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Hidden in plain sight: phylogeography of an overlooked parasitoid species *Trioxys sunnysidensis* Fulbright & Pike (Hymenoptera: Braconidae: Aphidiinae)

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Abstract

1 The bird cherry-oat aphid *Rhopalosiphum padi* is a major cereal pest with an almost cosmopolitan distribution. As one of the main groups of biocontrol agents for aphids, numerous Aphidiinae are associated with *R. padi*, including the genera *Binodoxys* and *Trioxys*.

2 As a recently described species parasitizing *R. padi*, *Trioxys sunnysidensis* is recorded from Europe for the first time based on morphological and molecular data. Specimens from North America, Europe and New Zealand were used in the analysis of the cytochrome *c* oxidase subunit I to explore genetic variation among populations. The analysis revealed one of the highest haplotype diversities in Aphidiinae so far, with 25 haplotypes detected. The two most common haplotypes are shared across groups of populations, whereas all of the others are found either in North America or Europe.

3 Because the genetic structure of populations is an important factor to consider when choosing a biocontrol agent, the results obtained in the present study may be helpful in guiding potential biocontrol attempts.

Keywords Aphidiinae, biological control, haplotype diversity, phylogeography, *Trioxys*.

Introduction

Rhopalosiphum padi (Linnaeus 1758) (Hemiptera: Aphididae) is a major pest of temperate cereal crops on a global scale reflecting damage caused both by feeding on plants, as well as by its role in the transmission of plant viruses (Van Emden & Harrington, 2017). It is a vector of numerous viruses, including the Barley yellow dwarf virus group, cereal yellow dwarf virus, filaree red leaf virus, maize leaf fleck virus, rice giallume virus, oat yellow leaf disease and onion yellow dwarf virus (Van Emden & Harrington, 2017).

Given the damage that cereal aphids inflict on economically important plants, research on their natural enemies has been extensive. As one of their most important groups of natural enemies, some species of Aphidiinae have been widely used as biocontrol agents against cereal aphids (Starý, 1981; Tomanović *et al.*, 2008), with numerous taxonomical and ecological studies having been conducted to assess their effectiveness (Starý, 1981; Powell, 1982; Pike & Starý, 1995; Sigsgaard, 2002; Kavallieratos *et al.*, 2005; Traugott *et al.*, 2008; Kos *et al.*, 2011; Plećaš *et al.*, 2014). According to Yu *et al.* (2012), there are currently 44 known aphidiine parasitoids of *R. padi*, including species of *Binodoxys* Mackauer, 1960 and *Trioxys* Haliday 1833.

With 74 species described worldwide, *Trioxys* is one of the most diverse genera in the subfamily Aphidiinae (Yu *et al.*, 2012). It is classified into the Trioxina subtribe based on morphological characters (Mackauer, 1961; Yu *et al.*, 2012). The most prominent morphological characters for differentiation of the genus are paired accessory prongs on the last abdominal sternite, together with the absence of secondary tubercles on the petiole. Although the later characteristic apparently separates *Trioxys* and *Binodoxys*, the relationship between these genera is not clear. Mackauer (1960) classified *Binodoxys* as a subgenus of *Trioxys* but later raised it to a genus (Mackauer, 1961, 1965) based on its possession of secondary tubercles on the petiole.

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Starý (1981) treated them as subgenera based on the same trait but noted that the grouping of species into *Trioxys* and *Binodoxys* was more for identification purposes than a reflection of their nomenclatural rank. Most of the later work did not consider their taxonomic status or relationships but, instead, focused on individual species or the position of the whole Trioxina subtribe within the subfamily. Based on past molecular analyses, *Trioxys* has been placed in the separate tribe Trioxini (Belshaw & Quicke, 1997) or viewed as a basal subtribe within the Aphidiini (Smith *et al.*, 1999; Kambhampati *et al.*, 2000; Sanchis *et al.*, 2000; Shi & Chen, 2005).

To date, 33 species of *Trioxys* from Europe (van Achterberg, 2013) and 22 from North America (Fulbright & Pike, 2007) have been reported. Most parasitize arboricolous aphids, although some parasitize aphids in steppe habitats (Tomanović & Kavallieratos, 2002). Except for the three species employed as biocontrol agents of aphids [*Trioxys complanatus* Quillis 1931/*Therioaphis trifolii* (Monell 1882); *Trioxys pallidus* (Haliday 1833)/*Chromaphis juglandicola* (Kaltenbach 1843) and *Myzocallis coryli* (Goeze 1778); *Trioxys curvicaudus* Mackauer 1967/*Eucallipterus tilliae* (Linnaeus 1758)], other *Trioxys* species have only been collected sporadically (Starý, 1988; Tomanović & Kavallieratos, 2002; Davidian, 2005).

