan (1977) who, based on detailed analysis of morphological and morphometric characteristics of gametocytes and their host cells, declared 7 species of leucocytozoids to be junior synonyms of this parasite. *Leucocytozoon toddi* gametocytes develop in either fusiform host cells or, less commonly, roundish host cells (Valkiūnas, 2005). A study of *L. toddi* in the Northern Sparrowhawk (*Accipiter nisus*) in Britain showed that transmission was typically from parents to their offspring (Ashford et al., 1990). Although it is still unclear which species of simuliid flies are responsible for transmission of this parasite, the studies showed that birds are likely to remain infected for life (Ashford et al., 1990).

Previous studies have shown the prevalence of *Leucocytozoon* spp. in birds of the family Accipitridae to be 31.3% in North America (Greiner et al., 1975). The prevalence of infection in the Northern Sparrowhawk and species of the genus *Circus* was recorded to be 94% and 73%, respectively, in the Palearctic (Valkiūnas, 2005). Twenty-four percent of Sharp-shinned Hawks (*Accipiter striatus*) were infected with *L. toddi* in a study of migratory birds captured in New Mexico (Smith et al., 2004). Seventeen of 19 (89%) Cooper's hawks (*Accipiter*

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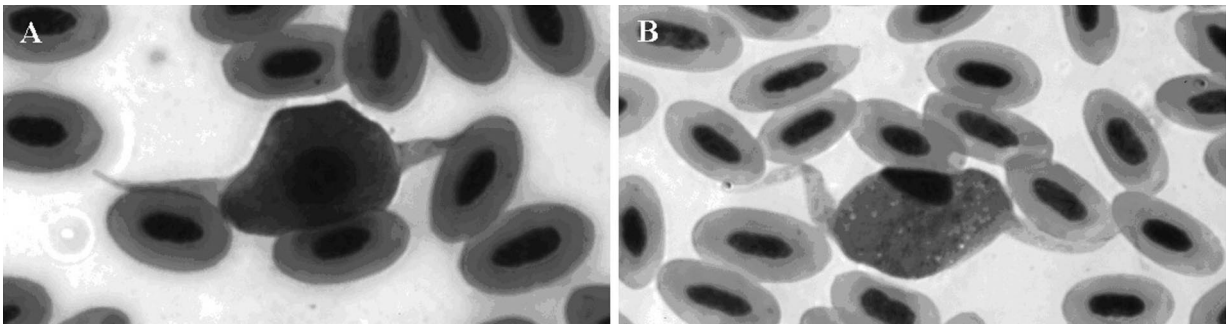

FIGURE 1. Photomicrographs of gametocytes of *Leucocytozoon* species seen in (**A**) *Accipiter cooperii* and (**B**) *Buteo jamaicensis* in California. Both host species harbor morphologically similar parasites identified as *L. toddi* based on the morphology of gametocytes and their host cells.

cooperii) were infected with *L. toddi* in a study of avian hematozoa from Wisconsin (Taft et al., 1994). All these studies relied on the detection of infection by microscopic examination of stained blood films. Here our objective was to implement PCR and DNA sequence data to determine the prevalence and phylogeography of *L. toddi* in diurnal raptors of Europe and California.

MATERIALS AND METHODS

Collection of blood samples and extraction of DNA

The blood samples used in this study were collected at the Golden Gate Raptor Observatory (GGRO) (37°40′N, 122°20′W), Sausalito, California, the Lindsay Wildlife Museum, Walnut Creek, California (37°N, 121°W), or the UC Davis Raptor Center, Davis, California (38°N, 121°W). In addition, 1 sample was collected at the Biological Station of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55°05′N, 20°44′E), and 18 from northern Kazakhstan (49°24′N, 46°48′E) in 2004. The blood samples collected from the GGRO were taken from apparently healthy active birds that were trapped with avian lures. They were captured during the autumn migration season from September to November 2003 and 2004. The samples collected from the other 2 locations in California were of birds brought in for rehabilitation after being found injured or sick, also during the fall seasons of 2003 and 2004. In Europe, the active birds were caught with mist nets and large Rybachy traps during their autumnal seasonal migration. The avian taxonomy used conforms to Sibley and Monroe (1990). Birds captured at GGRO were weighed, measured, marked with an aluminum numbered band for ongoing demographic studies, bled, and released. None of them was recaptured. Blood samples from all birds (50–100 μl) were collected from the medial metatarsal vein and stored in lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM ethylene-diaminetetraacetic acid, 2% sodium dodecyl sulphate) for subsequent molecular analysis (Sehgal et al., 2001). To obtain total DNA, the blood was extracted following a DNeasy kit protocol (Qiagen®, Valencia, California). Blood smears were made on site and air-dried and subsequently fixed in methanol and stained with Giemsa (Godfrey et al., 1987). To examine the parasites morphologically, a representative number were viewed microscopically at ×200, ×400, and ×1,000 for 20–50 min.

