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ORIGINAL ARTICLE

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DNA from museum samples of a parasitoid wasp genus (Braconidae: *Syntretus*) offers novel insights into host-parasitoid interactions

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Abstract

1. Parasitoid wasps are a large group of species-rich superfamilies within the order Hymenoptera which form an essential part of terrestrial ecosystems. Many species hold additional value as natural enemies of agricultural pests. Considering their ecological and economic importance, it is perhaps surprising that a significant proportion of these insects are understudied.
2. Here we focus on one genus of parasitoid wasp, *Syntretus*, due to its parasitism of short-haired bumblebee (*Bombus subterraneus*) queens from Sweden, investigated as part of a reintroduction programme in the United Kingdom (UK).
3. We used a genome-skimming approach to recover mitochondrial and nuclear DNA from single legs of *Syntretus* museum specimens, to elucidate the metagenomic content of archival samples and assess their suitability for use in phylogenetic analysis. Utilising 6.7 kb of genomic DNA, we recovered two clades within *Syntretus*, a possible consequence of host choice.
4. Our analyses also recovered commensal polydnavirus sequences, which is the first time this endogenous virus has been identified in this parasitoid genus. These commensal virions are likely used by egg-laying female *Syntretus* to circumnavigate host immune responses so that larvae can develop. Our polydnavirus Bayesian

Ian Barnes, Mark J.F. Brown and Selina Brace contributed equally to this study.

Stefan Schmidt died as the manuscript was being completed. We would like to remember all he did for Hymenoptera systematics.

phylogeny suggests that the viral genomes may have different evolutionary histories to their *Syntretus* hosts, which we infer from the lack of support for co-speciation in this symbiosis.

5. Finally, we elucidate a novel host-parasitoid relationship by identifying that *S. politus* parasitises *B. subterraneus* in Sweden. Combined, our results demonstrate the value of museum collections in undertaking detailed host-parasitoid studies, which can, in turn, inform conservation strategies.

KEYWORDS
cytochrome c oxidase I, insect conservation, metagenomics, phylogenetics, short-haired bumblebee

INTRODUCTION

Parasites, in the broad sense, are ubiquitous (de Meeûs & Renaud, 2002) and have negative impacts on both host fitness (Hudson et al., 1992) and populations (Orr, 1988; Schmidt et al., 2003). Nonetheless, parasitic organisms are essential for ecosystem composition and function (Bale et al., 2008; Brown, 2022; Hudson et al., 2006; Jervis et al., 1993; Lafferty et al., 2006). Partly because their presence is often concealed for at least part of their life-cycle, they can be easily overlooked, which can lead to undervaluation of their ecological and economic significance.

Unlike parasites, in the strict sense, so-called parasitic wasps (Insecta: Hymenoptera) kill their host, which is almost always an insect (rarely a spider), and are better termed parasitoids. It is only the larval stage that has an association with the host; the free-living adult female places egg(s) on or in the host, typically specialising on one or a narrow range of host species. Due in part to their species richness and relative abundance, parasitoid wasps are integral components of terrestrial ecosystems and have many incidental functions aside from the reduction of host numbers (Forbes et al., 2018; Polaszek & Vilhemsen, 2023; Veijalainen et al., 2012). Parasitoid wasps may be a reliable food supply for a variety of animals (Quicke, 2015), provide essential pollination services (Jervis et al., 1993; Menz et al., 2015) and help regulate host population densities (Orr, 1988; Quicke, 2015; Schmidt et al., 2003). Given their substantial ecological significance, it is surprising that parasitoid wasps are broadly understudied or neglected (Shaw & Hochberg, 2001).

There is a major dichotomy in the developmental biology of parasitoids: either the host is permanently paralysed or killed at the time of oviposition (idiobiont parasitoids), or the host is able to carry on with its life while the parasitoid larva(e) develops (koinobiont parasitoids) (Askew & Shaw, 1986). Most koinobionts are internal parasitoids and tend to be more host-specialised than idiobionts (which more often feed externally on a concealed host). Although most hymenopteran parasitoids develop on early stages of the host, members of one subfamily of Braconidae, Euphorinae, parasitise the adult stages of a variety of insects (Shaw & Huddleston, 1991). This subfamily includes the genus *Syntretus* (Braconidae: Euphorinae). Until recently it was believed that species of *Syntretus* are exclusively koinobiont endoparasitoids of adult apocritan Hymenoptera, species with known biology being either solitary parasitoids of Ichneumonidae (Cole, 1959) or gregarious parasitoids of bumble bees (Alford, 1968). However, a new species of *Syntretus*, from North America, has been identified as a parasitoid of adult *Drosophila* (Diptera: Drosphilidae) (Moore et al., 2024). This discovery will need to be taken into account in future analyses of the genus, though its relevance to our study is slight. To circumnavigate host immune responses and ensure larvae can develop, some clades of Ichneumonoidea, including *Syntretus* spp., utilise a commensal endogenous polydnavirus (*Polydnaviridae*: Bracovirus) (Espagne et al., 2004). These viruses occur in a number of koinobiont clades (Strand & Burke, 2014) and are incorporated into the parasitoid's genome (Bézier et al., 2009). When a gravid female lays eggs inside an insect host, virions are injected along with the egg and can be expressed in the host to interrupt cellular defences which would otherwise impact parasitoid egg/larval development. Consequently, a single host may be infected with multiple larvae yet appear relatively healthy. Nevertheless, when larvae leave their host to pupate and emerge as adults, they kill their host in the process, making *Syntretus* highly effective parasitoids (Alford, 1968; Brown et al., 2017). However, there is a paucity of information regarding *Syntretus* host-parasitoid interactions, largely because the koinobiont endoparasitoid larvae, for which morphological species delimitation (van Achterberg & Haeselbarth, 2003) is at present impossible, are rarely reared through to adulthood. One solution is to use a genomic approach, which can differentiate species (Hebert et al., 2003; Turvey et al., 2019) and elucidate host-parasitoid interactions in the absence of reliable field data or reared material (Heintzman et al., 2014). While there is a distinct lack of genomic data for many species of parasitoid wasps, previous phylogenetic work on Euphorinae has taken a combined mitochondrial and nuclear DNA approach to help delineate clades, although this was based on relatively few species per genus (Stigenberg et al., 2015).

