



Genomic Variation among Strains of *Crithidia bombi* and *C. expoeki*

Evgeny Gerasimov,^{a,b} Niklaus Zemp,^c Regula Schmid-Hempel,^d Paul Schmid-Hempel,^d  Vyacheslav Yurchenko^{a,e}

^aMartsinovskiy Institute of Medical Parasitology, Tropical and Vector Borne Diseases, Sechenov University, Moscow, Russia

^bFaculty of Biology, M. V. Lomonosov Moscow State University, Moscow, Russia

^cGenetic Diversity Centre, ETH Zürich, Zürich, Switzerland

^dInstitute of Integrative Biology, ETH Zürich, Zürich, Switzerland

^eLife Science Research Centre, Faculty of Science, University of Ostrava, Ostrava, Czech Republic

ABSTRACT In this study, we sequenced and analyzed the genomes of 40 strains, in addition to the already-reported two type strains, of two *Crithidia* species infecting bumblebees in Alaska and Central Europe and demonstrated that different strains of *Crithidia bombi* and *C. expoeki* vary considerably in terms of single nucleotide polymorphisms and gene copy number. Based on the genomic structure, phylogenetic analyses, and the pattern of copy number variation, we confirmed the status of *C. expoeki* as a separate species. The Alaskan populations appear to be clearly separated from those of Central Europe. This pattern fits a scenario of rapid host-parasite coevolution, where the selective advantage of a given parasite strain is only temporary. This study provides helpful insights into possible scenarios of selection and diversification of trypanosomatid parasites.

IMPORTANCE A group of trypanosomatid flagellates includes several well-studied medically and economically important parasites of vertebrates and plants. Nevertheless, the vast majority of trypanosomatids infect only insects (mostly flies and true bugs) and, because of that, has attracted little research attention in the past. Of several hundred trypanosomatid species, only four can infect bees (honeybees and bumblebees). Because of such scarcity, these parasites are severely understudied. We analyzed whole-genome information for a total of 42 representatives of bee-infecting trypanosomatids collected in Central Europe and Alaska from a population genetics point of view. Our data shed light on the evolution, selection, and diversification in this important group of trypanosomatid parasites.

KEYWORDS *Crithidia*, *Trypanosomatidae*, genomics

Monoxenous trypanosomatid flagellates (with one host, usually an insect, in their life cycle) have, for a long time, been considered dull and not-so-interesting cousins of the economically or medically important dixenous kin that have two hosts in their life cycle, an insect and a vertebrate or a plant (1, 2). This view has been challenged recently when insect parasites were found to be coinfecting vertebrates (3–5) or as their ancestral role in the evolution of dixeny became recognized (6, 7).

The monoxenous trypanosomatid parasites of bees are rather unique in many regards. This group of *Leishmaniinae* spp. (8) (which includes *Crithidia bombi*, *C. expoeki*, *C. mellifcae*, and *Lotmaria passim*) are the only trypanosomatids able to infect hymenopteran insects. *Crithidia mellifcae* and *Lotmaria passim* infect honeybees (*Apis* spp.) (9, 10), whereas *C. bombi* and *C. expoeki* are common parasites of bumblebees (*Bombus* spp.) (11, 12). All of these parasites have a worldwide distribution.

Bumblebees are social insects, and individual infections are acquired outside the nest by the foraging workers when visiting flowers that had been previously visited by

Citation Gerasimov E, Zemp N, Schmid-Hempel R, Schmid-Hempel P, Yurchenko V. 2019. Genomic variation among strains of *Crithidia bombi* and *C. expoeki*. mSphere 4:e00482-19. <https://doi.org/10.1128/mSphere.00482-19>.

Editor Ira J. Blader, University at Buffalo

Copyright © 2019 Gerasimov et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Paul Schmid-Hempel, paul.schmid-hempel@env.ethz.ch, or Vyacheslav Yurchenko, vyacheslav.yurchenko@osu.cz.

Received 6 July 2019

Accepted 26 August 2019

Published 11 September 2019

an infected bee or by contact with contaminated food or nest material inside the colony (13). In general, the infection has mild effects on the workers, but it can have severe consequences for the founding queens in spring, since they become unable to found a colony (14). There are concerns that the presence of this infectious pathogen contributes to the decline in the populations of wild bumblebees in the United States (15), South America (16), or the United Kingdom (17).

A particular hallmark of this biological system is the strong effect of the host and parasite genotypic background on a success of infection. Bumblebees colonies vary considerably in their susceptibility to infection and, vice versa, different genotypes ("strains") of *Crithidia* spp. vary in the range of different host colonies they are able to infect (18). This effect can be explained by variation in the genotypes (19, 20) and the corresponding gene expression patterns (21, 22). Among other factors, genotypic variation in parasite populations is determined by recombination during genetic exchange among coinfecting strains (23, 24) and maintained by selection during passage within colonies (25–27). As a result, natural bumblebee populations harbor a wide range of parasite's genotypes, even to the extent that each newly typed infection can be another unique genotype (28, 29).

The genomes of *Crithidia bombi* and *C. expoeki* were sequenced recently and demonstrated a considerable synteny in their organization (30). Several dozen orthologous gene groups, defined across a set of trypanosomatid species, revealed signatures of positive selection in the branch leading to *Crithidia*. Genes, putatively involved in the host-parasite interaction, were shown to coevolve. Remarkably, in *C. bombi* the biosynthesis of cell surface components, which are important for host-parasite interactions, deviates from that in most other eukaryotes, indicating the effects of rapid host-parasite coevolution (30).

Although previous studies, based on microsatellite polymorphism, have shown that genotypic variation is a major element of the natural interactions of these parasites with their bumblebee hosts, the extent of genomic differences among different strains of *Crithidia* has not yet been investigated in detail. Here, we compared the full genome sequences of 40 strains of *C. bombi* and *C. expoeki* that were collected at different sites, plus the genomes of the two type strains. We show that the analyzed strains form clusters with shallowly separated branches, show variation in gene copy numbers, and possess genes under positive selection.

RESULTS

Strain collection and sequencing. Previously, we have sequenced two strains of two *Crithidia* spp. using a combination of PacBio, Illumina, and Roche 454 technologies, and we assembled two high-quality reference genomes (30). We will further refer to BJ08_175 as a type strain for *C. expoeki* and to 08_076 as a type strain for *C. bombi*.

We sequenced 41 genomes using the Illumina platform with paired-end sequencing protocol and high coverage. Of these, one genome (BJ08_175a) was a repetition of the genome of the same strain, leaving 42 different strains (40 + 2 type strains) available (see Table S1 in the supplemental material). These strains were collected mainly in Switzerland (plus two samples collected on the Mediterranean islands of Corsica and Sardinia that were included in the "Switzerland strain" group based on their position in the phylogenetic tree, as described below) and in Alaska. The technical parameters of processing the data for newly sequenced strains are summarized in Table S2. The average coverage is about 95×, which is adequate for both single nucleotide polymorphism (SNP) calling and copy number variation (CNV) analyses (except for strain BJ08_068, with an average coverage of 51×, for which the CNV analysis was not performed).

Species assignment and read mapping. Previously, *18s rRNA*, *ggapdh* (glycosomal glyceraldehyde-3-phosphate dehydrogenase), and mitochondrial *cytB* (cytochrome B) sequences were used to assign the strains to groups (11). These markers defined two distinct lineages A and B, where lineage A corresponded to *Crithidia bombi* and lineage B represented a distinct, newly described species: *C. expoeki*. Here, we first checked