PCR and sequencing

Extracted DNA was used in nested PCR reactions to amplify a portion of the cytochrome *b* gene using the following methods. For the first amplification, we used primers DW2: 5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′, and DW4: 5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′, which are identical to primers described by Perkins and Schall (2002). The first PCR reaction was performed using the following conditions. Twenty-five-microliter reaction mixtures contained 10–100 ng of genomic DNA (2 μl of template DNA), 0.5 units of Qiagen Taq DNA Polymerase (Qiagen®), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl^2$, 0.4 μM of each primer, 0.4 mM of each dNTP, and 5 μl of Q buffer (Qiagen Inc., Valencia, California). The cycling profile consisted of denaturation at 94 C for 3 min, followed by 35 cycles of 94 C denaturation for 30 sec, 52 C annealing for 30 sec, and 72 C extension for 1 min. The samples were then extended at 72 C for 10 min.

For the second PCR reaction, the following primers were developed based on homology among aligned sequences of published *Leucocytozoon* cytochrome *b* sequences: LeucoF: 5′-TCTTACTGGTGTATTATTAGCAAC-3′, and LeucoR: 5′-AGCATAGAATGTGCAAATAAACC-3′. Two microliters of the first reaction were taken to seed the second reaction. The reaction conditions using the second set of primers were identical to the first round. The resulting PCR products of 865 bp were visualized on agarose gels. For evaluation of specificity, we tested the PCR protocol on raptor samples harboring known infections of the related parasite *Haemoproteus* spp., and no positive amplifications were observed.

PCR products were purified using a Qiagen® kit following the manufacturer's instructions. Bidirectional sequencing of the PCR fragments was performed using the primers LeucoF and LeucoR in an ABI Prism 377 automated sequencer (Applied Biosystems, Inc., Foster City, California). We used double-stranded cycle sequencing with dye-terminator fluorescent labeling, and electrophoresed sequenced products through a 5% Long Ranger gel. Sequences are deposited in GenBank™ with the following accession numbers DQ177235-DQ177273. Published sequences for the cyt *b* genes of *Leucocytozoon* spp. were obtained from GenBank™. Accession numbers AY684973 for *Leucocytozoon toddi* from the Frances' Sparrowhawk (*Accipiter francesii*) and AY733090 for *Plasmodium relictum* from the Amakihi (*Hemignathus virens*) are shown in Figure 2.

Phylogenetic analysis

We based our phylogenetic analysis of *Leucocytozoon* spp. on sequences (720 nucleotides) of the cytochrome *b* gene from 39 individuals, plus 1 sequence from GenBank™ (from Frances' Sparrowhawk). *Plasmodium relictum* was used as an outgroup. Phylogenetic analyses using maximum-parsimony techniques were conducted using PAUP*4.0b10 (Swofford, 2002). Searches used the bootstrap search option with 500 stepwise addition replicates using the TBR branch-swapping algorithm. In addition, we performed distance analyses using the Kimura 2-parameter distance model, and taxa were joined using neighbor-joining. Simple consensus trees were constructed to summarize the results. Genetic divergences between lineages were estimated using the distance setting HYK85, which yielded similar results as the uncorrected ''p'' setting.