The North American species *Trioxys sunnysidensis* Fulbright & Pike, 2007 was collected and described from *R. padi* in Washington State (Fulbright & Pike, 2007), which makes it just the second known species in this genus that parasitizes cereal aphids, in addition to *Trioxys auctus* (Haliday 1833) on *R. padi* (Starý, 1976, 1981, 2006).

In the present study, we report the first records of an overlooked cereal aphid parasitoid *T. sunnysidensis* from Europe after a long-term sampling campaign from cereal agroecosystems. We discuss its host range, the patterns of genetic variation among its populations across the world and its potential as a biocontrol agent.

Materials and methods

Adult parasitoids were dissected and slide-mounted for examination. The external morphology of the specimens was studied using a Discovery V8 stereomicroscope (Carl Zeiss, Germany). Species identification was performed in accordance with Fulbright and Pike (2007) and Fulbright *et al.* (2007). The material used in the morphological analysis and identification comprised: Germany, Göttingen, reared from *R. padi* on *Triticum aestivum*, two females, two males (coll. Vesna Gagić); Canada, Ontario, Wellington County, Guelph, 5. IX 2013, one female; 27. IX 2013, one male (coll. BIO Collections Staff); Walkerton, 4. X 2013, one female (coll. Meridith White); Sudbury, 3. X 2014, one male (coll. Brigitte Prevost).

Details on collection, nondestructive DNA extraction and amplification of the barcode region of the mitochondrial cytochrome *c* oxidase subunit I (COI) of two specimens of *T. sunnysidensis* reared from *R. padi* on *T. aestivum* from Germany (TRspA_331 and TRspA_333) are provided in Ye *et al.* (2017).

Sequences of other *Trioxys* and *Binodoxys* species used for phylogenetic analyses were obtained from previous studies by the authors (Ye *et al.* 2017; unpublished) and from GenBank (https://www.ncbi.nlm.nih.gov/genbank) and the Barcode of Life Database (BOLD) (http://www.boldsystems.org). *Trioxys* species were chosen because of their morphological similarity to *T. sunnysidensis* or because they parasitize cereal aphids. *Binodoxys* species were included in the analysis to examine relationships with *Trioxys* and evolutionary divergence rates between the two genera.

Sequences were edited using FINCHTV, version 1.4.0 (www.geospiza.com). Alignment of sequences was conducted using CLUSTALW integrated in MEGA, version 5 (Tamura *et al.*, 2011). Sequences were trimmed to a length of 564 bp. A phylogenetic tree was constructed using MRBAYES, version 3.1.2 (Ronquist & Huelsenbeck, 2003). The best fitting model of sequence evolution based on the Akaike information criterion was the Hasegawa–Kishino–Yano model with a gamma distribution and a fraction of invariable sites (HKY+G+I). Bayesian analysis was conducted running two Markov chain Monte Carlo searches, each with one cold and three heated chains. The analysis ran for two million generations, sampling every 1000 generations, and the first 250 trees were discarded as burn-in. Convergence of the parameters was confirmed with TRACER, version 1.5.0 (Rambaut & Drummond, 2007), whereas FIGTREE, version 1.3.1 (Rambaut, 2006) was used to view the consensus tree with posterior probabilities.

Calculation of average genetic distances between sequences was performed in MEGA, version 5 (Tamura *et al.*, 2011) using Kimura's two-parameter method of base substitution (Kimura, 1980).

Genetic diversity of T. sunnysidensis. In addition to the sequences for two *T. sunnysidensis* specimens from Germany reared from *R. padi* that were also used in the morphological analysis (TRspA_331 and TRspA_333), 314 sequences of *T. sunnysidensis* from BOLD were used to examine patterns of genetic variation among populations (Hebert *et al.*, 2016); sequences from BIN AAU8585 (https://dx.doi.org/10.5883/BOLD:AAU8585) on BOLD are provided in the Supporting information (Table S1). These sequences were trimmed to a length of 504 bp. The number of haplotypes was calculated using DNASP, version 6 (Rozas *et al.*, 2017). A median-joining network (Bandelt *et al.*, 1999) was constructed using NETWORK, version 5.0.0.1 (http://www.fluxus-engineering.com/).