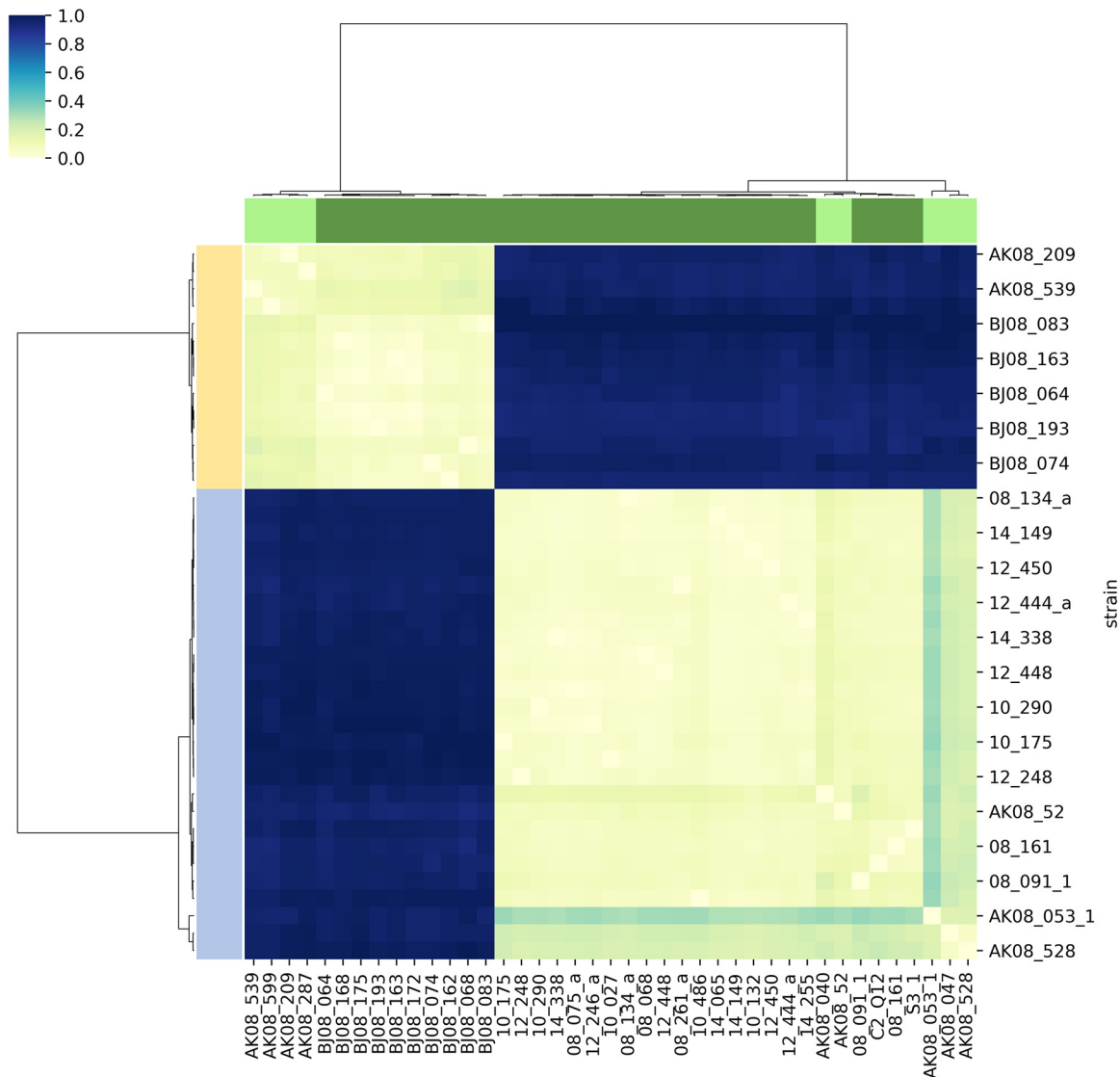


FIG 1 Clustering of sequenced strains based on reference-free SNP calling. Hierarchical clustering of sequenced strains based on distance matrix. Distance matrix obtained from SNP data set generated by DiscoSNP++ software. Distance is scaled to "(0, 1)". On dendrograms, dark green denotes Switzerland strains, light green denotes Alaskan strains, light blue denotes strains previously genotyped as lineage A, and the moccasin color depicts strains previously genotyped as lineage B (11).

whether our previous species assignment remained correct at the whole-genome level. To do that, we called SNPs using raw unassembled reads data for all collected strains with DiscoSNP++ software, which does not rely on a reference genome. This cluster analysis clearly splits the strains into two groups, which correlates with the previous assignments (Fig. 1). Accordingly, the extent of variation within these two species is much lower than between species. The clustering map also revealed that Alaskan strains form separate clades inside the *C. expoeki* clade (previously lineage B [11]) and inside the *C. bombi* clade (lineage A [11]). However, for *C. bombi* the situation was more complex: three Alaskan strains clearly branched out into a separate group, whereas two other Alaskan strains branched in a smaller clade inside the group of European strains.

The codon usage pattern of *C. bombi* clearly differs from that of *C. expoeki* (Fig. 2). The extent of this diversity is comparable to the difference documented between any of the two *Crithidia* spp. and their closest relative, *Leptomonas pyrrocoris*. Moreover, the genomic variation between strains does not influence the codon usage patterns, even within the Alaskan strains the usage remains the same. This supports an idea that codon usage is a species-specific trait.

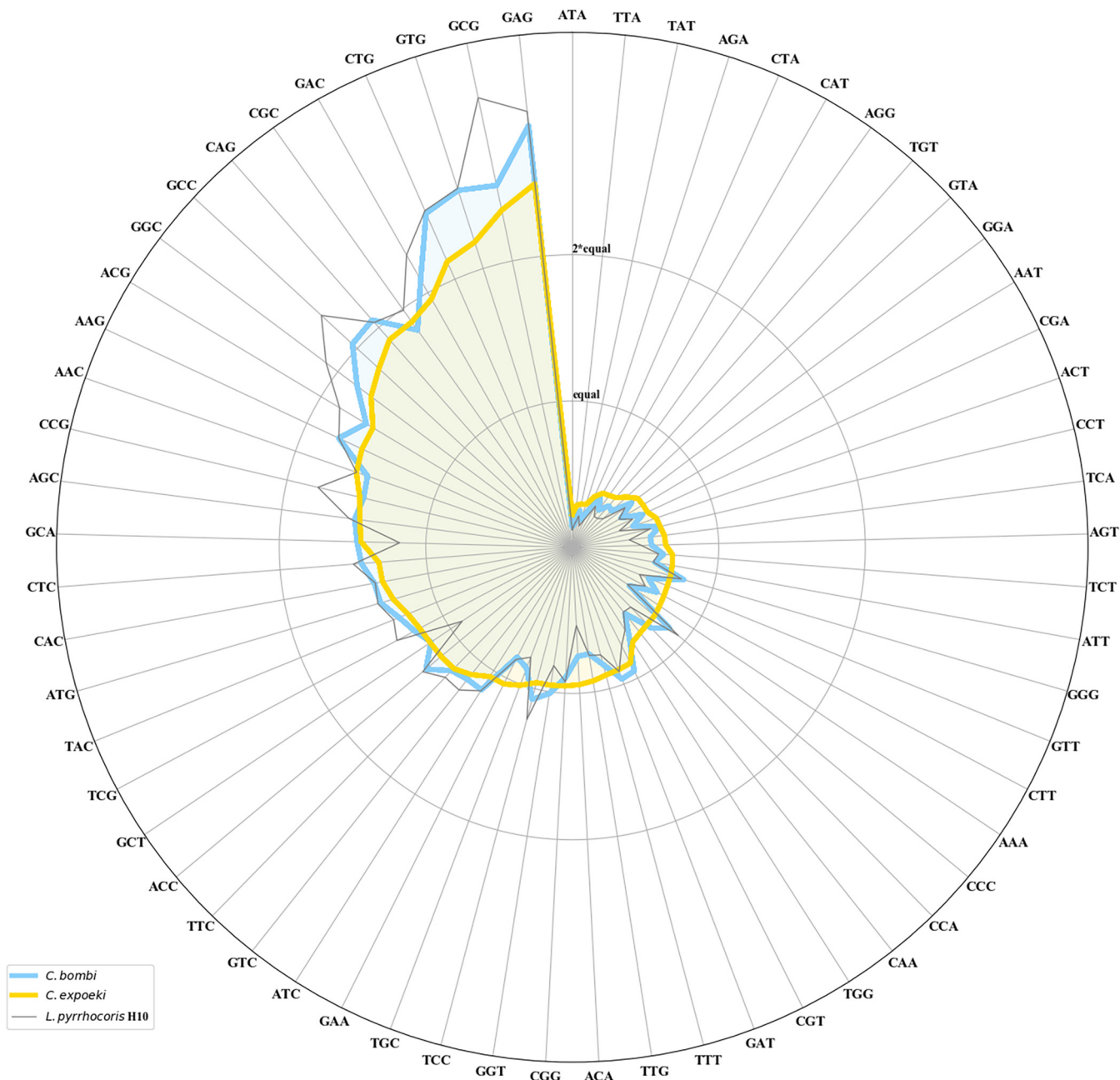


FIG 2 Codon usage plot. Frequencies of codons are plotted on polar coordinate system. Each spike represents a codon. Codons are sorted (counterclockwise) by decrease of frequency used in *C. expoeki* (yellow line). Each strain’s codon usage was plotted individually to show that there is no difference in codon usage between strains. To put the codon usage in context, the usage in *L. pyrrochoris*, a species from the closest sister group, is plotted in gray.

