Geographic Distribution, Phylogeny, and Genetic Diversity of the Fruit- and Blood-Feeding Moth *Calyptra thalictri* Borkhausen (Insecta: Lepidoptera: Erebidae)

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GEOGRAPHIC DISTRIBUTION, PHYLOGENY, AND GENETIC DIVERSITY OF THE FRUIT- AND BLOOD-FEEDING MOTH *CALYPTRA THALICTRI* BORKHAUSEN (INSECTA: LEPIDOPTERA: EREBIDAE)

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ABSTRACT: Facultative blood feeding on live animals or carrion is widespread within Lepidoptera. Male moths within the genus *Calyptra* are known to use their fruit-piercing mouthparts to occasionally feed on mammalian blood. The Palearctic species *Calyptra thalictri* is known to exhibit differential feeding behaviors that appear to be based on geographic location. This species is known to pierce fruit throughout its range but has recently been reported to also feed on human blood under experimental conditions in the Russian Far East. Here we document the distribution of this widespread species, reconstruct its evolutionary history, and calculate its genetic diversity for the first time. Recently collected samples are combined with museum specimens to model suitable environments for this taxon. Our findings suggest that while the blood-feeding populations are not monophyletic, there is geographical structure. Our analysis of macroclimate variables suggests that altitude and precipitation are the environmental variables most critical to habitat suitability in this lineage.

Approximately 14,000 insect species from 5 Orders (Diptera, Hemiptera, Lepidoptera, Phthiraptera, Siphonaptera) engage in hematophagy, feeding upon animal blood (Adams, 1999). Of these, at least 400 regularly feed on humans and livestock, and many are capable of transmitting disease (Adams, 1999; Lehane, 2005). For this reason, research attention is highly focused on medically and agriculturally important arthropods and their associations with humans and other animals. The acquisition of blood feeding over evolutionary time has likely arisen progressively through regular contact with nutrient-rich blood developing into a facultative hematophagic strategy, followed in some cases by obligate hematophagy (Waage, 1979). To date, species demonstrating facultative blood feeding have received less attention than their obligate counterparts. Facultative hematophagy occurs in a few adult butterflies and moths (Lepidoptera). Unlike most of the other hematophagic insects, which use blood primarily as a source of protein, Lepidoptera have been observed sucking blood from the wounds of animals, and even imbibing fluids from carrion, presumably in the quest for sodium (Clark, 1932; Reed, 1958; Payne and King, 1969; Downes, 1973). These salt acquisition strategies in butterflies and moths appear to be influenced by several factors including humidity (Beck et al., 1999), temperature (Molleman et al., 2005), and precipitation (Launer et al., 1996).

In most cases, facultative hematophagous behavior in Lepidoptera is not accompanied by overt morphological or physiological adaptations, as has been documented for hematophagous insects in other orders. However, in the genus *Calyptra*, (a.k.a. vampire moths; Fig. 1), facultative hematophagy is accompanied by pre-adaptations for fruit feeding, or frugivory: a stout proboscis with specialized microstructures (Bänziger, 1970; Zaspel et al., 2011). *Calyptra* comprises 18 described species and possibly 1 subspecies that are distributed throughout the Old World with 1 species, *Calyptra canadensis* (Bethune 1865), known to occur in the northern United States and Canada (Bänziger, 1983; Zaspel and Branham, 2008). Both sexes of all *Calyptra* species pierce fruit. Adults feed upon and damage a variety of both soft-skinned (e.g., *Rubus*, *Vitis*) and thicker-skinned fruits (e.g., *Ficus* L., *Citrus* L.) in subtropical and tropical Asia (Hattori, 1969; Bänziger, 1970, 1982, 2007). Thus, these moths are a rare example of a lepidopteran lineage that uses its fruit-piercing mouthparts to occasionally pierce the skin of vertebrate animals (Bänziger, 1970; Zaspel et al., 2012).

Of the 10 *Calyptra* species that are facultative hematophages, at least 3 exhibit patterns of differential feeding behavior depending on their geographic location. Limited evidence suggests that expression of hematophagous behavior, as well as host preference, may be linked to macroclimate or geographic location. For example, Bänziger (1989) provides anecdotal evidence that *Calyptra fasciata* (Moore 1882) preferentially pierced human skin and fed on blood at high elevation (1,000–1,600 m) field sites from India to Thailand, but only pierced non-human animals (e.g., Indian elephant and pig) at lower elevations (600 m). The species *Calyptra minuticornis* (Guenée 1852) fed on animal hosts (e.g., Asian elephant, *Elephus maximus* L.) in northern Thailand (800–1,470 m; Bänziger, 1986). Its putative subspecies, *Calyptra minuticornis novaepommeraniae* (Strand 1917), found in Papua New Guinea and northern Australia, has never been reported feeding on blood at any altitude (Bänziger, 1986). In 2007 the first case of blood feeding by a temperate species, *Calyptra thalictri* Borkhausen 1790, was reported from far eastern Russia (Zaspel et al., 2007). This Palearctic species is known to pierce thin-skinned fruits such as raspberry in Switzerland (Bänziger, 2007) but is not considered a fruit-piercing pest of economic importance. In 2008 additional field-based feeding trials were conducted at the same locations in Russia and corroborated the blood-feeding phenomenon (Fig. 1). Thus, expression of adult hematophagous behavior varies among species and within species, possibly correlated with elevation and perhaps with unknown habitat or macroclimate variables.