Haplotype and nucleotide diversity were determined in ARLEQUIN, version 3.5 (Excoffier & Lischer, 2010). Genetic differentiation between populations was measured by computing pairwise Φ_{ST} statistics. To infer differentiation between groups of populations, we conducted an hierarchical analysis of molecular variance (AMOVA) using genetic distances and tested significance by running 10 000 permutations, both on each population and on geographical groups of populations. Demographic history was inferred by applying two neutrality tests: Tajima's *D* (Tajima, 1989) and Fu's F_s (Fu & Li, 1993; Fu, 1997). All calculations were performed in ARLEQUIN, version 3.5.

Sampling data for specimens used in the reconstruction of relationships between *Trioxys* and *Binodoxys* are shown in Table 1, whereas the data for specimens used in the population analysis are provided in the Supporting information (Table S1). Specimens of *T. sunnysidensis* used in the population analysis have

Table 1 Collection data for specimens used for the reconstruction of relationships between *Trioxys* and *Binodoxys*

Code	Country	Collection date	Host plant	Aphid	Parasitoid	Accession number
JN620603	France	27 April 2008		*Aphis urticata*	*Binodoxys angelicae*	JN620603
Be15_230	Belgium	29 May 2015	*Sambucus nigra*	*Aphis sambuci*	*Binodoxys angelicae*	KY912707
Be15_195	Belgium	15 June 2015	*Tanacetum parthenium*	*Aphis fabae*, *Brachycaudus cardui*	*Binodoxys angelicae*	KY912706
SWE14_24	Sweden	2 July 2014	*Malus* sp.	*Aphis pomi*	*Binodoxys angelicae*	MK080159
S14_37	Serbia	28 June 2014	*Robinia pseudoacacia*	*Aphis* sp.	*Binodoxys acalephae*	MK080160
Fi16_03_2	Finland	22 July 2016	*Vicia cracca*	*Aphis craccae*	*Binodoxys acalephae*	MK080161
S11_860_1	Montenegro	7 August 2013	*Pastinaca* sp.	*Cavariella aegopodii*	*Binodoxys heraclei*	MF287648
S11_749_1	Montenegro	27 July 2012	*Sanicula europaea*	*Hyadaphis foeniculi*	*Binodoxys brevicornis*	MF287649
S11_749_2	Montenegro	27 July 2012	*Sanicula europaea*	*Hyadaphis foeniculi*	*Binodoxys brevicornis*	MK080162
KF597735	China	15 May 2012	*Glycine max*	*Aphis glycines*	*Binodoxys communis*	KF597735
JN620612	France	25 June 2009		Unknown	*Binodoxys centaureae*	JN620612
KJ848479	Iran				*Trioxys complanatus*	KJ848479
KR074105	Iran				*Trioxys pallidus*	KR074105
JN620696	France	24 June 2008		*Tuberculatus* sp.	*Trioxys pallidus*	JN620696
S12_868	Serbia	31 May 2013	*Alisma plantago-aquatica*	*Rhopalosiphum nymphaeae*	*Trioxys auctus*	MK080163
TRspA_331	Germany		*Triticum aestivum*	*Rhopalosiphum padi*	*Trioxys sunnysidensis*	KY887944
TRspA_333	Germany		*Triticum aestivum*	*Rhopalosiphum padi*	*Trioxys sunnysidensis*	KY887945
S11_480	Serbia	5 August 2011	*Gallium verum*	*Hydaphias molluginis*	*Trioxys parauctus*	MK080164
Be15_74	Belgium	11 May 2015	*Prunus avium*	*Myzus cerasi*	*Ephedrus persicae*	KY213710

been deposited in Mountain Agriculture Research Unit, Institute of Ecology, University of Innsbruck, Innsbruck, Austria; Centre for Biodiversity Genomics, Guelph, Ontario, Canada; NTNU University Museum, Department of Natural History, Trondheim, Norway; University of Oslo, Natural History Museum, Oslo, Norway; Research Collection of Beverly McClenaghan, Trent University, Peterborough, Ontario, Canada; Landcare Research, New Zealand Arthropod Collection, Aukland, New Zealand; and SNSB, Zoologische Staatssammlung Muenchen, Munich, Germany.