The genomes of the two type strains were used as references for mapping the reads and for the further reference-based SNP calling. Reads for 27 *C. bombi* strains (from a total of 29 samples; one strain was sequenced in two biological replicates, and one strain had insufficient coverage [see Table S1]) and 14 *C. expoeki* strains were taken as independent samples and mapped on their respective genome references. The overall statistics are summarized in Table S2.

Variant calling and divergence between strains. Variant calling revealed a total 165,124 variable genome positions in 27 strains of *Crithidia bombi* and 47,725 positions in 14 *C. expoeki* strains. The SNP densities (measured in numbers of nucleotides per SNP and indicating the average distance in nucleotides between SNPs) for *C. bombi* in

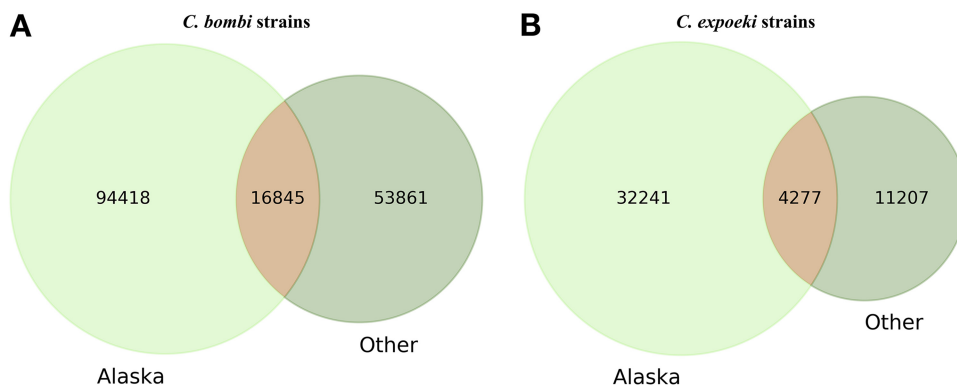


FIG 3 SNP distribution among populations. Venn diagrams for *Crithidia bombi* (A) and *C. expoeki* (B) show the numbers of SNPs coming from Alaskan (light green) or Switzerland (green) strains or the numbers of SNPs present in both populations (orange).

Switzerland and in two Alaskan strains (AK08_040 and AK08_52) strains were 1,835 and about 1,300 nucleotides (nt), respectively. Of note, three remaining Alaskan strains have much higher SNP densities of approximately 400 nt. The transition/transversion ratio was also higher for the latter three strains (2.74 compared to the average of 2.47 for *C. bombi*; Table S2).

At the same time, *C. bombi* strains demonstrated higher variation than *C. expoeki*. The average SNP distance between any two Switzerland strains is 15,100 SNPs for the former, whereas the SNP density is much lower (1 SNP per 5,500 nt) for the Switzerland strains of *C. expoeki*, with an average distance between two strains of ~6,000 SNPs. Similar to the situation in *C. bombi*, Alaskan strains of *C. expoeki* have ~4 times higher SNP density than the Switzerland strains.

For *C. expoeki*, 2,405 genes contained only Alaska-specific SNPs, 652 genes contained only Switzerland-specific SNPs, and 1,836 genes contained both Alaska-specific and Switzerland-specific SNPs. For *C. bombi* these values were 1,243, 799, and 5,084, respectively.

For both species, the Alaskan strains contributed around two-thirds of the observed overall genome variability (Fig. 3). For example, 94,418 variable genome positions (of a total 165,124 positions in the *C. bombi* genome) came from SNP calling in 5 Alaskan strains, while only 53,861 variable positions were from 22 Switzerland strains.

The mitochondrial genome is usually considered to be more variable than its nuclear counterpart and thus is often used as a sensitive genetic marker for distinguishing phylogenetically related species. Surprisingly, the mitochondrial genomes of Switzerland populations of both *Crithidia* spp. analyzed in this study have a very low SNP density (about 8,000 nt) and, as such, are even more conserved than nuclear genomes of the same strains. Of note, this analysis was performed using only coding regions of maxicircles and ignoring so-called divergent regions (31). For *C. expoeki*, the Alaskan strains have higher SNP densities in the mitochondrial genome (about 500 nt) compared to that of the nuclear genome. For most Alaskan strains of *C. bombi* SNP density is only slightly increased relative to that in the Switzerland population. Only one strain (AK08_053) exhibits the highest SNP density of 1 per 207 nt. This strain is also the most divergent by markers from the nuclear genome.

Phylogenetic analysis. A whole-genome-level phylogenetic analysis was done by building a phylogenetic tree based on a set of 2,876 protein-coding genes (Fig. 4). All four used algorithms produced trees with similar topology. A few algorithm-dependent branch rearrangements were noted within Switzerland and Alaskan clades of both species, but the following conclusions can be drawn based on all four tree-building algorithms: (i) the Alaskan strains occupy a basal position on the tree; (ii) the type strains are basal to all other Switzerland strains; (iii) two *C. bombi* strains (C2_Q12 and S3_1, collected in Corsica and Sardinia) always form a clade, basal to all Switzerland

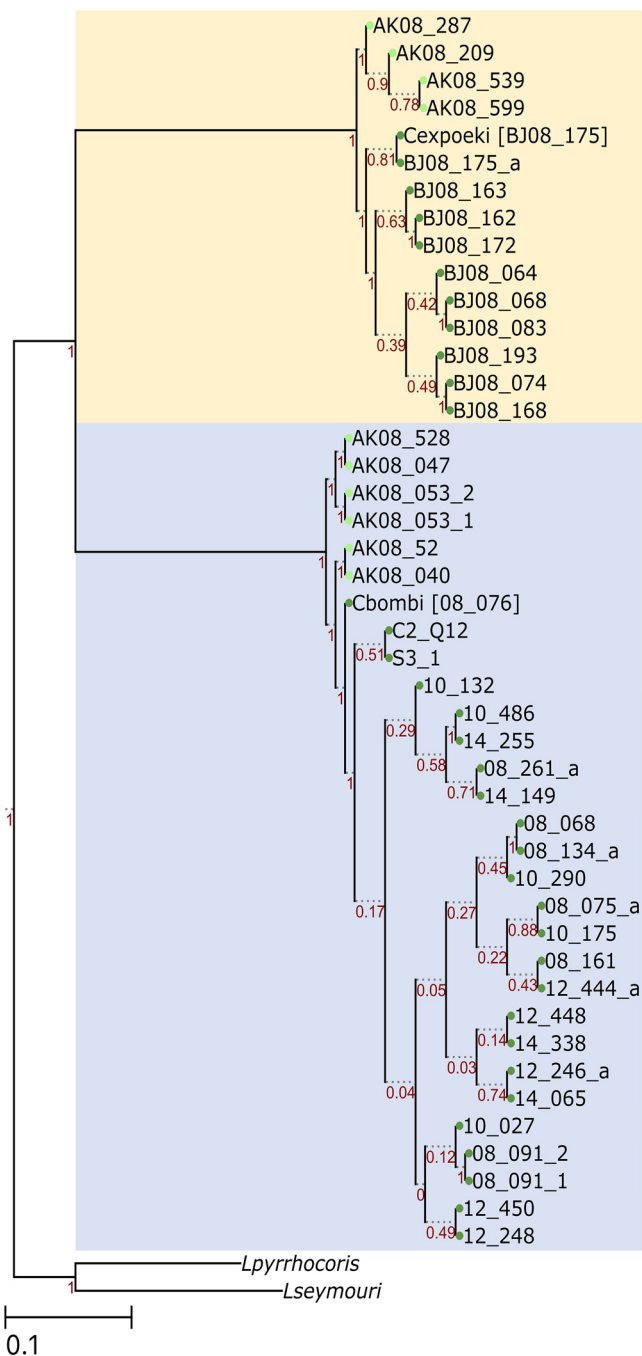


FIG 4 Phylogenetic analyses. A RaxML phylogenetic tree of two studied *Crithidia* strains is presented. *Leptomonas pyrrhocoris* H10 and *L. seymouri* were used as outgroups. *Crithidia bombi* and *C. expoeki* strains are on blue and moccasin backgrounds, respectively. The nodes of Alaskan and Switzerland strains are marked with light and dark green circles, respectively.

strains, but the type strain B08_176 (a possible artifact of a reference mapping bias due to low SNP); and (iv) low bootstrap support values and topology dependency on the algorithm used within Alaskan and Switzerland clades of both *Crithidia* spp. indicate that these strains are very close and hard to resolve. A basal position of the Alaskan strains of both species may provide a hint regarding the origin of these strains.