Until now, few studies have examined potential linkages between phylogenetic patterns, genetic diversity, and climate variables in a facultative blood-feeding lepidopteran lineage. In this study, we used a phylogeographic approach to investigate whether blood-feeding populations of *Calyptra thalictri* represent a cryptic species that correlates with hematophagy. Second, we

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FIGURE 1. *Calyptra thalictri* feeding on human blood, Russian Federation 2008.

examined nucleotide diversity, within and among populations of *Calyptra thalictri* in order to test whether localized facultative hematophagy can be linked with geography. Finally, we used presence-only distribution data, climate variable layers, and ecological niche modeling to predict possible occurrences and discuss the potential for continued range expansion of *Calyptra thalictri* in the Palearctic region.

MATERIALS AND METHODS

Sampling and gene amplification

We sampled 25 *Calyptra thalictri* from localities spanning its range from Scandinavia and southern Europe to the Russian Far East (RFE). Samples from the RFE (Primorye Territory) were collected from 3 separate localities: Kraunoka River Valley (KRV), Gornotayeznaya Biological Station (GBS), and Glee Factory (GF) (Table I). Three specimens of *Calyptra lata* Butler from GBS were included for outgroup comparison (Table I). Whole bodies of the moths were stored in 95% ethanol at –20 C prior to DNA extraction and are permanently stored in the Purdue Entomological Research Collection (frozen tissue collection). DNA was isolated from 2 or 3 legs using the DNeasy tissue extraction kit (Qiagen, Valencia, California) following the manufacturer's instructions. Each individual was sequenced for 2 mitochondrial markers (cytochrome oxidase I, COI; cytochrome oxidase *b*, Cyt*B*), 1 nuclear ribosomal RNA gene region (28S rRNA D2 region), and 1 nuclear ribosomal protein S5 (RpS5) for a total of 2,659 bp. Polymerase chain reaction (PCR) and sequencing protocols follow Wahlberg and Wheat (2008). The nDNA marker was selected based on similar studies within Lepidoptera (e.g., Kodandaramaiah et al., 2013). Cleaned PCR products (Qiagen) were sent to the Biomedical Genomics Center for sequencing (University of Minnesota, St. Paul, Minnesota). Resulting chromatograms were checked, sequences were assembled into contigs and DNA sequences were aligned using the MAFFT plugin in the program Geneious R6 (Geneious R6 Created by Biomatters).

Phylogenetic analyses

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) analyses of the partitioned, concatenated dataset. The optimal data partitioning strategy and model of evolution for each partition (Table II) were determined by analyzing the concatenated dataset in PartitionFinder v1.0.1 (Lanfear et al., 2012) using the Bayesian information criterion (BIC; Schwarz, 1978). The ML analysis was conducted using the default settings in RAxML–HPC2 v7.4.4 (Stamatikis, 2006; Stamatikis et al., 2008) with the exception that the GTR + G model of evolution was applied to each partition for both tree reconstruction and bootstrapping. One thousand bootstrap (BS) pseudoreplicates (Felsenstein, 1985) were used to calculate the nodal support. These values were then mapped onto the best scoring ML tree. The BI analysis was conducted using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). When possible, the best-fit model of evolution identified by PartitionFinder (Table II) was used in the analysis. However, when the limitations of MrBayes v 3.1.2 prevented the implementation of the best-fit model, the closest overparameterized model was used. In the Bayesian analysis, 2 simultaneous and independent runs of 20,000,000 generations were conducted, each consisting of 4 chains (1 cold and 3 hot) using the default temperature settings. Following every 1,000 generations, samples were taken from the cold chain, and 5,000,000 generations were discarded as "burn-in." To confirm that the runs had converged (SD $<$ 0.05), the probabilities of the 2 runs were summarized and the potential scale reduction factor (PSRF; Gelman and Rubin, 1992) was calculated. The PSRF is expected to approach 1.0000 as the runs converge. Clade credibility values were provided by the posterior probabilities (PP), which were mapped to the majority rule consensus tree obtained by summarizing the tree. The CIPRES Science Gateway (Miller et al., 2010) was used to conduct both the ML and BI analyses. FigTree v 1.3.1 (Rambaut, 2010) was used to visualize the trees from both analyses. The trees obtained from both types of analysis were rooted using *Calyptra lata* (CtAP055).

Population genetic analyses

An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was conducted in ARLEQUIN v3.5.1.3 (Excoffier and Lischer, 2010) to calculate the variance components within and among the following 4 *Calyptra thalictri* populations from Russia: KRV, GBS, GF, and Urals, as well as within and among the blood-feeding versus non-blood-feeding populations (Table III). Each of these analyses was conducted on the nuclear gene fragments and the combined mitochondrial fragments. The sequences for each gene fragment were trimmed to an equal length and ambiguity coding present in the sequences was treated as missing data. The trimmed length of each fragment was COI (582 bp), Cyt*B* (641bp), and RpS5 (587 bp). The significance of the AMOVA components was assessed using 1,000 permutations. These trimmed files were also used to estimate genetic polymorphism (e.g., number of haplotypes, number of polymorphic sites, nucleotide and haplotype diversity) using DnaSP v 5.10.1 (Librado and Rozas, 2009). These statistics were calculated for each gene marker (nuclear and mitochondrial) and the combined mitochondrial fragments. In addition, within each of the markers, these statistics were calculated for each population that was represented by more than 1 individual.