RESULTS

Prevalence and morphology of *Leuocotyozoon* spp. in California diurnal raptors

Using PCR, we surveyed the prevalence of *Leucocytozoon* spp. in 8 species of birds from 3 sites in California, 1 site at the Curonian Spit (Baltics), and 1 site in Kazakhstan. Table I

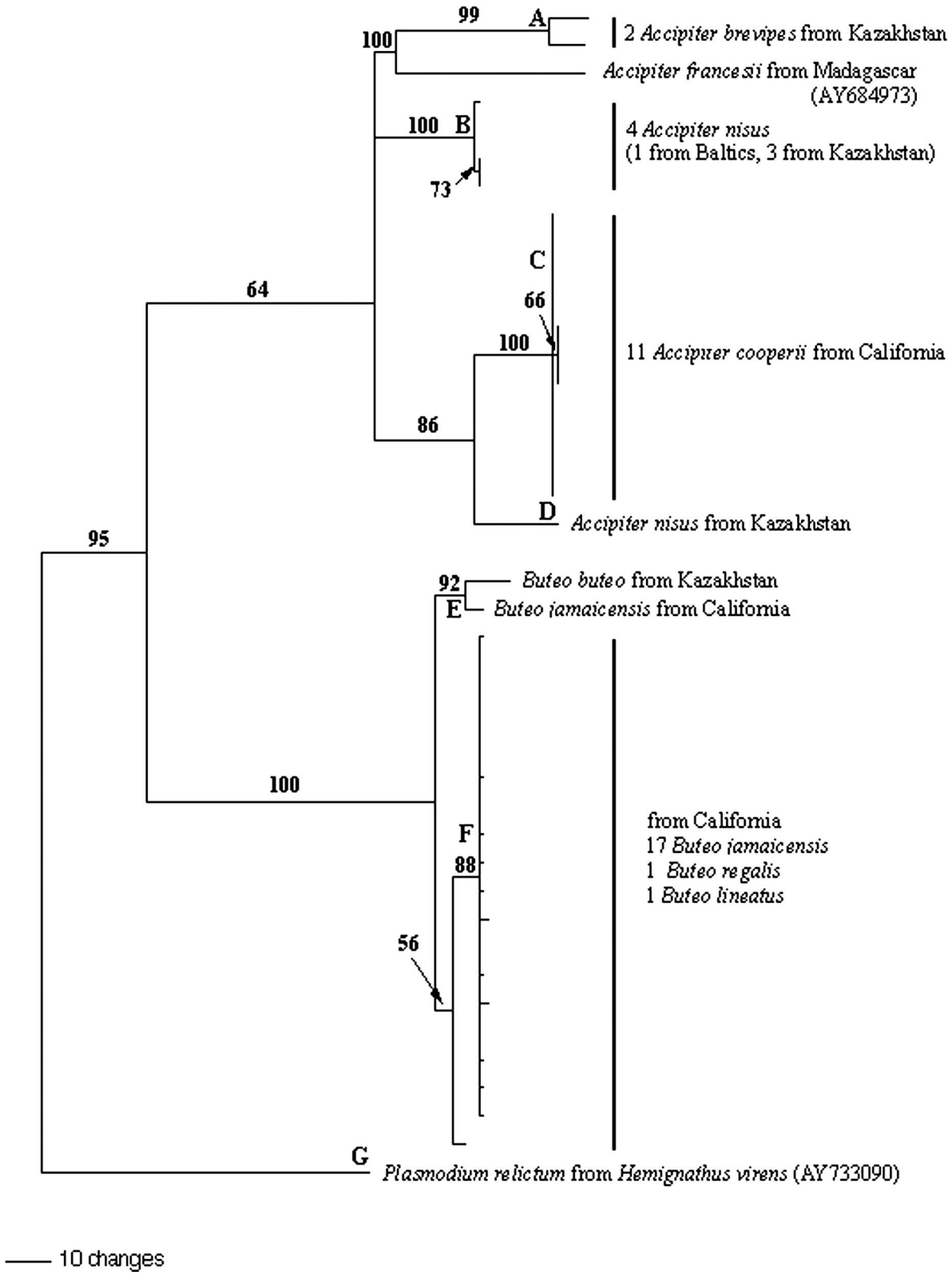

FIGURE 2. Consensus Maximum Parsimony Bootstrap tree based on sequences (720 bp) of *Leucocytozoon* spp. cytochrome *b* gene sequences from 40 individual avian hosts. The tree was rooted by *Plasmodium relictum* from the Hawaiian Amakihi (*Hemignathus virens*). Bootstrap percentages of clades (500 iterations) are shown above internal nodes. Of the 39 novel sequences, there are 22 distinct haplotypes. The clade of leucocytozoids found in birds of the genus *Accipiter* includes samples taken from California, the Baltics, Kazakhstan, and Madagascar. The clade of leucocytozoids found in species of *Buteo* includes samples from California and one from Kazakhstan. Although the *Leucocytozoon* lineages identified in *Buteo* spp. and *Accipiter* spp. are sympatric in California, they form distinct monophyletic clades. Letters A–G identify clades for comparison of genetic sequence divergence as described in the text. Distance analyses using neighbor-joining yielded an identical topology (data not shown).

TABLE I. Prevalence of *Leucocytozoon* in diurnal raptors as determined by PCR.

Species	Number tested	Number infected	Prevalence
California			
Buteo jamaicensis	458	119	26.0
Buteo lineatus	40	15	37.5
Buteo regalis	3	3	100.0
Accipiter cooperii	82	39	47.6
Circus cyaneus	8	1	12.5
Total	591	177	29.9
Kazakhstan and Baltics			
*Accipiter nisus**	12	6	50.0
Accipiter brevipes	6	5	83.3
Buteo buteo vulpinus	1	1	100.0
Total	19	12	63.2

* The one individual tested from the Baltics was infected.

lists these results. Examination of blood films revealed that the *Leucocytozoon* species found in all the California and European diurnal raptors are morphologically classified as *L. toddi*. Figure 1A shows a microphotograph taken from the blood of a Cooper's Hawk (*Accipiter cooperii*), and 1B, a parasite seen in the blood of a Red-tailed Hawk (*Buteo jamaicensis*), both from California. The gametocytes of *L. toddi* and their host cells are morphologically similar in both raptor species.

Phylogeography of *Leucocytozoon* spp. of diurnal raptors