Results

Parasitoids reared from *R. padi* collected in Germany (two females, two males) were identified as *T. sunnysidensis* based on morphological characters, apparently representing the first records for this species from Europe.

However, their COI sequences showed a 98–100% match with 337 sequences from specimens identified as Aphidiinae or *Trioxys* sp. and collected in Germany, Norway, U.K. and Canada in GenBank and BOLD (Hebert *et al.*, 2016). Among those sequences, 52 derived from specimens collected in Europe (Germany, Norway and U.K.) during 2012 and 2014. To confirm our identification and to identify unidentified specimens with records on BOLD, we obtained four additional specimens from Canada (Ontario, Wellington County, Guelph, 5. IX 2013, one female; 27. IX 2013, one male (coll. BIO Collections Staff); Walkerton, 4. X 2013, one female (coll. Meridith White); Sudbury, 3. X 2014, one male (coll. Brigitte Prevost)), all of which morphologically resemble *T. sunnysidensis* after Fulbright and Pike (2007) and Fulbright *et al.* (2007).

The phylogenetic tree constructed in MRBAYES to examine relationships between *T. sunnysidensis* and its congeners, as well as *Binodoxys* species, is shown in Fig. 1. Although all sequences cluster with their conspecifics (when at least two sequences of the same species were included), the situation is not so clear with respect to the grouping of genera. Despite being morphologically most similar and parasitizing the same aphid species, *T. auctus* and *T. sunnysidensis* do not cluster together; instead, *T. sunnysidensis* haplotypes form a sister clade to that containing all *Binodoxys* and other *Trioxys* species, whereas *T. auctus* forms a separate branch on the tree. The genetic distance between *T. sunnysidensis* and *T. auctus*, although very high, has one of the lowest values among the species analyzed (8.3%) (Table 2).

Population analysis of *T. sunnysidensis* revealed 25 haplotypes (H1–H25), with 90.2% of the 316 analyzed sequences belonging to two haplotypes, H1 (126 sequences; 39.9%) and H2 (159 sequences; 50.3%). Estimation of a haplotype network produced a single network with no ambiguous connections (Fig. 2).

The distribution of haplotypes showed geographical variation; haplotypes H1 and H2 are the only two shared between North America, Europe and New Zealand (just H2). Haplotype H2 was detected in all populations except those from Alberta. Haplotypes H3–H16 occur only in Europe, with H3 present only in Germany, whereas H4–H16 are recorded only in Norway 1. Most of the haplotypes occurring only in Europe show the closest connection to haplotype H2 (except H15 and H16). Haplotypes H17–H25 are found only in North America (Canada) without any sharing among populations: H17–H21, Ontario and Quebec; H23, British Columbia; and H22, H24, H25, Nova Scotia and New Brunswick (Fig. 3; see also Supporting information, Table S1). The Canadian haplotypes appear to be distributed more or less evenly with respect to the grouping with H1 or H2 haplotypes.

The overall haplotype and nucleotide diversities are 0.59 ± 0.02 and 0.0034 ± 0.0022, respectively (Table 3). All populations are characterized by relatively low nucleotide diversity, whereas haplotype diversity varies from 0.0 to 0.78 ± 0.06