To determine whether feeding behavior influenced genetic polymorphism, all specimens representing the KRV population were examined in combination and separately based on the recorded feeding behavior, i.e., hematophagous (BF) or non-hematophagous (NBF). We used Tajima's D statistic (Tajima, 1989) to test whether our nucleotide sequence data are consistent with selective neutrality (Kimura, 1983) and Fu's F_s (Fu, 1997) to test whether *Calyptra thalictri* populations are expanding. These analyses were also calculated for each gene fragment and the population groups examined in the genetic polymorphism analysis using DnaSP v. 5.10.1. However, these statistics could only be assessed in populations represented by more than 4 individuals that possessed at least 1 polymorphic site.

Climate data and variable selection

Calyptra thalictri is Palearctic in distribution. The species has been recorded from areas as far east as the Kraunoka River Valley in eastern Russia and as far west as Órgiva in the Granada province of Spain. In addition, a single record has been reported from Golestan province in Iran (Ebert and Hacker, 2002). All 246 museum-specimen records for *Calyptra thalictri* were used. The data were mapped using Google Maps in combination with Lat-Long Crosshairs (Canadensys) for visual inspection, and the Georeferencing Calculator was used to determine the maximum error for each record (Wieczorek et al., 2001; Wieczorek and Bloom, 2011; see Supplemental Materials 1). For the period 1950–2000, world climate data were obtained from WorldClim v1.4 (Hijmans et al., 2005) using the generic grid format with a grid cell resolution of 2.5 arcminutes. These climate data layers were produced through interpolation of average monthly climate data from weather stations located on a

TABLE I. Complete list of specimens used in the phylogenetic analysis including the collection locality, sample and tree IDs, and the gene fragments that were successfully amplified. BF = blood feeder, NBF = non-blood feeder. Subscripts for Primorye: 1, Kraunoka River Valley; 2, Gornotayeznaya Biological Station; 3, Glee Factory.

Species/region	Locality and behavior code	Specimen code	GenBank accession nos.			
			COI	Cyt*b*	28S rRNA	RpS5
Calyptra thalictri						
Russia						
$Primorye_1$	(KRV-BF)	CtAP001	KF542971	KF542998	KF542935	KF543020
$Primorye_1$	(KRV-BF)	CtAP002	KF542957	KF542993	KF542934	KF543019
$Primorye_1$	(KRV-BF)	CtAP003	KF542968	KF542995	KF542945	KF543028
$Primorye_1$	(KRV-BF)	CtAP004	KF542965	KF542997	KF542952	KF543018
$Primorye_1$	(KRV-BF)	CtAP005	KF542967	KF542990	KF542943	KF543010
$Primorye_1$	(KRV-NBF)	CtAP011	KF542975	KF542994	KF542930	KF543016
$Primorye_1$	(KRV-NBF)	CtAP012	KF542964	KF542999	KF542939	KF543022
$Primorye_1$	(KRV-NBF)	CtAP013	KF542978	KF542988	KF542928	KF543008
$Primorye_1$	(KRV-NBF)	CtAP014	KF542960	KF542986	KF542931	KF543024
$Primorye_1$	(KRV-NBF)	CtAP015	KF542969	KF543003	KF542950	KF543014
$Primorye_2$	(GBS-NBF)	CtAP021	KF542966	KF542985	KF542938	PF
$Primorye_2$	(GBS-NBF)	CtAP022	KF542963	KF542992	KF542933	KF543025
$Primorye_2$	(GBS-NBF)	CtAP023	KF542972	KF542984	KF542937	KF543013
$Primorye_2$	(GBS-NBF)	CtAP024	KF542958	KF542996	KF542949	KF543026
$Primorye_2$	(GBS-NBF)	CtAP025	KF542970	KF542989	KF542946	KF543030
$Primorye_3$	(GF-NBF)	CtAP035	KF542977	KF543004	KF542940	KF543027
$Primorye_3$	(GF-NBF)	CtAP036	KF542977	KF543004	KF542929	KF543027
Ekaterinburg	(Middle Ural-NBF)	CtAP037	KF542974	KF542983	KF542941	KF543017
Ekaterinburg	(Middle Ural-NBF)	CtAP038	KF542959	KF542987	KF542936	KF543031
Ekaterinburg	(Middle Ural-NBF)	CtAP039	KF542979	KF542991	KF542942	KF543012
Krasnoschekovsky	(Tigiretsky Nat. Res.–NBF)	CtAP298	KF542961	PF	KF542951	KF543011
Iran						
Province of Golestan	(JZ001-NBF)	CtAP299	KF542973	KF543002	KF542948	KF543009
Ukraine						
Ivano-Frankivsk	(CLFMNH-NBF)	CtAP296	KF542976	PF	KF542944	KF543021
Finland						
Parikkala Ristimaki	(F1-NBF)	CtAP040	KF542956	KF543000	KF542932	KF543015
Sweden						
Rotskar	(SSR2-NBF)	CtAP297	PF	PF	KF542947	KF543029
C. lata						
Russia						
$Primorye_2$	(GBS-NBF)	CtAP054	KF542955	KF542982	KF542925	KF543005
$Primorye_2$	(GBS-NBF)	CtAP055	KF542953	KF542980	KF542926	KF543007
$Primorye_2$	(GBS-NBF)	CtAP056	KF542954	KF542981	KF542927	KF543006