Copy number variation. A copy number variation analysis of *C. bombi* and *C. expoeki* strains revealed 306 and 259 genome loci with CNVs, respectively. Locus deletions were as common as locus multiplications, and CNVs were rarely associated with gene bound-

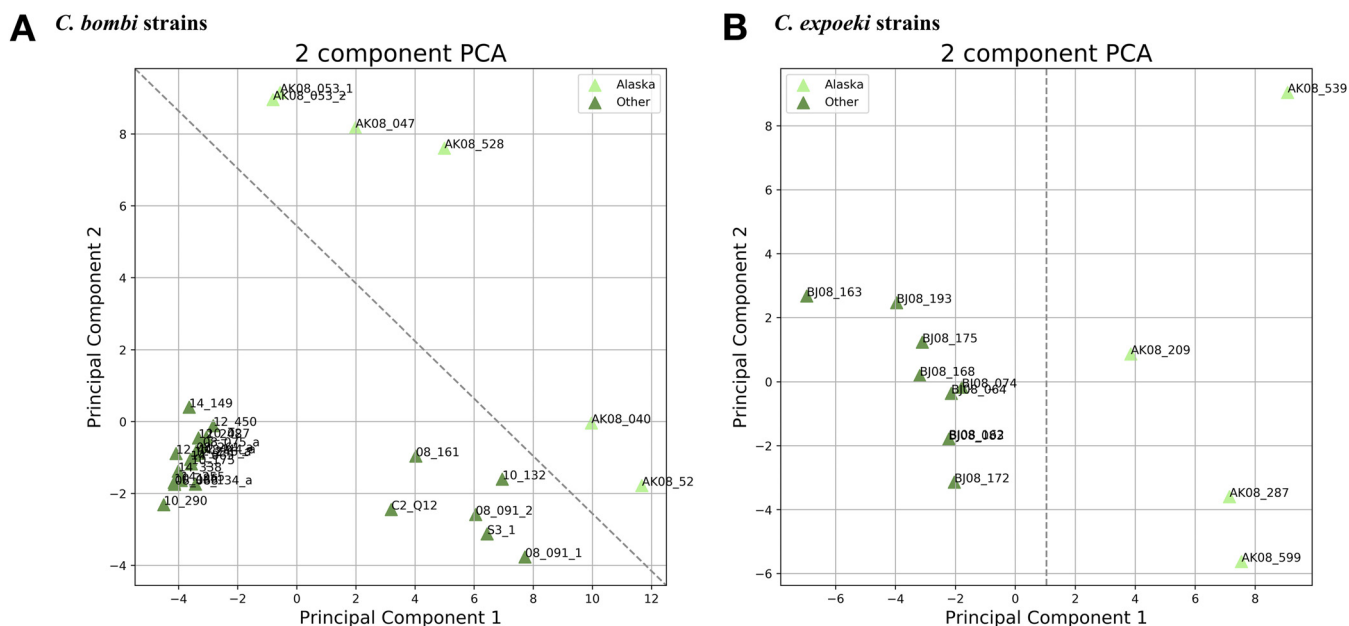


FIG 5 PCA for CNV analysis. A scatterplot of *Crithidia bombi* (A) and *C. expoeki* (B) strains shows the first and second principal components of PCA based on results of CNV analysis. Alaskan and Switzerland strains are marked with light and dark green triangles, respectively.

aries, most of them not overlapping with genes. Principal component analyses (PCAs) demonstrated that the Alaskan strains can be easily separated from the Switzerland strains and tend to be more scattered on the planes of the first and second PCA axes (Fig. 5). In this rendering, the Switzerland strains of *C. bombi* formed two clusters. Whereas most strains were compactly clustered together, forming a first cluster, another small group of strains (08_161, 10_132, 08_091, S3_1, and C2_Q12) formed a separated second cluster. Notably, the Mediterranean strains (S3_1 and C2_Q12) also belonged to this second cluster (Fig. 5A). As with SNP density, the overall amount of variation between the strains was greater for the Alaskan strains.

Signatures of selection and GO enrichment. The nonsynonymous (*dN*) and synonymous (*dS*) substitution rates were estimated for nested M8 versus M7 models and revealed 990 and 430 genes with signs of positive selection for *Crithidia bombi* and *C. expoeki*, respectively.

GO enrichment analysis results are summarized in Table S6. Positively selected genes (Table S6, parts A and B) were enriched with GO terms that are associated with core genome maintenance. These are likely involved in DNA replication, chromosome segregation, and DNA reparation processes. In particular, the GO definitions “DNA metabolic process,” “microtubule-based movement,” “transcription, DNA-templated,” and “microtubule motor activity” were significantly enriched for both species, but the enrichment was more pronounced for *C. bombi*, probably due to the higher overall number of genes under positive selection. Note that “microtubular” genes are associated with the flagellum and its movement. Although not a proof, the importance of these genes was corroborated by a recent study showing that certain nectar compounds, taken up by the bees, can remove the flagellum and reduce the virulence of *C. bombi* (32).

We also analyzed an enrichment in gene lists based on Alaska-specific SNPs (Table S6, part D [*C. bombi*] and part I [*C. expoeki*]), Switzerland-specific SNPs (Table S6, part C [*C. bombi*] and part F [*C. expoeki*]), and SNPs from both populations (Table S6, part E [*C. bombi*] and part J [*C. expoeki*]). The last group is composed of genes that evolve in all strains of *Crithidia* spp. and therefore should reflect common pattern of genome evolution of that species. This enrichment pattern was similar for *C. bombi* and *C. expoeki* and resembles the overall pattern observed for positively selected genes,

indicating the importance of genes involved in DNA repair, DNA replication, chromosome segregation, and cell division processes. The list of enriched genes according to the common SNP analysis largely overlaps the list of positively selected genes according to the M8/M7 LRT analysis.

The enrichment pattern was more complex when we compared lists based on Switzerland-specific and Alaska-specific SNPs only. Alaska-specific *C. bombi* genes showed marginal enrichment with GO-terms connected with the metabolism of fatty acids and genome maintenance, whereas in the Switzerland populations there was a strong enrichment of genes connected with “oxidation-reduction process” and “oxidoreductase activity.” For *C. expoeki*, Alaska-specific genes showed no terms connected with genome maintenance; instead, they were slightly enriched with genes involved in cytochrome complex assembly. Switzerland-specific genes were highly enriched with terms connected to the mitochondrial respiratory chain function (“NADH dehydrogenase activity,” “ubiquinone biosynthetic process,” and “mitochondrial electron transport”). We therefore propose that the evolution of genes involved in mitochondrion electron transport chain functioning is a specific feature of Switzerland populations of both species.

We also analyzed a selected set of genes (*gp63* and *amastins*) from the previous study (30) and found that none of the *gp63*-annotated genes was under positive selection. Three *amastins* from *C. expoeki* (Ce.1.12300, Ce.1.52410, and Ce.1.71020) were under positive selection, and the respective sites with an omega of >1 were Alaska specific. None of the *C. bombi amastins* appeared to be under positive selection.

DISCUSSION

The intestinal parasite of the genus *Crithidia* and their social bumble bee hosts have been studied for many years with respect to behavior, ecology, and genetics (29, 33–35). This host-parasite interaction is characterized by a strong parasite-versus-host colony genotype interaction effect on infection success, such that only a few parasite strains (genotypes) are able to infect a given host colony and, vice versa, a colony is refractory to a range of strains and will only succumb to a few (18, 19, 25, 34). Such host-parasite systems tend to diversify genetically, and we demonstrate here that different strains of *Crithidia bombi* and *C. expoeki* vary considerably in terms of SNP and CNV. We showed that the SNP density is between 1,300 and 1,800 nt for *C. bombi* and ~5,500 nt for *C. expoeki*; it is usually higher in Alaskan strains of both species. These values fit well in the range, known from other trypanosomatids (36, 37). *Crithidia bombi* proved to be more variable also when comparing the average number of variants per strain (6,116 versus 3,409 in *C. expoeki*). Most of the variants in *C. bombi* were found in the Alaskan samples. Interestingly, on a per-strain basis, *C. expoeki* (variations at 18.5 genomic loci/strain) contained more sites with CNV (gene duplications and gene losses) than *C. bombi* (11.3 genomic loci/strain). Taken together, the average genomic distance among strains was higher in the Alaskan samples than in those from the Central Europe, and this was the case for both *Crithidia* spp. (Fig. 5).