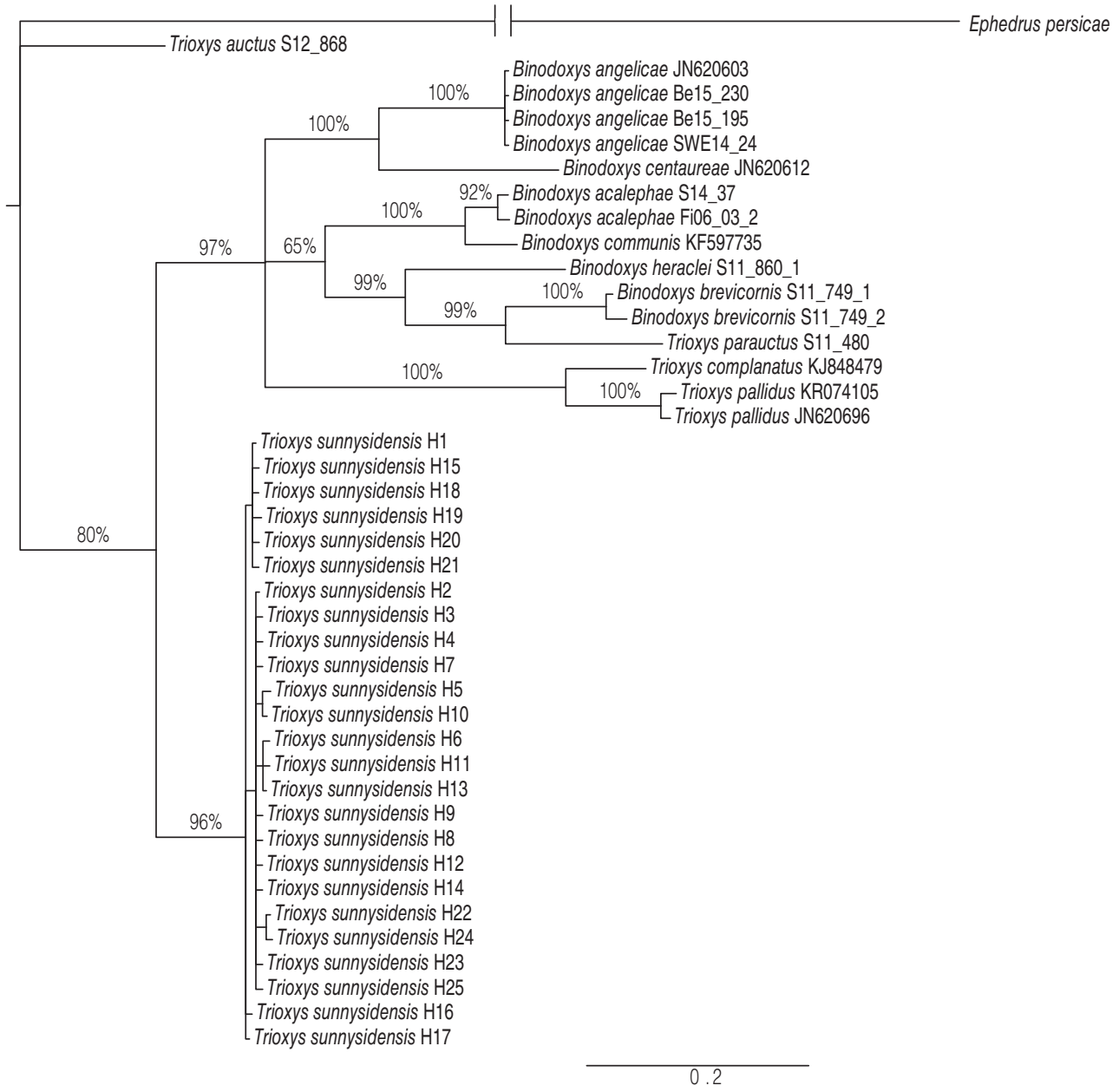

Figure 1 Bayesian inference phylogram for cytochrome *c* oxidase subunit I mitochondrial DNA sequences for species of *Binodoxys* and *Trioxys*. Bayesian posterior probabilities above 50% are shown. The scale bar indicates substitutions per site. *Ephedrus persicae* was used as an outgroup.

(Table 3). Three main geographical groups (North America, Europe and New Zealand) significantly differ in haplotype diversity. The New Zealand group with only one recorded haplotype lacks diversity, whereas the highest diversity was recorded for the European group of populations (Table 3).

Neutrality tests were performed for populations with more than five specimens. Tajima's *D* statistics were not significant for New Zealand or for any North American populations, whereas they showed significance for one population from Norway, indicating population expansion. The results of Fu's F_s test were identical (negative and significant only for Norway 1), which was as expected considering that this test is more sensitive to singletons in a sample (Table 3).

The results of the AMOVA indicate that there is no genetic partitioning among groups and that the majority of genetic variation occurs among specimens within populations (Table 4).

Pairwise Φ_{ST} values of group pairs are shown in Table 5, whereas pairwise Φ_{ST} values of population pairs are shown in Table 6 (results are shown only for populations with more than five specimens).