30 arcsecond grid and represent monthly precipitation and temperatures (minimum, maximum, and median) as averages, and an additional 19 bioclimatic variables determined by summarizing monthly climate data. Each of these layers was imported into DIVA-GIS v 7.5 (Hijmans et al., 2012) and trimmed to represent only the Palearctic Region (coordinates used: $x = -12.844575$, 149.73607 and $y = 22.038123$, 82.565982).

Distribution modeling

To predict suitable environmental conditions for all of the 246 reported occurrences of *Calyptra thalictri* in the Palearctic, MaxEnt v 3.3.3k was used (Phillips et al., 2006, 2009, available at www.cs.princeton.edu/~schapire/maxent/; Phillips and Dudik, 2008). The trimmed world climate layers were imported into MaxEnt v 3.3.3k along with decimal degree data

TABLE II. Optimal partitioning scheme selected by PartitionFinder v 1.0.1 (Lanfear et al., 2012) using the BIC selection criterion. The model of evolution for each partition, the number of subsets, parameters, and the log likelihood for the scheme are included.

Optimal scheme	Model of evolution	Subset no.	Parameter no.	ln *L*
28S = 1–694	F81	5	76	−4536.58951
COI position1, CytB position1 = 695–1351\3, 1352–1999\3	TRN			
COI position2, CytB position2 = 696–1351\3, 1353–1999\3	F81			
COI position3, CytB position3 = 697–1351\3, 1354–1999\3	TRN+I			
RpS5 all positions = 2000–2659\3, 2001–2659\3, 2002–2659\3	K80+I			

TABLE III. Analysis of molecular variance (AMOVA) for concatenated mtDNA and RpS5 gene fragments of *Calyptra thalictri* from the Russian Federation. BF = blood feeding, NBF = non-blood feeding.

Source of variation	d.f.	Variance	Percentage of variation	*P* value
mtDNA (KRV, GBS, GF, Ural)				
Among populations	3	0.04978	5.47	0.24340
Within populations	16	0.86042	94.53	
RpS5 (KRV, GBS, GF, Ural)				
Among populations	2	1.81271	59.36	0.00684
Within populations	13	1.24103	40.64	
mtDNA (BF and NBF)				
Between BF and NBF populations	1	0.10864	11.31	0.13587
Within populations	18	0.85185	88.69	
RpS5 (BF and NBF)				
Between BF and NBF populations	1	−0.08737	−3.91	0.57478
Within populations	14	2.32338	103.91	

for each reported occurrence of *Calyptra thalictri*. A jackknife analysis was completed to measure the importance of each of the climate layers. The analysis was conducted using default settings, and the data were output as cumulative and logistic. Regions with a cumulative output greater than 1–20 are predicted to be suitable locations for *Calyptra thalictri*. The logistic output was used to estimate the probability of presence at a given location.

Quantum GIS v 1.8.0 (Quantum GIS Development Team, 2013) was used to plot the range of *Calyptra thalictri* based on the current collection data. The coordinates in decimal degrees for the 246 records of *Calyptra thalictri* were imported into a general map for the Palearctic within Quantum GIS v 1.8.0. A buffer with a radius of 125 km was placed around each data point. The buffers were then merged to form groups where records overlapped. These merged buffers were then clipped to include only areas over land and then shaded. To produce a map illustrating the final range based on the data, the original data points were removed.

RESULTS

Phylogenetic analyses

Optimal topologies were recovered using ML (Fig. 3a) and BI analyses (Fig. 3b). Both approaches recovered *Calyptra thalictri* as a strongly supported clade (BS = 100; PP = 1); however, in the BI analysis, there was little resolution among *Calyptra thalictri* populations (compare Fig. 3a, b). For example, relationships among hematophagous *Calyptra thalictri* from RFE could not be discerned from the BI analysis, nor could individuals from that population (KRV) be placed within clades comprising non-hematophagous *Calyptra thalictri* from the same location (Fig. 3b). Although the BI analysis did provide support for a clade comprised of all non-blood-feeding individuals from the KRV population, except CtAP015 (PP $\geq$ 0.94, Fig. 3b), relationships among the other representatives of the Primorye Territory populations were unresolved or only weakly supported. In addition, the BI results produced strong support for a clade comprising individuals from Scandinavia, Western Europe, Ukraine, Iran, and 2 individuals from the Middle Urals (PP $\geq$ 0.99; Fig. 3b). Finally, in the BI analysis, support for a monophyletic outgroup taxon, *Calyptra lata*, was not recovered although weak support was provided for 2 of the 3 outgroup sequences used. While the ML analysis produced a well-resolved topology when compared with the BI tree, support for some ingroup relationships was weak (BS $\leq$ 50; Fig. 3a). A hematophagous (BF) *Calyptra thalictri* individual from GBS was basal to remaining ingroup populations (CtAP005; Fig. 3a). Next, a clade comprising individuals from 2 separate populations within the Primorye Territory was recovered as sister to a clade with remaining *Calyptra thalictri* individuals. Two additional major groupings were recovered from the ML analysis. One monophyletic clade comprised of remaining BF *Calyptra thalictri* individuals and also those from both the KRV and the GBS in RFE (Fig. 3a). Within this clade, moderate support (BS = 89) for a group comprised of all but 1 non-hematophagous NBF individual (CtAP015) from the KRV was recovered (Fig. 3a). Sister to the assemblage of both BF and individuals from the KRV and representatives of the GBS, a clade comprising individuals from the RFE, Middle Urals, Western Europe, and Scandinavia was recovered (Fig. 3a). Within this grouping, there was weak support (BS = 71) for a clade comprising individuals representing populations from Finland, Iran, Ukraine, Sweden, and the Middle Urals (Fig. 3a). In contrast to the BI analysis, in the ML analysis, the outgroup taxon, *Calyptra lata*, formed a well-supported, monophyletic clade (BS =100).