The differences between the two locations (Alaska and Central Europe [combining Switzerland and the Mediterranean]) are the most striking. The two cases of genomic variation analyzed here (SNP and CNV) result from mutation events changing single positions or leading to gene duplication or losses. On the other hand, the standing variation, observed on a population level, typically reflects the rate at which new variants emerge versus the rate, at which they are removed (or maintained) by selection. In fact, CNV has been suggested to be adapted to different local selection regimes in *Leishmania* (38) or in trypanosomatids in general (39). The actual selection pressure remains unknown in most cases, but copy numbers determine the level of gene expression. This trait is particularly relevant because of the peculiarities of the genome organization and gene expression in trypanosomatids (31). Previous work on *C. bombi* has demonstrated that the genotypic background of different host colonies exerts selection on which the parasite genotype is able to persist in the host and eventually get transmitted (27, 40), and a part of this variation is due to expression

differences (22). Thus, we must assume that selection is an essential process determining the standing genomic variation of the natural parasite populations. Furthermore, with a more variable genetic background of a host, the parasite population is likely to become more genetically diverse as well. Some evidence for such an effect came from the field studies of *Crithidia bombi* and its host communities, where the host species identity was a significant factor, facilitating the distribution of strains among hosts (28). Host species also affected growth dynamics of the parasites and the seasonality of their epidemics, yielding a given diversity of the strains (29). As such, selection by a diverse host community may be one factor that could explain the higher parasite diversity in Alaskan populations (where there is higher host species diversity) versus Central European populations (where there is lower host species diversity). In addition, one should consider an effect of different ecological factors in these two regions. Host populations are less dense in Alaska than in Central Europe. Consequently, the infection prevalence is also lower in the former (ca. 6%) than in the latter (ca. 33%), simply because there are fewer opportunities to transmit when hosts are rarer. This also means that coinfections of a single host individual by more than one parasite genotype (and also the opportunities to exchange genes among genotypes of *Crithidia*) is lower in Alaska than in Central Europe. In line with this, the effect of niche overlap (the degree to which bees of different species utilize the same flowering plant species) is also different. Niche overlap is known to play a particularly important role for the genetic structure of the infecting parasite population in a given host community (28). In fact, transmission among host individuals from the same or different species is known to occur when they visit the same flower (13). However, even when the same flower is visited, the likelihood of picking up an infection decreases over time since the parasites were last deposited by an infected bee (18). This “waiting time” is much higher in low-density host populations. Thus, the role of a niche overlap for transferring an infection is less prominent in Alaska. Taken together, the diversifying selection by variable hosts and ecological factors suggests that parasite strains in the Alaskan populations should be more separated from each other (Fig. 5) and more variable as a whole (Fig. 3), which was confirmed in the present study.

The population structures of *C. bombi* and *C. expoeki* are typical for most trypanosomatids; i.e., they are somewhere on a continuum of freely mixing, sexual and clonal populations (41, 42). High rates of genetic exchange, and thus sexual reproduction with segregation according to the Mendelian rules, leading to the emergence of novel genotypes, was demonstrated for *C. bombi* (23). Based on some earlier observations, *Crithidia expoeki* is suspected to be more clonal than *C. bombi*, which would fit the overall differences between species observed in this work. Because genetic exchange (which primarily affects the nuclear genome) must be less common in the Alaskan populations, and thus populations will be more clonal, this might also help to explain why the variation in mitochondrial genome was lower than that of its nuclear counterpart in the high-transmission, more sexual populations of Central Europe, compared to the low-transmission, more clonal populations of Alaska. More evidence is needed to establish the causes and consequences of these processes with certainty.

Based on the genomic structure (Fig. 1), codon usage (Fig. 2), phylogenetic analyses (Fig. 4), and pattern of CNV (Fig. 5), the status of *C. expoeki* types as separate species is confirmed in our current work. At the same time, the Alaskan populations appear to be clearly separated from those of Central Europe, according to SNP and SNV data. Codon usage pattern, in contrast, does not separate Alaskan strains from the strains of Central Europe and appears to be a species-specific feature. Within each region and species, the phylogeny is flat, and clustering is also independent of the collection year. This pattern fits a scenario of rapid host-parasite coevolution, where the selective advantage of a given parasite strain is only temporary. Interestingly, parasite strains from Alaska, as well as the type strains of both species, appear to be basal in the rooted tree (Fig. 4). We have no ready explanation for the latter observation, since the type strains were chosen arbitrarily. In contrast, the more basal position of the Alaskan strains may reflect a genuine biological process. Given the close phylogenetic proximity of *Crithidia bombi*

and *C. expoeki* to *Leptomonas* spp. (6, 30), it is plausible to suggest that these *Crithidia* strains entered the *Bombus* host via horizontal transfer from other insects that happened to visit the same plants, just as is observed today with spillovers of parasites from bumblebees to honeybees (43). Of note, mixed infections (facilitating horizontal transfer) are very common in trypanosomatids (26, 44–46). To elaborate a scenario, the *Bombus* clade has emerged in the mountains of Western China approximately 25 Mya ago, in a period of global cooling at the Eocene–Oligocene boundary (47). These insects spread to the East into the Americas and to the West into Europe. Although we have no definitive information on the age of the bumblebee-infecting *Crithidia*, it is possible that they have entered their new host group during the Eastern expansion of the bumblebees, which would render the Alaskan populations basal, as observed here.

It has been postulated that nuclear genome is more conserved compared to its mitochondrial counterpart, probably due to a higher overall mutation rate, determined by the presence of reactive oxygen species. However, this rule is not without exceptions in several lineages of eukaryotes, most prominently, in yeasts (48, 49).

Our study identified a number of genes with a signature of positive selection (Table S6). Several identified categories, such as DNA repair, DNA replication, or chromosome segregation, implicate cell division. The doubling time of *C. bombi* is on the order of 10 to 16 h (50), and a such fast-growing strain can be transmitted to a new host within a few days after infection (51). In culture, a faster-dividing strain almost invariably outgrows its slower competitors (44), but the outcome *in vivo* often depends on the host (26). This implies that other than speed of multiplication factors may influence a parasite's survival in insects. For example, an improved metabolic performance is likely to add to the competitiveness against other strains. These GO-terms are enriched in the Swiss populations, where multiple infection and thus competition among strains within a host is more common than in the Alaska setting. As was implicitly assumed above, selection appears to play a more important role in the Alaska populations than the genetic exchange. In line with this argument, three *amastins*, genes encoding surface proteins implicated in the parasite-host interaction, seem to have originated in the Alaskan populations of *C. expoeki* and were presumably kept under the positive selection pressure.

In summary, our analyses of genomic variation among strains of the bumblebee-infecting *Crithidia* spp. give helpful insights into possible scenarios of selection and diversification of trypanosomatid parasites.

MATERIALS AND METHODS

Sample collection and whole-genome sequencing. *Crithidia* spp. were examined for the presence of trypanosomatids (52) either during field trips at various sites or in the context of sampling spring queens for experimental work in the Zürich lab (Table S1). Samples were either collected and frozen as described previously (11) or collected from fresh feces and processed immediately. In each case, samples were submitted to fluorescence-activated cell sorting to differentiate single cells and produce clonal lines, which were analyzed further (50). Libraries were produced using an Illumina TruSeq kit and sequenced on the Illumina HiSeq 2500 and HiSeq 4000 platforms (Illumina, San Diego, CA) at the Functional Genomic Center, Zürich, Switzerland (Table S1).

Read mapping and processing. Reads were trimmed for quality and sequencing adaptors with Trimmomatic v.0.36 (53) and quality controlled using FastQC v.0.11.8 (54). Trimmed reads were mapped with Bowtie2 v.2.3.4.1 (55) using the “–very-sensitive,” “–end-to-end” options. Downstream processing was done with SAMtools v.1.9 (56, 57) and in-house Python scripts or Linux core utilities (*grep*, *awk*, *sort*, and *uniq*). Uniquely mapped reads were selected for further analyses with *grep* (picking properly aligned pairs by searching the “YT:Z:CP” pattern and discarding reads with secondary alignments by discarding lines with the “XS:i:” pattern), and PCR duplicates were removed with SAMtools. Final sorted .bam files with correctly mapped unique read pairs were used for all downstream analyses.