Museum collections have a central role in species identification, as they contain voucher specimens which have been authoritatively determined (or can be reidentified). Although DNA quality in museum specimens is invariably poorer than in fresh material, protocols and laboratory methods designed to work with ancient DNA (aDNA) can facilitate the retrieval of DNA from archival samples (Mullin et al., 2022; Sampaio et al., 2023). Consequently, museum collections may be co-opted to undertake detailed molecular phylogenetic and autecological analysis that can inform our understanding of elusive host-parasitoid interactions (Emery et al., 2009; Heintzman et al., 2014).

The short-haired bumblebee (*Bombus subterraneus* (L.)), which can be parasitised by *Syntretus* spp., was declared locally extinct in the United Kingdom (UK) in 2000 (Gammans & Allen, 2014; Nieto et al., 2014). Between 2012 and 2016, inclusive, a reintroduction programme was undertaken in the UK by the Short-haired Bumblebee project (SHBP), coordinated by the Bumblebee Conservation Trust (BBCT). In brief, wild caught Swedish *B. subterraneus* queens were collected and held in quarantine in the UK to screen for parasites and parasitoids, before being released at a field site in Dungeness, Kent, UK (Brown et al., 2017). During the reintroduction programme, infection with *Syntretus* spp. larvae, which could not be identified to species level using morphology, was recorded as the proximate cause of death in 16.1% of the quarantined bumblebee queens (67/415). Here, we analyse taxonomically validated collections specimens, using aDNA methods, to establish the identity of *Syntretus* spp. larvae recovered during the *B. subterraneus* UK reintroduction, and contribute to future conservation efforts. More broadly, we explore the potential for genome skimming data from collections material (Raxworthy & Smith, 2021) to conduct both phylogenetic and metagenomic analyses on a subset of the *Syntretus* genus.

METHODS

Next-generation sequencing of adult *Syntretus* voucher specimens

We worked with 28 voucher specimens representing 12 *Syntretus* species, courtesy of the National Museum Scotland (NMS) and the Bavarian State collection of Zoology, Germany (ZSM), upon request of the Short-Haired Bumblebee Project. All specimens were collected from across Europe (1961–1992) using Malaise traps, and were subsequently kept in a variety of conditions not suited to DNA preservation, before being stored in an 80% alcohol solution (Supplementary Table 1). A single middle leg (including femur, tibia and tarsus, and sometimes coxa), which has not been used for morphological identification (van Achterberg & Haeselbarth, 2003), was used for molecular analysis through genome skimming.

All (pre-indexing) laboratory work (DNA extraction and library building) for the archival voucher specimens was carried out in a dedicated aDNA laboratory at the Natural History Museum (London, UK). Genomic DNA was extracted using the QIAamp DNA micro kit (Qiagen, UK) with a modified version of the tissues protocol by conducting the final eluting step twice (40 µL each). Overnight lysis was conducted for 20 h in a heated orbital incubator at 56°C. Using 20 µL DNA extract for each sample, libraries were constructed using a modified version of the Meyer and Kircher (2010) protocol, without the initial DNA fragmentation step. Samples were double-indexed, and the index PCR was set for 20 cycles with three PCR reactions conducted per library. Index libraries were then diluted to an equimolar concentration and pooled. The multiplexed samples were sequenced using 2 × 75 bp paired read kits on an Illumina NextSeq platform, using a single lane on a paired-end flow cell. The resulting.fastq (file containing sequence data and quality scores for each nucleotide) files were quality checked in CLC Genomics Workbench (Qiagen) and used for de novo assembly.

De novo assembly was carried out using SPAdes v3.14.1, with a minimum contiguous sequence (hereafter referred to as contig) size set to 200 bp and a k-mer length of 31. Assembled contigs for each sample were then saved as individual local BLAST databases. The National Centre for Biotechnology Information (NCBI) GenBank database was then used to identify available *Syntretus* COI, 18 and, 28 s sequences and a full mitogenome from the closely related subfamily Helconinae (Supplementary Table 2). Finally, a bracovirus sequence was downloaded from GenBank to recover polydnavirus commensal sequences (Supplementary Table 2). These Genbank sequences were then used as seeds to search each local BLAST database for matches (Altschul et al., 1990) (gap penalty and mismatch costs were increased to maximum). Matched contigs for each sample were concatenated using CLC Genomics Workbench (Qiagen), leading to nine identified genes (7 × mitochondrial [mt] [ATP6, COI, COII, Cytb, ND1, ND4, ND5] and 2 × nuclear [18, 28 s]), representing 6.7 kilobases (kb) of sequence data. We also recovered approximately 1 kb of bracovirus sequence for use in phylogenetic analysis. For each sample the original merged reads were mapped back to the produced contig to give a figure for average coverage depth using a combination of Burrows-Wheeler Aligner v0.7.13 and SAMtools v1.9. Where de novo assembly was unsuccessful, presumably due to the low preservation of insect-specific DNA in a sample, a successful de novo-assembled concatenated contig was used as a scaffold to map reads to. Read coverage for all sequences was visually inspected in Tablet v1.19.09.03.