Population genetic analyses

The results from the AMOVA of Russian *Calyptra thalictri* populations, based on our mtDNA markers, suggest that variation is higher within than among the 4 populations sampled, although the result was not significant ($P = 0.243$). The AMOVA results based on the nuclear protein-coding marker RpS5, however, indicated that there is more variation among *Calyptra thalictri* populations than within ($P = 0.00684$). To determine the extent to which genetic differentiation occurred between individuals expressing the hematophagous habit and those that do not, we compared these groups using both mtDNA and nuclear datasets. For the AMOVA based on mtDNA markers, the molecular variation was higher within feeding groups than between (result not significant). Structured differentiation based on the nuclear marker RpS5 was not recovered between or within BF and NBF groups (Table III).

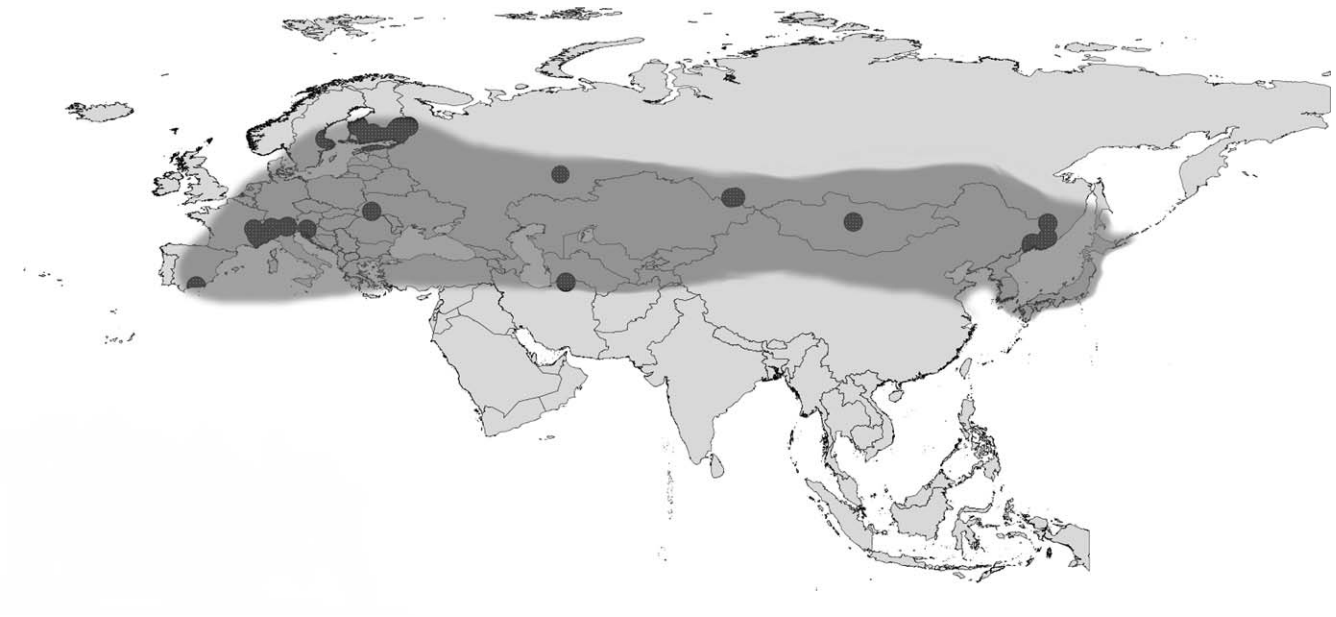

FIGURE 2. Distribution map of *Calyptra thalictri* based on approximately 200 specimen records.