SNP calling. Variant calling was done using bcftools/SAMtools v1.9. Only SNPs were taken for analysis; indels were filtered out with the “–remove-indels” option. The resulting VCF was used to build distance matrices and generate consensus fasta files using the VCF consensus tool. Gene sequences with SNPs typical for each strain were extracted with a Python script using gene coordinates from the gff annotation (PRJEB21109 and PRJEB21108). Alaska-specific and Switzerland-specific SNPs were determined with a Python script: SNP was counted as Alaskan specific if it was observed only in one or more Alaskan strains but not in any Switzerland strain (and the reverse was used for Switzerland-specific positions). Genes that contain only Alaska-specific SNPs or only Switzerland-specific SNPs were determined with BEDTools v.2.16.2 (58) and an in-house Python script.

Reference-free SNP calling and clustering. DiscoSnp++ (59) was used to obtain SNPs for all sequenced strains (and all strains including previously sequenced type strains 08_076 and BJ08_175 in a separate run) with an algorithm that does not rely on genome reference assembly and therefore can compare samples from different species. Single-end reads were used for 08_076, BJ08_175 (PRJEB21109 and PRJEB21108), and paired-end reads generated in frame of this study were used for all other strains.

Maxicircle assembly. Sequences of the mitochondrial maxicircles of *C. bombi* and *C. expoeki* were assembled from PacBio sequencing data (PRJEB21109 and PRJEB21108), which were used previously for nuclear genome assembly (30). A subset of reads with BLASTN hits to *12s* or *nd5* genes was compiled from the total PacBio data set. This subset was then assembled with the Canu assembler v1.8 (60). Full-length circular molecule was obtained for *C. expoeki*, for which numerous of reads, matching our subsetting criteria, were found. For *C. bombi*, the overall read coverage of maxicircle was much lower and, thus only coding regions with short flanks were assembled. Assembly quality was checked with Illumina paired-end reads mapping (mapping with Bowtie2, average insert size plotting with a Python script). Coding regions were annotated with BLASTN, followed by manual alignment curation of *12s* and *nd5* gene sequences. Coding regions with 500-nt flanks were extracted and used for read mapping. Reads were mapped and processed as described above using the pipeline for the nuclear genome.

Copy number variation analysis. Copy number variation analysis was done with the cn.MOPS package for R (61). Segmentation algorithm was “DNACopy,” and window lengths were 200, 300, 400, or 500 nt. Window length did not change the results greatly, so we selected “WL = 300 nt” for the final analysis. Regions with significant CNV detected were summarized in Tables S2 to S4. A value of 2 corresponds to the normal diploid locus. A value of 1 indicates the loss of a single allele, and a value of 0 signifies the complete loss of this locus. Values of >2 correspond to possible locus multiplications. The states of all detected variable loci were used as feature vectors for clustering and PCA (performed in Python, scikit-learn, and Seaborn packages).

Phylogenetic analysis. Orthologous groups (OGs) of genes were taken from (30). A total of 2,876 OGs containing a single gene per species (1-to-1 orthologues) were selected for this analysis. Two data sets were compiled to build the tree. The first set included all studied *Crithidia* strains, along with the two phylogenetically related species, *Leptomonas seymouri* (44) and *Leptomonas pyrrocoris* (36), to root the tree. This data set is referred to as “Lept.” The second set (referred to as “Tryp”) also included *Blechnomonas ayala*, *Crithidia fasciculata*, and *Trypanosoma brucei* as outgroup species (see references 30 and 62 for details on the outgroups). For the Tryp and Lept data sets, the orthologues for each gene were aligned with mafft v.7.305 (63) with default parameters. These alignments were combined into multilocus alignments and treated with Gblocks v.0.91b (64, 65). Phylogenetic trees were built with the “ETE3 build” from the ETE3 package v.3.1.1 (66) using PhyML (67), RAxML (68), BIONJ (69), and FastTree (70) algorithms. Topology of the other obtained trees was compared to that tree and is accessible in Newick format in Table S5.

For the final alignment of 2,876 genes, 3,795,880 positions (85% of the total alignment length) in 18,508 selected blocks for the Lept data set, and 3,185,151 positions (67%) in 34,891 selected blocks for the Tryp data set, were left after Gblocks polishing. Phylogenetic trees obtained for the Tryp data sets confirmed that two *Leptomonas* species are the closest outgroup for the *Crithidia* spp. studied here. In further analyses, the trees obtained from the Lept data set (based on a greater number of genomic loci) were used. From the four congruent trees, built with different algorithms, we present only the RAxML tree.

Analysis of selection. A total of 7,808 sets of aligned gene sequences for each strain and for each gene of *C. bombi*, as well as 7,851 comparable sets for *C. expoeki* strains, were prepared. The Ete3 python package as a wrapper around PAML’s codeml to search for selection was used. The best model was identified using likelihood ratio test (LRT) for the best-fitting model among pairs of nested models in two tests: (i) M2 versus M1 and (ii) M8 versus M7. Both models were site models and detected positive selection acting on sites. LRT results for M2 versus M1 and for M8 versus M7 were very similar; hence, we used the predictions made with M8 versus M7 for further discussions. For genes in which the LRT was significant, we looked at positively selected sites requesting a Bayes Empirical Bayes posterior probability of >0.95.

GO enrichment. GO enrichment on gene subsets was calculated with the top.GO v.1.0 R package (71). The GO enrichment was performed for subsets of genes that were found to be positively selected in *C. bombi* and in *C. expoeki* strains.

Lists of genes carrying only Alaska-specific SNPs, only Switzerland-specific SNPs, and both types of SNPs were produced. These gene sets were used to discriminate between genes that evolve only in Alaskan strains, genes that evolve only in Switzerland strains (with respect to type strain), and genes that evolve both in Alaskan population and in Switzerland population but have different patterns of nucleotide changes. Top.GO software was used to demonstrate functional enrichment in genes. Enriched GO categories and their definitions are listed in Table S6.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphere.00482-19>.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, PDF file, 0.5 MB.

TABLE S3, PDF file, 0.7 MB.

TABLE S4, PDF file, 0.3 MB.

TABLE S5, XLSX file, 0.01 MB.

TABLE S6, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

This study was financially supported by a Swiss NSF grant (3100A0-116057), an ERC advanced grant (268853 RESIST to P.S.-H.), an ERD Funds Project (OPVVV 16_019/0000759 to V.Y.), and a Russian Science Foundation grant (19-15-00054 [bioinformatics analyses] to V.Y. and E.G.). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We thank Ben Sadd for the samples from Corsica and Sardinia and Derek Sikes (Museum of the North, University of Alaska, Fairbanks) for help with the collection of Alaskan strains in 2008. Data analyzed here were generated in collaboration with the Genetic Diversity Centre, ETH Zurich, Switzerland.

Individual author contributions were as follows: planning of the study and sample collection, P.S.-H. and R.S.-H.; sequencing, N.Z. (Genetic Diversity Centre); data analysis, bioinformatics, and planning of the genomic study, E.G., V.Y., and N.Z.; and writing of the manuscript, E.G. and P.S.-H., with additions from all other authors.