Metagenomic analysis of archival *Syntretus* samples

To assess the metagenomic composition of each sample, the first 10% of generated de novo contigs per sample were used as megaBLAST input files. This ensured the largest contigs were used and removed any false signal that might occur from the use of shorter contigs (analysed contig range 502–15,892 bp). The resulting megaBLAST taxonomic assignment file was visualised in MEGAN v.4.70.3 (Huson et al., 2011). Input parameters were set to minScore = 50, minComplexity = 0.44, topPercent = 10, winScore = 0 and minSupport = 5 as recommended in Heintzman et al. (2014) to ensure contigs with low quality BLAST hits were excluded. Taxonomic assignment was taken for the rank of Class for comparison across samples. The metagenomic graphical output was created in ggplot2 (Wickham, 2016) using R programming language (R Core Team, 2018).

Syntretus larval detection and cytochrome c oxidase I (COI) characterisation

Four hundred and fifteen free-flying, Swedish *B. subterraneus* queens were collected and quarantined in the UK between 2012 and 2016

inclusive, as previously described (Brown et al., 2017, MJFB unpublished data). In total, 67 queens died where *Syntretus* infection was determined as the proximate cause of death (Brown et al., 2017). All *Syntretus* larvae, which were always gregarious, recovered from dead queens were stored at -20°C until they were required for molecular analysis. For DNA extraction, larvae ($n = 33$; individual larvae came from separate queens, therefore, queens sampled = 33) were removed from −20°C storage and submerged briefly in 70% ethanol, before being submerged in distilled water to remove any remaining host tissue which may contaminate the extraction procedure and confound sequencing results. Genomic DNA was extracted from samples using 400 μL of Chelex®, preheated to 100°C, in an Eppendorf tube containing a 5 mm ball-bearing to lyse tissue. Sterilised forceps were used for each larva to minimise the risk of contamination. Samples were then placed in a Qiagen Tissue Lyser II for 3 min at 30 Hz. Lysed samples were then incubated at 85°C for 8 min, before spinning in a centrifuge for 3 min at 16.356 *G*, and assessing DNA yield using a nanodrop.

The invertebrate-based, broad-spectrum primers (LCO1490 & HCO2198) were used to amplify a 685 base pair (bp) region of the Cytochrome c oxidase I (COI) from each larval sample where DNA extraction was successful ($n = 32$) (Folmer et al., 1994). Cycling conditions were set to 94°C for 1 min, five 1-min cycles at 94°C, 1.5 min at 45°C and 1.5 min at 72°C, before 35 1-min cycles at 94°C, 1.5 min at 50°C, 1 min at 72°C and a final step of 5 min at 72°C. Resulting amplicons were visualised on 1.5% agarose gel to confirm amplification product size, before being sent for Sanger sequencing c/o Source Biosciences (Cambridge, UK). The resulting sequences ($n = 32$) were imported, and quality checked in Geneious v7.0, resulting in a 572 bp region of the COI gene. Thirty-one COI sequences were identical; the other sequence had adenine instead of guanine at base 254, which was attributed to a low-quality base call score. Therefore, one 572 bp consensus sequence, not including the erroneous sequence, was used for all downstream analysis.

Phylogenetic analysis to identify *Syntretus* larvae and investigate DNA preservation in archival samples

We ran three separate Bayesian phylogenetic analyses. For each phylogeny, sequences were aligned in Mafft v7.471 before being imported into BEASTv1.10.4. Appropriate nucleotide substitution models were selected using jModelTest v2.1.10 (substitution partitions as follows, combined mt sequence HKY + I + G, combined nuclear K80 + I) and MCMC chains were run for 1×10^7 generations, sampling every 1×10^4 generations, unless otherwise stated. To elucidate the identity of the *Syntretus* larvae recovered during the *B. subterraneus* reintroduction programme, a COI phylogeny was created using the Sanger-derived larval sequence (described above), three GenBank sequences and 12 sequences generated in this investigation using de novo assembly. We used *Eumacrocentrus* sp. (Helconinae) (GenBank accession: KF385872) as an outgroup as this was the closest (phylogenetically), near-complete mitochondrial genome, to compare to *Syntretus* (Stigenberg et al., 2015). A second phylogeny was created for nine samples for which we were able to obtain 6.7 kb of sequence data (hereafter referred to as mt and nuclear phylogeny); as, before, we used *Eumacrocentrus* sp. an outgroup. Finally, we produced a phylogeny for the recovered 1 kb of polydnavirus sequence information; here we used a polydnaviral sequence recovered from *Cotesia sesamiae* (Cameron) (GenBank accession: HF586479) as an outgroup.