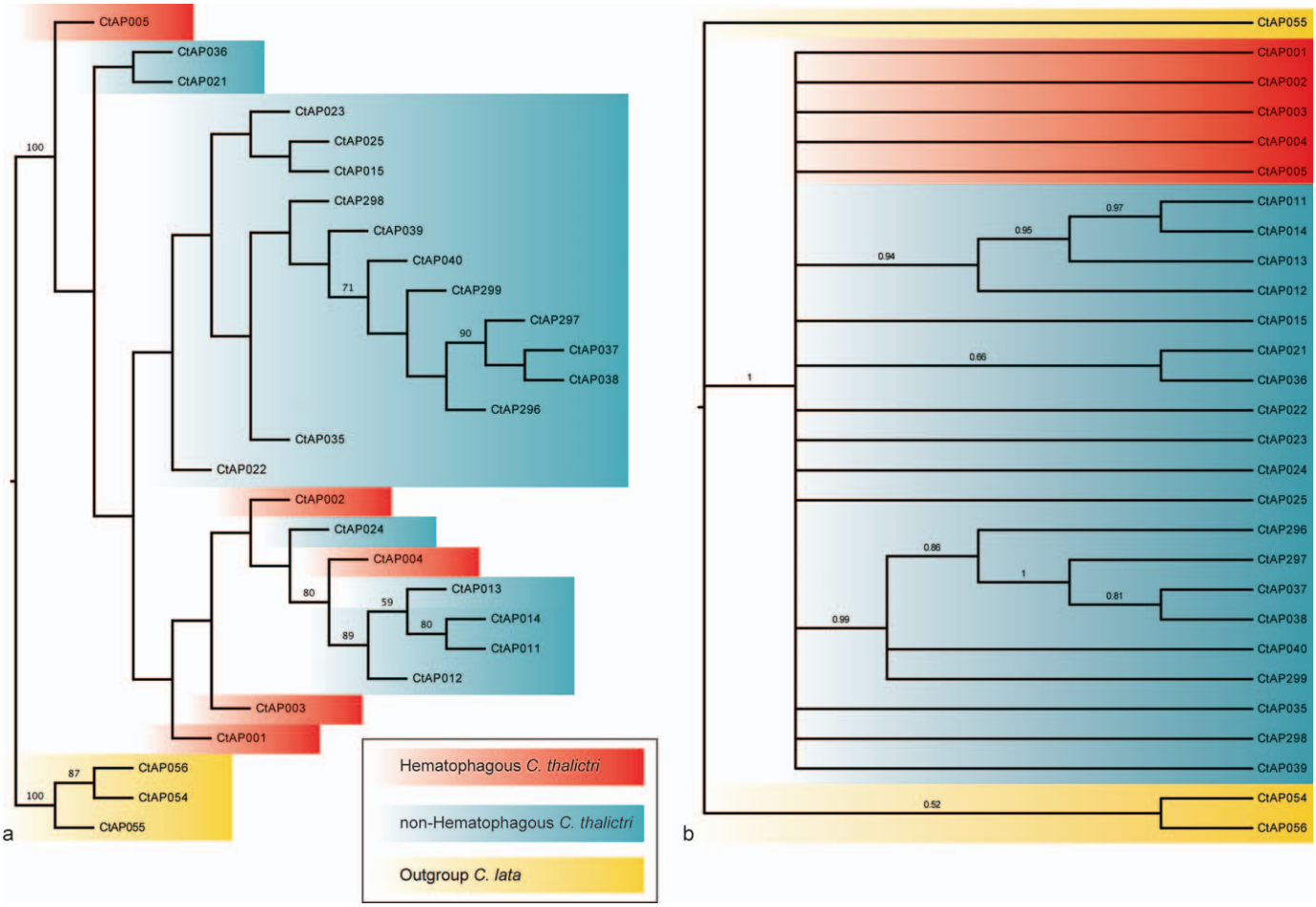

FIGURE 3. (**a**) ML phylogeny of *Calyptra thalictri* based on 4 molecular markers. Sequence alignments were analyzed using RAxML HPC2 v. 7.4.4. Node labels represent Bootstrap support values. (**b**) BI phylogeny of *Calyptra thalictri* based on 4 molecular markers. Sequence alignments were analyzed using MrBayes v. 3.1.2. Node labels represent posterior probability values.

TABLE IV. Summary of genetic polymorphism within populations* of *Calyptra thalictri*. Sample size (n), sequence length (L), number of different haplotypes (k), number of polymorphic sites (S), nucleotide diversity (Π), haplotype diversity (h), Fu's F_s statistic (F_s), and Tajima's D statistic (D).

Population	Locus	n	L (bp)†	k	S	$\Pi \pm$ SD	$h \pm$ SD	F_s‡	D‡
	28S	25	610	1	0	0.00000 ± 0.00000	0.000 ± 0.000	–	–
	mtDNA	22	1,220	9	10	0.00145 ± 0.00025	0.775 ± 0.081	−3.494	−1.19299
GBS		5	1,221	5	5	0.00197 ± 0.00051	1.000 ± 0.126	−2.680	0.00000
GF		2	1,223	2	4	0.00327 ± 0.00164	1.000 ± 0.500	–	–
KRV-All		10	1,222	3	4	0.00078 ± 0.00039	0.378 ± 0.181	0.390	−1.24468
KRV-BF		5	1,223	1	0	0.00000 ± 0.00000	0.000 ± 0.000	–	–
KRV-NBF		5	1,222	3	4	0.00147 ± 0.00050	0.700 ± 0.218	0.469	−0.41017
Ural		3	1,223	3	4	0.00218 ± 0.00081	1.000 ± 0.272	–	–
	RpS5	21	543	9	11	0.00467 ± 0.00112	0.681 ± 0.113	−2.218	−0.85247
GBS		3	585	1	0	0.00000 ± 0.00000	0.000 ± 0.000	–	–
KRV-All		10	582	3	3	0.00103 ± 0.00057	0.378 ± 0.181	−0.459	−1.56222
KRV-BF		5	586	3	3	0.00205 ± 0.00083	0.700 ± 0.218	−0.186	−1.04849
KRV-NBF		5	582	2	1	0.00069 ± 0.00041	0.400 ± 0.237	0.090	−0.81650
Ural		3	572	3	21	0.02506 ± 0.00775	1.000 ± 0.272	–	–