REFERENCES

- Maslov DA, Votýpka J, Yurchenko V, Lukeš J. 2013. Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. *Trends Parasitol* 29:43–52. <https://doi.org/10.1016/j.pt.2012.11.001>.
- Podlipaev SA. 2000. Insect trypanosomatids: the need to know more. *Mem Inst Oswaldo Cruz* 95:517–522. <https://doi.org/10.1590/s0074-02762000000400013>.
- Kostygov AY, Butenko A, Yurchenko V. 2019. On monoxenous trypanosomatids from lesions of immunocompetent patients with suspected cutaneous leishmaniasis in Iran. *Trop Med Int Health* 24:127–128. <https://doi.org/10.1111/tmi.13168>.
- Pacheco RS, Marzochi MC, Pires MQ, Brito CM, Madeira MD, Barbosa-Santos EG. 1998. Parasite genotypically related to a monoxenous trypanosomatid of dog's flea causing opportunistic infection in an HIV positive patient. *Mem Inst Oswaldo Cruz* 93:531–537. <https://doi.org/10.1590/S0074-02761998000400021>.
- Chicharro C, Alvar J. 2003. Lower trypanosomatids in HIV/AIDS patients. *Ann Trop Med Parasitol* 97(Suppl 1):75–78. <https://doi.org/10.1179/000349803225002552>.
- Lukeš J, Butenko A, Hashimi H, Maslov DA, Votýpka J, Yurchenko V. 2018. Trypanosomatids are much more than just trypanosomes: clues from the expanded family tree. *Trends Parasitol* 34:466–480. <https://doi.org/10.1016/j.pt.2018.03.002>.
- Lukeš J, Skalický T, Týč J, Votýpka J, Yurchenko V. 2014. Evolution of parasitism in kinetoplastid flagellates. *Mol Biochem Parasitol* 195: 115–122. <https://doi.org/10.1016/j.molbiopara.2014.05.007>.
- Kostygov AY, Yurchenko V. 2017. Revised classification of the subfamily *Leishmaniinae* (Trypanosomatidae). *Folia PARASIT* 64:20. <https://doi.org/10.14411/fp.2017.020>.
- Schwarz RS, Bauchan GR, Murphy CA, Ravoet J, de Graaf DC, Evans JD. 2015. Characterization of two species of *Trypanosomatidae* from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp. *J Eukaryot Microbiol* 62:567–583. <https://doi.org/10.1111/jeu.12209>.
- Ravoet J, Schwarz RS, Descamps T, Yanez O, Tozkar CO, Martin-Hernandez R, Bartolome C, De Smet L, Higes M, Wenseleers T, Schmid-Hempel R, Neumann P, Kadowaki T, Evans JD, de Graaf DC. 2015. Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. *J Invertebr Pathol* 130:21–27. <https://doi.org/10.1016/j.jip.2015.06.007>.
- Schmid-Hempel R, Tognazzo M. 2010. Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. *J Eukaryot Microbiol* 57:337–345. <https://doi.org/10.1111/j.1550-7408.2010.00480.x>.
- Graystock P, Goulson D, Hughes WO. 2014. The relationship between managed bees and the prevalence of parasites in bumblebees. *PeerJ* 2:e522. <https://doi.org/10.7717/peerj.522>.
- Durrer S, Schmid-Hempel P. 1994. Shared use of flowers leads to horizontal pathogen transmission. *Proc R Soc Lond* 258:299–302.
- Brown MJF, Schmid-Hempel R, Schmid-Hempel P. 2003. Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. *J Anim Ecol* 72:994–1002. <https://doi.org/10.1046/j.1365-2656.2003.00770.x>.
- Otterstatter MC, Thomson JD. 2008. Does pathogen spillover from commercially reared bumble bees threaten wild pollinators? *PLoS One* 3:e2771. <https://doi.org/10.1371/journal.pone.0002771>.
- Schmid-Hempel R, Eckhardt M, Goulson D, Heinzmann D, Lange C, Plischuk S, Escudero LR, Salathe R, Scriven JJ, Schmid-Hempel P. 2014. The invasion of southern South America by imported bumblebees and associated parasites. *J Anim Ecol* 83:823–837. <https://doi.org/10.1111/1365-2656.12185>.
- Whitehorn PR, Tinsley MC, Brown MJ, Darvill B, Goulson D. 2011. Genetic diversity, parasite prevalence and immunity in wild bumblebees. *Proc R Soc B* 278:1195–1202. <https://doi.org/10.1098/rspb.2010.1550>.
- Schmid-Hempel P, Pühr K, Kruger N, Reber C, Schmid-Hempel R. 1999. Dynamic and genetic consequences of variation in horizontal transmission for a microparasitic infection. *Evolution* 53:426–434. <https://doi.org/10.1111/j.1558-5646.1999.tb03778.x>.
- Baer B, Schmid-Hempel P. 2003. Bumblebee workers from different sire groups vary in susceptibility to parasite infection. *Ecol Lett* 6:106–110. <https://doi.org/10.1046/j.1461-0248.2003.00411.x>.
- Barribeau SM, Schmid-Hempel P. 2013. Qualitatively different immune response of the bumblebee host, *Bombus terrestris*, to infection by different genotypes of the trypanosome gut parasite, *Crithidia bombi*. *Infect Genet Evol* 20:249–256. <https://doi.org/10.1016/j.meegid.2013.09.014>.
- Brunner FS, Schmid-Hempel P, Barribeau SM. 2013. Immune gene expression in *Bombus terrestris*: signatures of infection despite strong variation among populations, colonies, and sister workers. *PLoS One* 8:e68181. <https://doi.org/10.1371/journal.pone.0068181>.
- Barribeau SM, Sadd BM, Du Plessis L, Schmid-Hempel P. 2014. Gene expression differences underlying genotype-by-genotype specificity in a host-parasite system. *Proc Natl Acad Sci U S A* 111:3496–3501. <https://doi.org/10.1073/pnas.1318628111>.
- Schmid-Hempel R, Salathe R, Tognazzo M, Schmid-Hempel P. 2011. Genetic exchange and emergence of novel strains in directly transmitted trypanosomatids. *Infect Genet Evol* 11:564–571. <https://doi.org/10.1016/j.meegid.2011.01.002>.
- Tognazzo M, Schmid-Hempel R, Schmid-Hempel P. 2012. Probing mixed-genotype infections II: high multiplicity in natural infections of the trypanosomatid, *Crithidia bombi*, in its host, *Bombus* spp. *PLoS One* 7:e49137. <https://doi.org/10.1371/journal.pone.0049137>.
- Wilfert L, Gadau J, Baer B, Schmid-Hempel P. 2007. Natural variation in the genetic architecture of a host-parasite interaction in the bumblebee *Bombus terrestris*. *Mol Ecol* 16:1327–1339. <https://doi.org/10.1111/j.1365-294X.2007.03234.x>.
- Ulrich Y, Schmid-Hempel P. 2012. Host modulation of parasite compe-