For all phylogenies, nodal support was determined by approximate posterior probabilities and log files were analysed in Tracer v1.7.1 to check effective sample size. A 10% burn-in was included for each analysis (TreeAnnotator v1.10.4), before tree visualisation and annotation in FigTree v1.4.4.

RESULTS

Metagenomic data analysis of archival *Syntretus* samples

Insect-specific content ranged between 42.6% and 92% across 16 sequenced archival *Syntretus* samples. Known symbionts of the Braconidae were identified in low proportions. Notably, *Wolbachia* (recovered in all 16 sequenced samples) and Polydnaviridae sequences (9/16) were identified (Figure 1). Samples contained a variety of potential contaminant contigs, including plant, fungal and indirect human contamination typified by the presence of Actinobacteria and Gammaproteobacteria, for example.

Syntretus phylogeny using NGS data of archival samples

Genomic DNA was successfully extracted from 18 archival *Syntretus* samples. After quality check and filtering of raw sequencing reads we

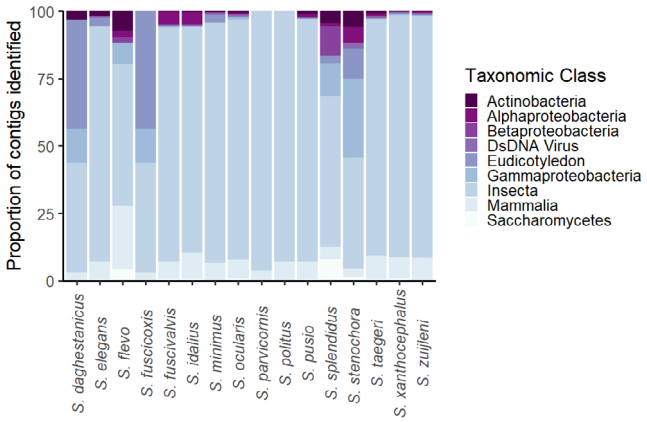

FIGURE 1 Metagenomic content of extracted DNA from 16 archival *Syntretus* samples. The nine most abundant classes identified are illustrated. Five samples (*S. daghestanicus*, *S. flevo*, *S. fuscicoxis*, *S. splendidus* and *S. stenochora*) showed relatively high levels of contamination, which is likely a consequence of poor sample preservation resulting in insect-specific DNA degradation.

obtained an average read length of 53 bp (range: 41–67 bp) and Adenine – Thymine (A-T) content ranging from 45.20% to 74.91% (Table 1). There was a strong positive correlation between the number of reads sequenced and number of contigs produced (PMCC $r = 0.75$, $n = 18$), as would be expected. Three archival *S. splendidus* samples (a known parasitoid of bumblebees; Alford, 1968) were used in an attempt to extract *S. splendidus* DNA. However, with low A-T content (which can be indicative of insect DNA) and a relatively low number of contigs produced ($n = 61$–1706), insufficient Braconidae-specific sequence was retrieved, so these were excluded from our mt and nuclear phylogenetic analysis (Figure 2). However, we did recover a *Syntretus*-specific COI sequence from one of the *S. splendidus* samples, which is why this species was included in the COI phylogeny (Figure 4). Incidentally, this sample had the highest A-T content, which

TABLE 1 Sequencing data for the 18 *Syntretus* voucher specimens that were used across our phylogenetic analyses.

Species	Geographic origin (collection date)	Number of reads	Number of contiguous sequences	Average fragment length	A-T content (%)
S. daghestanicus ♀	Italy (1980)	5,315,529	14,217	45.99	65.51
S. elegans ♀	Hungary (unknown)	9,008,590	18,727	43.72	64.35
S. flevo ♀	United Kingdom (1986)	8,432,301	15,896	65.47	61.86
S. fuscicoxis ♀	Denmark (1984)	3,826,904	658	40.62	61.67
S. fuscivalvis ♀	United Kingdom (1986)	10,289,555	155,201	67.73	64.61
S. idalius ♀	United Kingdom (1991)	11,615,151	203,963	74.91	65.76
S. minimus ♀	Germany (1985)	1,558,862	3307	56.50	60.14
S. ocularis ♀	United Kingdom (1985)	12,503,592	11,0618	61.35	64.03
S. parvicornis ♀	Germany (1985)	3,677,935	4294	48.87	60.34
S. politus ♀	Italy (1966)	5,909,038	5984	44.32	64.78
S. pusio ♀	United Kingdom (1986)	14,555,598	169,813	66.73	64.61
S. splendidus (*1*) ♀[a]	Italy (1966)	1,343,249	61	43.55	67.00
S. splendidus (*2*) ♀	Italy (1967)	859,937	79	43.27	57.10
S. splendidus (*3*) ♀	United Kingdom (1989)	5,947,164	1706	66.05	45.20
S. stenochora ♀	Germany (1974)	697,220	168	42.27	52.99
S. taegeri ♀	United Kingdom (1986)	28,178,549	173,355	61.93	66.51
S. xanthocephalus ♀	United Kingdom (1985)	18,072,123	178,035	70.18	67.05
S. zuijleni ♀	United Kingdom (1985)	11,096,845	156,904	65.11	64.30

[a]Used in phylogenetic analysis as a *Syntretus*-specific COI was recovered. All other *S. splendidus* samples were excluded from phylogenetic analysis.