* Populations represented by a single individual were not analyzed for genetic polymorphism but were included in the analysis of the genetic diversity of the species.
† Sequence length in bp excludes sites with gaps of missing data.
‡ F_s and D require at least 4 individuals in a population to be calculated, and at least 1 polymorphic site must be present.

The analysis of genetic variation implemented in DnaSP assessed the polymorphism present in the *Calyptra thalictri* individuals (Table IV). The ribosomal gene fragment (28S) was identical for all individuals, whereas RpS5 had the greatest genetic variation (9 haplotypes and 11 polymorphic sites). The combined mitochondrial gene fragments had an equivalent number of haplotypes (=9) but 1 less polymorphic site (=10) than the RpS5 fragment. To determine if more fine-grained genetic differentiation had occurred among individuals expressing hematophagous behavior or whether structured differentiation existed primarily at the larger geographic scale, we conducted a series of comparisons among populations (GBS, GF, KRV-all, and Urals) and feeding behaviors (KRV-BF and KRV-NBF). The mitochondrial haplotype varied within all populations. However, all individuals from the GBS population shared the same haplotype for the RpS5 fragment. The Ural Mountains population had the greatest RpS5 variation (21 polymorphic sites). Comparisons between the 2 feeding behaviors (e.g., hematophagous and non-hematophagous) that occur in the KRV population indicated that all hematophagous individuals had identical mitochondrial haplotypes, whereas the non-hematophagous individuals possessed some variation (3 haplotypes and 4 polymorphic sites; Table IV). However, the individuals representing these 2 feeding behaviors had similar levels of variation for the RpS5 fragment (e.g., 3 haplotypes and 3 polymorphic sites among the hematophagous individuals and 2 haplotypes and 1 polymorphic site for the non-hematophagous individuals).

The selective forces acting on each gene fragment were also assessed for all individuals as well as among populations and feeding behaviors. For both the mtDNA and the RpS5 gene, Fu's F_s and Tajima's D values were negative (mtDNA: $F_s = -3.494$ and $D = -1.19299$; RpS5: $F_s = -2.218$ and $D = -0.85247$; Table IV). However, neither value was significant for either of the markers. Among individual populations, both positive and negative values were recovered for the Fu's F_s of mitochondrial haplotypes (KRV-all $F_s = 0.390$ and GBS $= -2.680$). All populations had negative F_s values for the RpS5 fragment. The Tajima's D value was negative for each of the populations and both markers. Between the feeding behaviors, the F_s and D values could not be compared for the mtDNA because all hematophagous individuals shared the same haplotype. With the RpS5 fragment, the *Fs* and D value were negative for the hematophagous individuals –0.186 and –1.04889, respectively; Table IV). However, the F_s value for the non-hematophagous individuals was positive (0.090), while the D value for this feeding behavior was also negative (–0.81650).

Predictive distributions for *Calyptra thalictri*

The precipitation variable (precip11) was found to be the most informative variable for predicting the suitable conditions and presence at a location. The altitude variable (alt_a) provided the most information on suitability and occurrence that was not present in the other variables examined. As expected, the locations from which *Calyptra thalictri* has previously been collected, were predicted to have the most suitable conditions (Fig. 4). In addition, the MaxEnt analysis identified the areas surrounding and joining these regions as being equally suitable. This analysis also identified several locations as suitable for *Calyptra thalictri* that are not contiguous with these collection locations (e.g., the Greater Caucasus mountain range and Hokkaido). The map estimating the probability of *Calyptra thalictri* occurring at a given location (Supplemental Materials 2) was congruent with the distribution map (Fig. 2) produced using Quantum GIS.

DISCUSSION

Our phylogenetic analyses show support for the monophyly of *Calyptra thalictri* across its geographic range. Although our tree topology does not necessarily reflect the molecular underpinnings of differential feeding behaviors in *Calyptra thalictri*, the results of our analysis do suggest that geographical structure is occurring between far eastern and western European populations. For