There are significant differences between North America and both Europe and New Zealand, although the difference between North America and New Zealand is only marginally

Table 2 Estimates of evolutionary divergences between sequences used in the analysis (Kimura's two-parameter method)

	Binodoxys angelicae	*Binodoxys acalephae*	*Binodoxys heraclei*	*Binodoxys brevicornis*	*Binodoxys communis*	*Binodoxys centaureae*	*Trioxys complanatus*	*Trioxys pallidus*	*Trioxys auctus*	*Trioxys sunnysidensis*	*Trioxys parauctus*
Binodoxys angelicae											
Binodoxys acalephae	0.101										
Binodoxys heraclei	0.114	0.098									
Binodoxys brevicornis	0.112	0.103	0.097								
Binodoxys communis	0.101	0.032	0.096	0.105							
Binodoxys centaureae	0.087	0.117	0.118	0.123	0.115						
Trioxys complanatus	0.142	0.123	0.134	0.148	0.121	0.136					
Trioxys pallidus	0.140	0.124	0.134	0.140	0.125	0.130	0.060				
Trioxys auctus	0.118	0.098	0.123	0.128	0.094	0.123	0.132	0.137			
Trioxys sunnysidensis	0.083	0.092	0.101	0.119	0.088	0.108	0.128	0.137	0.083		
Trioxys parauctus	0.117	0.106	0.103	0.081	0.094	0.111	0.141	0.122	0.113	0.117	
Ephedrus persicae	0.228	0.232	0.241	0.233	0.226	0.242	0.237	0.232	0.195	0.198	0.233

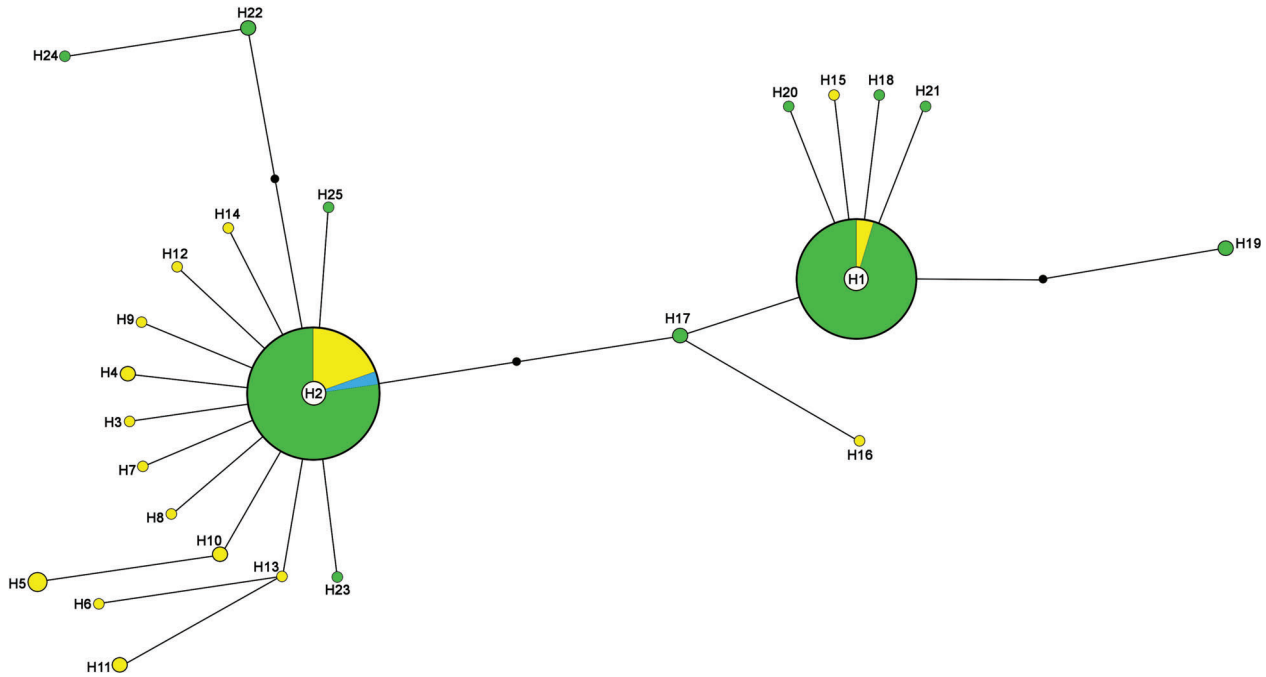

Figure 2 Haplotype network based on 316 mitochondrial DNA cytochrome *c* oxidase subunit I sequences. Circles represent different haplotypes with the size of the circle representing the number of individuals with that haplotype. Small filled circles represent missing haplotypes. [Colour figure can be viewed at wileyonlinelibrary.com].

significant. Differences at a population level reflect patterns recorded between groups, where the population from Norway is significantly different from all North American populations. Also, the Φst values show mainly significant differences between North American populations, with the only nonsignificant Φst value being recorded for Ontario and Quebec versus Nova Scotia and New Brunswick (Table 6).