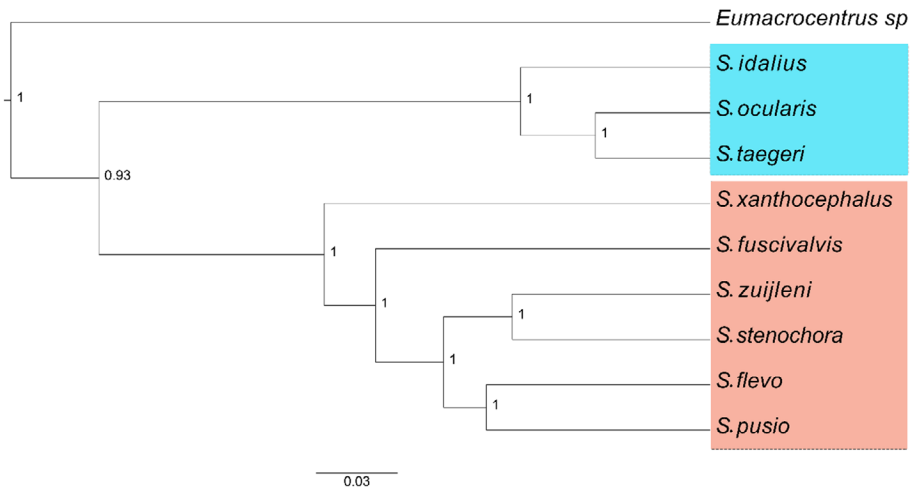

FIGURE 2 Bayesian phylogeny of *Syntretus* archival samples, using 6.7 kb of sequence consisting of ATP6, COI, COII, Cytb, ND1, ND4, ND5, 18 and 28 s genes. There are two distinct, well supported clades, which have been highlighted, identified within the phylogeny. Node labels represent posterior probability and scale bar represents substitutions per site. *Eumacrocentrus* sp. has been included as an outgroup.

was similar to the other adult samples from which we successfully extracted *Syntretus*-specific DNA. The mt and nuclear phylogeny using 6.7 kb of sequence data recovered two distinct, novel, well-supported clades. Neither molecular clock nor net diversification estimates were undertaken due to incomplete sampling of the genus. There are 43 species currently recognised globally in *Syntretus*, with 20 recorded in Europe (van Achterberg & Haeselbarth, 2003), and only nine archival samples had sufficient sequence data for our phylogenetic analysis.

Polydnaviridae phylogeny derived from archival samples

Approximately 1 kb of polydnavirus sequence data was recovered from nine *Syntretus* archival samples. We were unable to recover polydnaviral sequence data from all samples included in the mt and nuclear phylogeny (6/9 species are the same). A phylogeny of endogenous *Bracovirus* virions was produced using *Cotesia congregata* (Say) bracovirus (GenBank accession number: HF586479) as an outgroup (Figure 3). This Bayesian phylogeny illustrates convincing sequence delimitation with strong nodal support. The *Bracovirus* phylogeny appears to have limited congruence in topology with the archival *Syntretus* phylogeny (Figure 2) presumably indicating a lack of co-speciation in this symbiosis.

Syntretus larval identification using cytochrome c oxidase I (COI)

A 573 bp region of the COI gene was successfully amplified and sequenced from unidentified *Syntretus* larvae that had infected Swedish *B. subterraneus* queens. In addition, matching COI regions were identified from Illumina reads for 12 of our archival samples. To increase the likelihood of identifying the larval sequence to species level, three GenBank COI sequences were also included in the alignment and resulting phylogeny (Figure 4). The unknown larval sequence formed a monophyletic group, with our de novo assembled COI region from *S. politus*, suggesting that they are the same species. Similar to the mt and nuclear phylogeny two distinct clades, with similar species composition, were recovered (Figure 2). Further, the use of additional sequence data in our mt and nuclear phylogeny increased the approximate posterior probability of nodes, confirming that using a combination of nuclear and mitochondrial DNA has produced stronger phylogenetic signal.

DISCUSSION

We applied ancient DNA methods to museum material to enhance our understanding of an ecologically important parasitoid wasp genus. We recovered sufficient sequence data to demonstrate that archival insect samples are suitable for detailed phylogenetic and metagenomic analysis, including identification of two well supported clades and the retrieval of commensal endogenous polydnavirus virion sequences. In addition, we co-opted our *Syntretus* COI phylogeny and identified a formerly unrecognised parasitoid of *B. subterraneus*, which was recovered during a UK reintroduction programme. Our results show that *S. politus*, which is known to occur in both Sweden and England (van Achterberg & Haeselbarth, 2003), had parasitized 33 bumblebee queens in Sweden between 2012 and 2016, ultimately resulting in their death during quarantine in the UK.