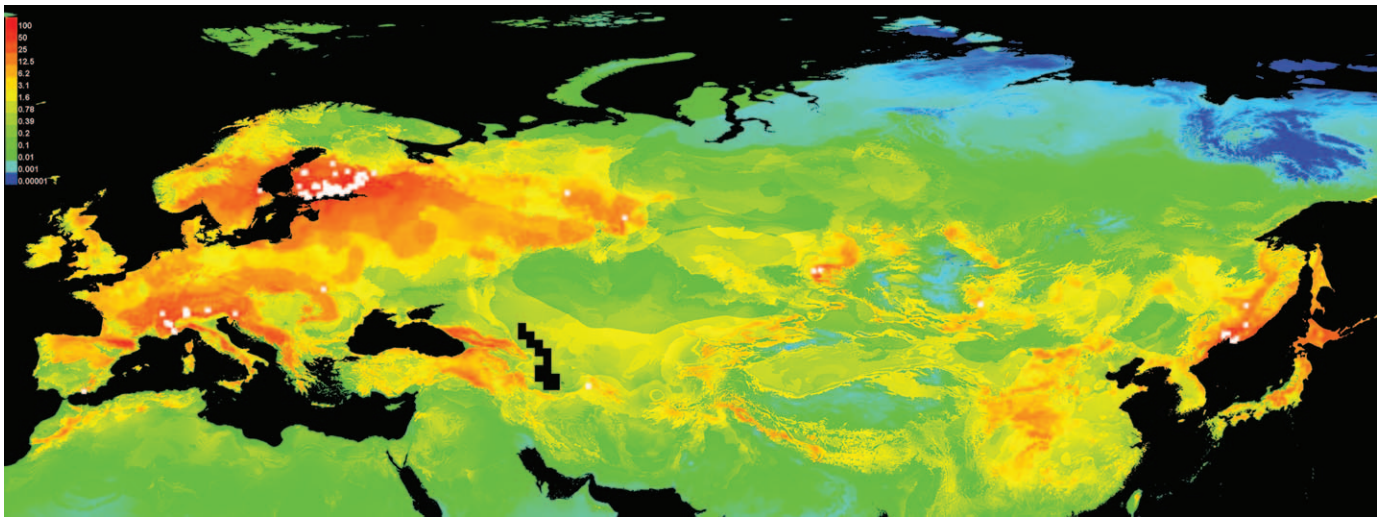

FIGURE 4. Predictive map illustrating regions with suitable climate conditions for *Calyptra thalictri* in the Palearctic region based on 21 environments. Red = highest probability, Blue = lowest probability.

example, a clade comprising NBF individuals from western Russia (Urals), Iran, Ukraine, and Scandinavia was recovered in the ML analysis, albeit without strong support (Fig. 3a) and a less resolved, but well-supported clade comprising individuals from Ukraine, Iran, and Scandinavia and 2 individuals from western Russia was recovered in the BI analysis (Fig. 3b). The relationships among Russian Far East populations of *Calyptra thalictri* are less clear. While blood feeding can be elicited within at least 1 of the 3 far eastern Russia populations sampled (KRV), our phylogenetic results do not suggest that the blood-feeding *Calyptra thalictri* represent a cryptic species. Rather, our data support our second hypothesis, that facultative blood feeding in this lineage is labile. Perhaps most interesting, is that while males of *Calyptra thalictri* from the Russian Far East readily pierced human skin and fed on blood for prolonged time periods, all but 1 individual of *Calyptra lata* from identical collecting and capture events refused. The behavior is sex-specific. Even though males exhibited hematophagous behaviors, *Calyptra thalictri* females never fed on blood when tested in Russia (Zaspel et al., 2007) and Sweden (S. R. Hill, unpubl. data). In summary, blood-feeding behavior cannot be elicited in all *Calyptra* species, regardless of shared environmental conditions and their ability to pierce fruit hosts.

Patterns of mitochondrial and nuclear diversity were in conflict in the AMOVA of eastern Russian populations. Our results showed that *Calyptra thalictri* mtDNA and 28S rDNA haplotypes are shared, even among geographically disparate populations, regardless of adult feeding behavior. The analysis of our nuclear locus (RpS5), however, revealed significant variation among those populations, suggesting the possibility of gender-biased dispersal. This pattern is consistent with the mating and feeding behaviors in this widespread species. In this system, *Calyptra* males are the hematophagous sex and would presumably travel longer distances in the search for salts, but has not yet been tested experimentally. Our estimates of Fu's F_s and Tajima's D values were negative and non-significant, which suggests little to no selection is occurring on the molecular markers we sampled. The low mtDNA diversity coupled with negative Fu's F_s and Tajima's D values could suggest a mtDNA selective sweep has occurred, although additional sampling will be needed to determine statistical support for this assertion.

Previous authors (Goater et al., 2003) asserted the Alps range was the northernmost extent of *Calyptra thalictri* species' range, with migrants reaching Finland only occasionally. However, the frequency of observations of this species in Finland has increased substantially since this species was first collected there in 2000 (Mikkola, 2007); new records were also reported from north of Uppsala, Sweden, in 2008 (Lindeborg, 2009). Precipitation and elevation were the macroclimate variables considered to be most important in determining suitability for the species; however, a climatic correlate specific to the hematophagous individuals was not found. This result suggests other factors such as microclimate, components of larval diet, and the availability of adult hosts could be influencing this highly localized feeding occurrence.

This work examined possible linkages between evolutionary history, geography, and facultative hematophagy in *Calyptra thalictri*. Our molecular data show evidence of geographic structure occurring in this species, although this geographic structure cannot be linked to shifts in feeding behavior from non-hematophagous to hematophagous at this time. Our climatic analysis identified areas to search for this species using data often overlooked in museum collections. This work provides insight into the evolution of arthropod blood feeding through illumination of potential heritable and environmental factors influencing facultative hematophagy in an insect lineage that commonly pierces plants.

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