We sequenced PCR products of the cytochrome *b* gene of *Leucocytoon* spp. from 39 infected individuals, and 23 haplotypes were identified. Phylogenetic analysis revealed 2 distinct monophyletic groups of parasites: 1 infecting *Accipiter* spp. and 1 infecting *Buteo* spp. (Fig. 2). The average genetic divergence between the 2 major clades was 10.9% (Figs. 2C–F). The percentage genetic divergences between the letters representing the clades in Figure 2 are A–F 8.5%, A–C 8.6%, B–C 8.5%, C–D 5.5%, E–F 3.5%, and F–G 20.0%. The *Leucocytozoon* species infecting *Accipiter* spp. taken from birds captured in California, the Baltics, Kazakhstan, and Madagascar grouped together. One individual *Leucocytozoon* infection obtained from the Common Buzzard (*Buteo buteo*) from Kazakhstan similarly grouped together with the *Buteo* spp. clade of *Leucocytozoon* sp. from California. Sequence divergence between *Leucocytozoon* lineages infecting the Levant Sparrowhawk (*A. brevipes*), the Cooper's Hawk, and the Northern Sparrowhawk averaged to 8.5%. The parasites of the Northern Sparrowhawk group together, even though 3 of the specimens were taken from Kazakhstan and 1 from the Baltics. Similarly, the *Leucocytozoon* sp. found in the Levant Sparrowhawk differs from those found in the Northern Sparrowhawk, even though they were taken from the same location in Kazakhstan.

DISCUSSION

PCR increasingly is becoming a common method to estimate prevalence rates and in some cases has been shown to be more sensitive and accurate than microscopy (Richard et al., 2002; Hellgren et al., 2004). The prevalence of *Leucocytozoon* sp. in California diurnal raptors as detected by PCR is within the range described in previous studies that used microscopy. In North America, Greiner et al. (1975) found 4 of 25 (16%) Red-tailed Hawks and 7 of 13 (53%) of Cooper's Hawks infected with *Leucocytozoon* sp. We found a similar prevalence of 47.6% in Cooper's Hawks (Table I). The prevalence of infection, however, was lower than that recorded in a study from Wisconsin, where 89% of the individuals tested harbored *Leucocytozoon* sp. (Taft et al., 1996). In our relatively large sample size of Red-tailed Hawks, we found a prevalence of 26%. It must be noted, however, that the prevalence of *Leucocytozoon* spp. infection can vary greatly with season and proximity to flowing streams, in which the simuliid vectors develop (Desser and Bennett, 1993; Valkiūnas, 2005).