Our results highlight the value of museum collections for molecular-based phylogenetic analysis, which, in turn, can inform both host-parasitoid studies (Heintzman et al., 2014) and conservation policy (Jensen et al., 2022; Turvey et al., 2019). We were able to characterise *Syntretus*-specific COI regions from 12 of our archival samples. The resulting COI phylogeny had clear species delimitation,

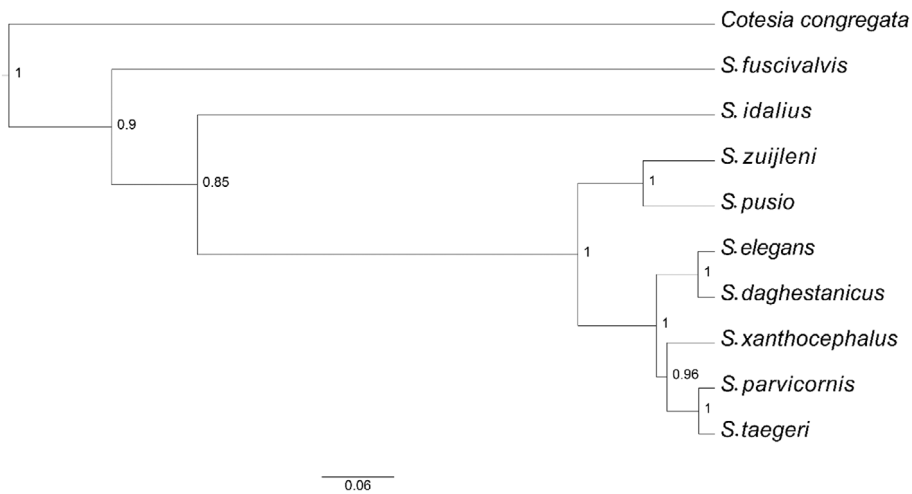

FIGURE 3 Bayesian phylogeny of 1 kb sequence of endogenous *Bracovirus* virions recovered from archival *Syntretus* samples. Node labels represent posterior probability and scale bar represents substitutions per site. A *Bracovirus* sequence recovered from *Cotesia congregata* (GenBank accession number: HF586479), which was used as a scaffold to map reads to, has been included as an outgroup.

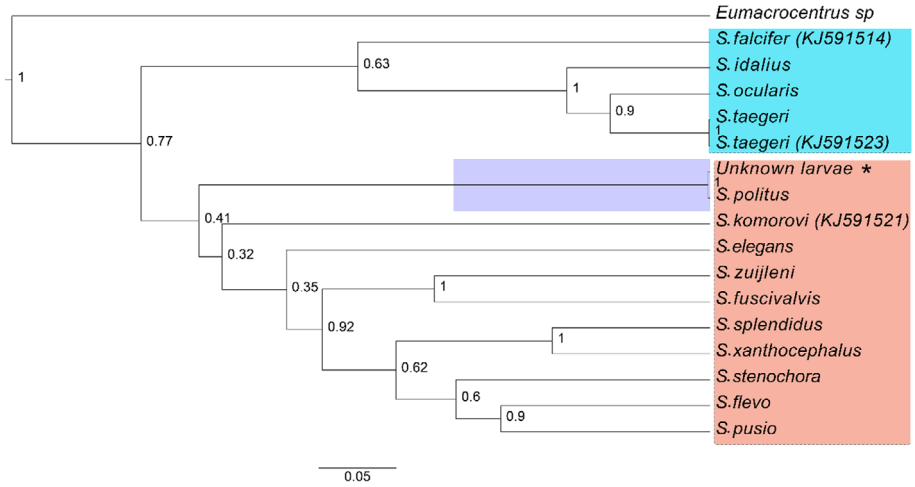

FIGURE 4 Bayesian phylogeny of *Syntretus* Cytochrome c oxidase I (COI) sequences. Sequences from GenBank have accession numbers included. The unknown larval COI (*) is identical to *Syntretus politus*, forming a clade, highlighted in lilac, indicating that the two are likely to be the same species. Congruent with our mt and nuclear phylogeny, there are two distinct, well supported, clades, which are highlighted as in Figure 2 to enable comparison. Node labels represent posterior probability and scale bar represents substitutions per site. *Eumacrocentrus* sp. has been included as an outgroup.

highlighting the well described value of COI 'barcoding' in phylogenetic analysis (Hebert et al., 2003), especially when combined with the use of voucher specimens (Pleijel et al., 2008). Our COI phylogeny recovered two distinct, well supported, groups, which have not previously been identified in *Syntretus* (Stigenberg et al., 2015), however, they do largely correspond to a major division in the key to Palaearctic species (van Achterberg & Haeselbarth, 2003), between species with a laterope on the first metasomal segment (the *splendidus* group) and those without. The only species in the COI phylogeny which is 'misplaced' according to this morphological character is *S. falcifer* (Tobias, 1965), which has been placed in a separate subgenus, *Falcosyntretus* (Tobias, 1965). The species included in both groups broadly have a Palaearctic distribution (van Achterberg & Haeselbarth, 2003) and there are no obvious geographic differences that may have contributed to this differentiation. Consequently, the two clades may be linked to host choice, which can drive parasite/parasitoid adaptation and ultimately speciation (Hafner & Nadler, 1988; Paplauskas et al., 2021). We note that more information on *Syntretus* host choice from field-infected insects is required to confirm this hypothesis. Furthermore, our COI analysis does not include all known *Syntretus* species and only one outgroup species, therefore it is not a definitive phylogenetic topology. However, the split between the two clades is relatively basal in origin and, therefore, it is likely that the differentiation would persist if further species were included. Indeed, our mt and nuclear phylogeny, which included analysis of 6.7 kb of both mitochondrial and nuclear sequence data, also recovered a basal split of *Syntretus* into two distinct groups. During our analysis of NGS derived genomic data we identified *Wolbachia* reads in all archival *Syntretus* samples. *Wolbachia* is an obligate intracellular pathogen of arthropods and can confound COI-derived phylogenetic interpretation (Bartoňová et al., 2021; Whitworth et al., 2007). However, while our mt and nuclear phylogeny included fewer species, where species were the same, they were partitioned into the same clades as the COI tree, suggesting accurate species delimitation, as the genes included in our mt and nuclear phylogenetic analysis are not impacted by *Wolbachia* in the same way as COI. In addition, as all 31 larval samples from different locations across Sweden over 4 years were identical, it is unlikely that *Wolbachia* infection has impacted the recovered COI sequences. However, by using more sequence data, approximate posterior probability support for species delimitation and the two groups was stronger in our mt and nuclear phylogeny when compared with using COI alone (Caravas & Freidrich, 2013; Springer et al., 2001).