- titition in multiple infections. *Proc Biol Sci* 279:2982–2989. <https://doi.org/10.1098/rspb.2012.0474>.
27. Ulrich Y, Sadd BM, Schmid-Hempel P. 2011. Strain filtering and transmission of a mixed infection in a social insect. *J Evol Biol* 24:354–362. <https://doi.org/10.1111/j.1420-9101.2010.02172.x>.
 28. Salathé RM, Schmid-Hempel P. 2011. The genotypic structure of a multi-host bumblebee parasite suggests a role for ecological niche overlap. *PLoS One* 6:e22054. <https://doi.org/10.1371/journal.pone.0022054>.
 29. Ruiz-Gonzalez MX, Bryden J, Moret Y, Reber-Funk C, Schmid-Hempel P, Brown MJ. 2012. Dynamic transmission, host quality, and population structure in a multihost parasite of bumblebees. *Evolution* 66:3053–3066. <https://doi.org/10.1111/j.1558-5646.2012.01655.x>.
 30. Schmid-Hempel P, Aebi M, Barribeau S, Kitajima T, Du Plessis L, Schmid-Hempel R, Zoller S. 2018. The genomes of *Crithidia bombi* and *C. expoeki*, common parasites of bumblebees. *PLoS One* 13:e0189738. <https://doi.org/10.1371/journal.pone.0189738>.
 31. Maslov DA, Opperdoes FR, Kostygov AY, Hashimi H, Lukeš J, Yurchenko V. 2019. Recent advances in trypanosomatid research: genome organization, expression, metabolism, taxonomy and evolution. *Parasitology* 146:1–27. <https://doi.org/10.1017/S0031182018000951>.
 32. Koch H, Woodward J, Langat MK, Brown MJ, Stevenson PC. Flagellum removal by a nectar metabolite inhibits infectivity of a bumblebee parasite. *Curr Biol*, in press.
 33. Baer B, Schmid-Hempel P. 2001. Unexpected consequences of polyandry for parasitism and fitness in the bumblebee, *Bombus terrestris*. *Evolution* 55:1639–1643. <https://doi.org/10.1111/j.0014-3820.2001.tb00683.x>.
 34. Baer B, Schmid-Hempel P. 1999. Experimental variation in polyandry affects parasite loads and fitness in a bumble-bee. *Nature* 397:151–154. <https://doi.org/10.1038/16451>.
 35. Schmid-Hempel P. 2001. On the evolutionary ecology of host-parasite interactions: addressing the question with regard to bumblebees and their parasites. *Naturwissenschaften* 88:147–158. <https://doi.org/10.1007/s001140100222>.
 36. Flegontov P, Butenko A, Firsov S, Kraeva N, Eliáš M, Field MC, Filatov D, Flegontova O, Gerasimov ES, Hlaváčová J, Ishemgulova A, Jackson AP, Kelly S, Kostygov A, Logacheva MD, Maslov DA, Opperdoes FR, O'Reilly A, Sádlová J, Ševčíková T, Venkatesh D, Vlček Č, Volf P, Votýpka J, Záhonová K, Yurchenko V, Lukeš J. 2016. Genome of *Leptomonas pyrrocoris*: a high-quality reference for monoxenous trypanosomatids and new insights into evolution of *Leishmania*. *Sci Rep* 6:23704. <https://doi.org/10.1038/srep23704>.
 37. Weir W, Capewell P, Foth B, Clucas C, Pountain A, Steketee P, Veitch N, Koffi M, De Meeus T, Kabore J, Camara M, Cooper A, Tait A, Jamonneau V, Bucheton B, Berriman M, MacLeod A. 2016. Population genomics reveals the origin and asexual evolution of human infective trypanosomes. *Elife* 5:e11473. <https://doi.org/10.7554/eLife.11473>.
 38. Bussotti G, Gouzou E, Côrtes Boité M, Kherachi I, Harrat Z, Eddaïkra N, Mottram JC, Antoniou M, Christodoulou V, Bali A, Guerfali FZ, Laouini D, Mukhtar M, Dumetz F, Dujardin J-C, Smirlis D, Lechat P, Pescher P, El Hamouchi A, Lemrani M, Chicharro C, Llanes-Acevedo IP, Botana L, Cruz I, Moreno J, Jeddi F, Aoun K, Bouratbine A, Cupolillo E, Späth GF. 2018. *Leishmania* genome dynamics during environmental adaptation reveal strain-specific differences in gene copy number variation, karyotype instability, and telomeric amplification. *mBio* 9. <https://doi.org/10.1128/mBio.01399-18>.
 39. Reis-Cunha JL, Valdivia HO, Bartholomeu DC. 2018. Gene and chromosomal copy number variations as an adaptive mechanism towards a parasitic lifestyle in trypanosomatids. *Curr Genomics* 19:87–97.
 40. Yourth CP, Schmid-Hempel P. 2006. Serial passage of the parasite *Crithidia bombi* within a colony of its host, *Bombus terrestris*, reduces success in unrelated hosts. *Proc R Soc B* 273:655–659. <https://doi.org/10.1098/rspb.2005.3371>.
 41. Tibayrenc M, Ayala FJ. 2013. How clonal are *Trypanosoma* and *Leishmania*? *Trends Parasitol* 29:264–269. <https://doi.org/10.1016/j.pt.2013.03.007>.
 42. Koffi M, De Meeus T, Sere M, Bucheton B, Simo G, Njiokou F, Salim B, Kabore J, MacLeod A, Camara M, Solano P, Belem AM, Jamonneau V. 2015. Population genetics and reproductive strategies of African trypanosomes: revisiting available published data. *PLoS Negl Trop Dis* 9:e0003985. <https://doi.org/10.1371/journal.pntd.0003985>.
 43. Fürst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJ. 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature* 506:364–366. <https://doi.org/10.1038/nature12977>.
 44. Kraeva N, Butenko A, Hlaváčová J, Kostygov A, Myšková J, Grybchuk D, Leštínová T, Votýpka J, Volf P, Opperdoes F, Flegontov P, Lukeš J, Yurchenko V. 2015. *Leptomonas seymouri*: adaptations to the dixenous life cycle analyzed by genome sequencing, transcriptome profiling and coinfection with *Leishmania donovani*. *PLoS Pathog* 11:e1005127. <https://doi.org/10.1371/journal.ppat.1005127>.
 45. Votýpka J, Suková E, Kraeva N, Ishemgulova A, Duží I, Lukeš J, Yurchenko V. 2013. Diversity of trypanosomatids (*Kinetoplastea: Trypanosomatidae*) parasitizing fleas (Insecta: Siphonaptera) and description of a new genus *Blechomonas* gen. n. *Protist* 164:763–781. <https://doi.org/10.1016/j.protis.2013.08.002>.
 46. Grybchuk-Ieremenko A, Losev A, Kostygov AY, Lukeš J, Yurchenko V. 2014. High prevalence of trypanosome coinfections in freshwater fishes. *Folia Parasitol* 61:495–504. <https://doi.org/10.14411/fp.2014.064>.
 47. Hines HM. 2008. Historical biogeography, divergence times, and diversification patterns of bumble bees (Hymenoptera: Apidae: *Bombus*). *Syst Biol* 57:58–75. <https://doi.org/10.1080/10635150801898912>.
 48. Freil KC, Friedrich A, Hou J, Schacherer J. 2014. Population genomic analysis reveals highly conserved mitochondrial genomes in the yeast species *Lachancea thermotolerans*. *Genome Biol Evol* 6:2586–2594. <https://doi.org/10.1093/gbe/evu203>.
 49. Ruan J, Cheng J, Zhang T, Jiang H. 2017. Mitochondrial genome evolution in the *Saccharomyces sensu stricto* complex. *PLoS One* 12:e0183035. <https://doi.org/10.1371/journal.pone.0183035>.
 50. Salathé R, Tognazzo M, Schmid-Hempel R, Schmid-Hempel P. 2012. Probing mixed-genotype infections. I. Extraction and cloning of infections from hosts of the trypanosomatid *Crithidia bombi*. *PLoS One* 7:e49046. <https://doi.org/10.1371/journal.pone.0049046>.
 51. Schmid-Hempel P, Schmid-Hempel R. 1993. Transmission of a pathogen in *Bombus terrestris*, with a note on division of labour in social insects. *Behav Ecol Sociobiol* 33:319–327. <https://doi.org/10.1007/BF00172930>.
 52. Votýpka J, d'Ávila-Levy CM, Grellier P, Maslov DA, Lukeš J, Yurchenko V. 2015. New approaches to systematics of *Trypanosomatidae*: criteria for taxonomic (re)description. *Trends Parasitol* 31:460–469. <https://doi.org/10.1016/j.pt.2015.06.015>.
 53. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
 54. Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.
 55. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9:357–359. <https://doi.org/10.1038/nmeth.1923>.
 56. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing Study. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* 25:2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>.
 57. Ramirez-Gonzalez RH, Bonnal R, Caccamo M, Maclean D. 2012. BioSAMtools: ruby bindings for SAMtools, a library for accessing BAM files containing high-throughput sequence alignments. *Source Code Biol Med* 7:6. <https://doi.org/10.1186/1751-0473-7-6>.
 58. Quinlan AR. 2014. BEDTools: the Swiss-Army tool for genome feature analysis. *Curr Protoc Bioinformatics* 47:11.12.1–11.12.34. <https://doi.org/10.1002/0471250953.bi1112s47>.
 59. Peterlongo P, Riou C, Drezén E, Lemaitre C. 2017. DiscoSnp++: *de novo* detection of small variants from raw unassembled read set(s). *bioRxiv* <https://doi.org/10.1101/209965>.
 60. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res* 27:722–736. <https://doi.org/10.1101/gr.215087.116>.
 61. Klambauer G, Schwarzbauer K, Mayr A, Clevert DA, Mitterecker A, Bodenhofer U, Hochreiter S. 2012. cn.MOPS: mixture of Poissons for discovering copy number variations in next-generation sequencing data with a low false discovery rate. *Nucleic Acids Res* 40:e69. <https://doi.org/10.1093/nar/gks003>.
 62. Opperdoes FR, Butenko A, Flegontov P, Yurchenko V, Lukeš J. 2016. Comparative metabolism of free-living *Bodo saltans* and parasitic trypanosomatids. *J Eukaryot Microbiol* 63:657–678. <https://doi.org/10.1111/jeu.12315>.
 63. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30:772–780. <https://doi.org/10.1093/molbev/mst010>.
 64. Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 17:540–552. <https://doi.org/10.1093/oxfordjournals.molbev.a026334>.

65. Talavera G, Castresana J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst Biol* 56:564–577. <https://doi.org/10.1080/10635150701472164>.
66. Huerta-Cepas J, Serra F, Bork P. 2016. ETE3: reconstruction, analysis, and visualization of phylogenomic data. *Mol Biol Evol* 33:1635–1638. <https://doi.org/10.1093/molbev/msw046>.
67. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59:307–321. <https://doi.org/10.1093/sysbio/syq010>.
68. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30:1312–1313. <https://doi.org/10.1093/bioinformatics/btu033>.
69. Gascuel O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Mol Biol Evol* 14:685–695. <https://doi.org/10.1093/oxfordjournals.molbev.a025808>.
70. Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 26:1641–1650. <https://doi.org/10.1093/molbev/msp077>.
71. Alexa A, Rahnenfuhrer J, Lengauer T. 2006. Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics* 22:1600–1607. <https://doi.org/10.1093/bioinformatics/btl140>.