Based on detailed analysis of morphological and morphometrical characteristics of gametocytes and their host cells, Greiner and Kocan (1977) proposed to consolidate the 7 species of *Leucocytozoon* of the Falconiformes to *L. toddi*. Since the intraspecific morphological criteria of *Leucocytozoon* spp. are variable, and the interspecific morphology of gametocytes and their host cells are frequently not taxonomically distinguishable, this appeared to be the most judicious treatment of the nomenclature and was accepted in all subsequent taxonomic studies. However, the basis of taxonomy on blood stages without information on merogonic and sporogonic stages may have underestimated the diversity of species of this genus. Sacchi and Prigioni (1982) maintained that a *Leucocytozoon* sp. found in the Common Buzzard appeared to differ from *L. toddi* and was more like the previously described *L. franchini*. Now, with sequence data, it has become evident that the nomenclature should be reassessed.

The phylogenetic reconstructions based on cytochrome *b* sequence data provide evidence that species of *Accipiter* harbor a different strain of *Leucocytozoon* sp. than species of *Buteo*. The sequence divergence of 10.9% measured between the 2 clades is much higher than the 3.1% observed between cryptic species of *Plasmodium azurophilum* found in *Anolis* spp. lizards of the eastern Caribbean (Perkins, 2000). Bensch et al. (2004) found that *Haemoproteus* haplotypes with divergences of less than 0.5% still associated with different sequences of the nuclear allele DHFR-TS, suggesting that these strains are isolated reproductively. Hellgren et al. (2004) found a sequence divergence of 8.4% between 2 lineages recorded in the same passerine host species in Sweden. Although in that study morphological descriptions of the parasite species were not available, it was suggested that the distance of 8.4% most likely reflected different parasite species.

Our data are more compelling when phylogeographic results are considered. Although the birds in California were caught at roughly the same locations, a significant difference is apparent between the *Leucocytozoon* sp. infecting species of *Buteo* and *Accipiter*. When samples from 2 distant European sites and Madagascar were included in the analysis, leucocytozoids from the *Accipiter* species grouped more closely with each other despite marked geographic distances than the parasites of the California *Accipiter* spp. clade did with the California *Buteo* spp. clade (Fig. 2). The sequence divergence between *Leucocytozoon* haplotypes found in the birds taken at the GGRO ranged from 1.4% found within the Cooper's Hawk to 10.9% found between the Cooper's Hawk and Red-tailed Hawk. Identical

haplotypes were found of *Leucocytozoon* sp. in the Red-shouldered Hawk (*B. lineatus*), the Ferruginous Hawk (*B. regalis*), and the Red-tailed Hawk (*B. jamaicensis*). In addition, the 1 sample of the Common Buzzard (*B. buteo*) in our collection harbored a *Leucocytozoon* lineage that grouped with the leucocytozoids of California *Buteo* spp. group. The sequence divergence between *Leucocytozoon* haplotypes of the Cooper's hawk and the Northern Sparrowhawk (collected in the Baltics) was 8.5%. The levels of cytochrome *b* sequence divergence that delineates species of leucocytozoids is unknown, and it is also possible that *Leucocytozoon* spp. may have different rates of evolution than other hemosporidian parasites. However, it is still striking that in one case, a parasite taken from a bird in Madagascar is more similar in sequence to one taken from a congeneric host in California than that host is to a sympatric con-familial host. We cannot rule out that there may be more species of *Leucocytozoon* in birds belonging to the Accipitridae. For example, the sequence divergence between 2 parasites found in the Levant Sparrowhawk and Northern Sparrowhawk in Kazakhstan was as high as 8.5%, although the birds were caught at the same location during seasonal migration, but may originate from different regions of the Palearctic. Additional sequence information from nuclear genes would help confirm that *L. toddi* may in fact be a group of cryptic species or subspecies.

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