The only *Syntretus* species previously known to parasitise bumblebees is *S. splendidus* (Alford, 1968). While this parasitoid was reported to have a relatively broad bumblebee host range, especially across short-tongued bumblebees (Alford, 1968), it was identified at species level at a time that the genus *Syntretus* had not been subjected to modern morphotaxonomic scrutiny, and now many additional European species have been recognised (van Achterberg & Haeselbarth, 2003). This unfortunately makes the determinations underlying Alford's (1968) work unclear, and suggest that a number of *Syntretus* species are involved in the parasitisation of bumblebees, not just *S. splendidus*. However, there is one voucher specimen in the Natural History Museum (London), reared by Alford from *Bombus pascuorum* (Scopoli), and this is *Syntretus splendidus*, as confirmed by van Achterberg and by GRB. Whether any other voucher specimens were deposited elsewhere, or indeed whether any more specimens were reared to adulthood, is unknown.

The identification of *S. politus* as the parasitoid of *B. subterraneus* from Sweden is somewhat unexpected. More generally, there is a paucity of data available on the prevalence and diversity of *Syntretus* infection in bumblebees. Primarily this is a consequence of the imago-endoparasitoid lifestyle of *Syntretus*, meaning that infections are often missed in the wild and, therefore, host-parasitoid relationships and

impact on host fitness are difficult to quantify. Consequently, our results enhance our understanding of *Syntretus* ecology by elucidating a novel host-parasitoid interaction in this elusive genus. Our results demonstrate that prevalence of *S. politus* infection in collected *B. subterraneus* queens in southern Sweden during early May was between 6.8% and 27% between 2012 and 2016, inclusive. Given that at this time of year in Sweden, *B. subterraneus* queens have emerged from their winter hibernacula and are actively foraging prior to colony founding, *Syntretus* infection will likely have a negative impact on annual population density as infected queens are unlikely to produce successful colonies (Alford, 1968). Although *S. politus* is known from the UK, there is limited information on specific geographic range, population density and host choice (van Achterberg & Haeselbarth, 2003). During the UK *B. subterraneous* reintroduction project, 16% of queens died due to *Syntretus* infection while under quarantine (Brown et al., 2017). Therefore, parasitoid infection reduced the viable population of *B. subterraneous* that were released in the UK, which, in turn, decreased the likelihood of successful reintroduction. Unfortunately, to date (winter 2024), there has been no confirmation that *B. subterraneous* has established in the UK following the reintroduction efforts. While *Syntretus* infection resulted in fewer animals being released in the UK, it is likely that other factors, such as predation or native parasites (Goulson et al., 2018) at the reintroduction site, may have also negatively impacted the reintroduction success. Future conservation efforts should investigate disease status of organisms prior to reintroduction and where possible, identify parasitoids and parasites to species level, as we have done here. This approach is key to safeguarding native species by reducing the likelihood of non-native parasitoid co-introduction (Miura et al., 2006) and ensuring healthy animals are used in the reintroduction. It is also recommended that parasitoids are reared to adulthood, where possible, as it makes morphological identification much more straightforward.

Archival samples used in our analysis were collected between 1966 and 1991 across Europe. These samples were collected and identified by Braconidae experts and, therefore, are presumably accurate species records. Here, by using an Illumina-based, shotgun sequencing methodology, we add to a growing body of evidence that highlights the value of museum collections for molecular investigations (Card et al., 2021; Heintzman et al., 2014; Jensen et al., 2022; Mullin et al., 2022; Sampaio et al., 2023; Turvey et al., 2019). Our results show that fragment-length distribution (range = 40.62–74.91) was reasonably conserved across all samples, but with older samples generally producing shorter fragments. However, the total number of reads produced, and putative contamination, differed between samples, which resulted in a disparity of contigs generated by de novo assembly. Given that sample storage history was not recorded in detail it is likely that the differences we have recorded in sequencing efficiency may be linked to sample preservation. Indeed, where we sequenced fewer reads, we typically generated fewer de novo sequences. Insect genomes are known to be A-T rich (Cameron, 2014) and our samples that had the lowest A-T content typically produced the fewest insect-specific DNA de novo contigs. It is likely that this reduction in A-T content is indicative of insect-specific DNA degradation in a sample, and may also indicate higher levels of contamination in shotgun-sequenced archival samples (der Sarkissian et al., 2014; Heintzman et al., 2014).

Alongside the value of museum collections for phylogenetic analysis, our results also demonstrate that shotgun sequencing can provide detailed inferences on the autecology of archival samples. Surprisingly, we were able to recover sequence data from endogenous viral commensals in nine of our archival *Syntretus* samples. These sequences originated from bracovirus virions, which are unique to female Braconidae wasps, such as *Syntretus*, and are transferred into an insect host by an egg-laying female (Espagne et al., 2004). Since these commensal viruses are vital for the success of *Syntretus* progeny development in their insect host, it is likely that they are maintained at relatively high titres in adult female *Syntretus* (Herniou et al., 2013). Therefore, polydnaviral DNA, in a similar paradigm to mitochondrial DNA, might be maintained at relatively high prevalence (Rizzi et al., 2012), which may have facilitated its detection in archival samples. We recovered 1 kb of bracovirus sequence data for phylogenetic analysis. The topology of this viral phylogeny, while well supported with good sequence delimitation, does not appear to be congruent with our *Syntretus* mt and nuclear phylogeny, suggesting that the *Bracovirus* genome may be under different selection pressures compared with *Syntretus*, and that co-speciation is presumably not driving the observed radiations within this symbiosis. For example, *S. taegeri* and *S. idalius* are contained within the same clade in our mt and nuclear phylogeny, however, in the bracovirus phylogeny their associated polydnaviral reads are in different clades. As our nine bracovirus DNA sequences were obtained directly (1:1) from nine *Syntretus* specimens, resulting in only nine known interactions, we were unable to calculate congruence to statistically check for co-speciation or lack thereof. However, we note that differential immune responses in the ultimate bumblebee host may require varying expression of bracovirus virulence genes (Chevignon et al., 2014). Therefore, it is likely that disparity in the ultimate host immune response is driving viral evolution in this host-virus-parasitoid paradigm and that this may be separate to the evolutionary pressures acting on the *Syntretus* itself (Katzelnick et al., 2021). An alternative explanation for these apparently non-congruent host-endogenous virus phylogenies is horizontal transmission of the symbiont. Sampling across more host species, and eco-immunological studies of the symbiont-*Bombus* interaction are needed to distinguish between these two explanations for our results.

Our results also elucidate the metagenomic content of archival samples. We were able to recover *Wolbachia* sequences in all 16 sequenced archival *Syntretus* samples. This monophyletic group of Alphaproteobacteria are obligatory intracellular pathogens that can infect the reproductive tissues of arthropods (Werren, 1997) and may be obligatory in some species for reproduction, including Braconid wasps (Dedeine et al., 2000), although the prevalence of this phenomenon requires further research. Therefore, while detecting *Wolbachia* may seem inconsequential in a known host, as our samples are historic with an unknown storage history, our results add to the evidence of persistence of *Wolbachia* in historic collection-based invertebrates

(Sakamoto et al., 2006; Strunov et al., 2023), and to our understanding of this unique host-pathogen complex. Given that *Wolbachia* may be involved in Braconidae reproduction (Dedeine et al., 2000) it is likely that, along with polydnavirus, it may be maintained at a relatively high prevalence in its insect host, facilitating its detection in our archival samples. Metagenomic profiling also identified obvious contaminants in samples. These contaminants, which included human skin bacteria such as *Propionibacterium acnes* (Actinobacteria), along with sequences of plant, fungal and other bacterial origins, highlight the importance of understanding the ecology of an organism to differentiate between true versus contaminant microbiota. For example, we identified firmicute bacteria of soil origin and eudicotyledon sequences, which may be attributed to the hibernation phase of bumblebees, but may equally be a human-derived contaminant which originated during handling. Only by undertaking detailed characterisation of each contiguous sequence would a comprehensive picture of exotic contamination versus true endemic microbiota be achieved.

Museum collections already contribute unique and irreplaceable knowledge to our understanding of global biodiversity. Our results add to a growing body of evidence that suggests they can be co-opted, through genome skimming, to improve our understanding of cryptic species, elucidate host–parasite relationships, and enhance the efficacy of conservation strategies.

AUTHOR CONTRIBUTIONS

Arran J. Folly: Investigation; writing – original draft; writing – review and editing; formal analysis; data curation; visualization. **Hannah-Rose Porter:** Investigation; writing – review and editing; formal analysis; data curation. **Jim A. M. Galloway:** Investigation; writing – review and editing; formal analysis; data curation. **Stefan Schmidt:** Conceptualization; writing – review and editing; data curation; resources. **Mark R. Shaw:** Conceptualization; writing – review and editing; resources; data curation. **Gavin R. Broad:** Writing – review and editing; data curation; resources; investigation. **Nikki Gammans:** Conceptualization; investigation; writing – review and editing; funding acquisition; resources. **Mark J. F. Brown:** Conceptualization; investigation; funding acquisition; writing – review and editing; project administration; supervision; resources; methodology. **Ian Barnes:** Conceptualization; investigation; funding acquisition; writing – review and editing; project administration; supervision; resources; methodology. **Selina Brace:** Conceptualization; investigation; funding acquisition; writing – review and editing; project administration; supervision; resources; methodology.

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CONFLICT OF INTEREST STATEMENT

The authors declare there are no competing interests.

DATA AVAILABILITY STATEMENT

All data used for this study are presented in the manuscript and supplementary files.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the Journal's author guidelines page, have been adhered to.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Supplementary Table 1. Provenance data for all *Syntretus* archival samples. Specimens with MRS/MJFB numbers are in the National Museum Scotland.

Supplementary Table 2. Concatenated genes and GenBank scaffold sequences used in phylogenetic analysis.

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