Discussion

Trioxys sunnysidensis *hiding in plain sight*

Trioxys sunnysidensis was described recently from Washington (U.S.A.) where it was reared from *R. padi* on artificially infested wheat (*T. aestivum* var. Alpowa) placed in *Phalaris arundinacea* habitats (Fulbright & Pike, 2007). Despite its supposed Nearctic origin (Fulbright *et al.*, 2007), there were no other records of this species from North America until now. The present study documents the presence of *T. sunnysidensis* in Europe (Germany) for the first time and from the same aphid host. Several ecological studies with huge field research campaigns on cereal aphid–parasitoids as a model system were performed in Germany and central Europe (Pankanin-Franczyk & Sobota, 1998; Adisu *et al.*, 2002; Thies *et al.*, 2005), although *T. sunnysidensis* was not collected. DNA barcoding allowed us to identify previously unidentified specimens in the BOLD database and to establish that *T. sunnysidensis* is broadly distributed in North America (Washington and Canada), Europe (Germany, Norway

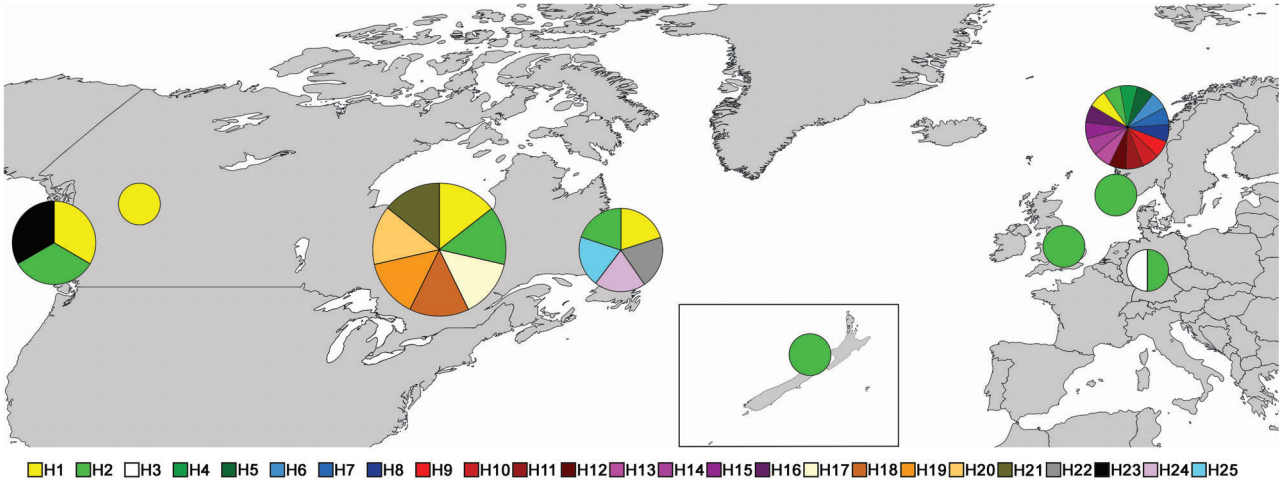

Figure 3 Map of the distribution area of *Trioxys sunnysidensis*; pie charts show the presence of certain haplotypes. The size of a pie chart reflects the number of specimens (small: < 5; medium: 5–50; large: > 50). [Colour figure can be viewed at wileyonlinelibrary.com].

Table 3 Genetic diversity and historical demographic results for populations of *Trioxys sunnysidensis* (populations with less than five samples are excluded from the analysis)

Area	Population	*N*	*H*	Haplotype diversity	Nucleotide diversity	Tajima's *D*	Fu's F_s
North America	ONQU	201	7	0.536 ± 0.013	0.003 ± 0.002	0.344	1.077
	BC	36	3	0.427 ± 0.076	0.002 ± 0.002	0.630	2.358
	NSNB	21	5	0.690 ± 0.066	0.004 ± 0.003	0.931	0.991
	Alberta	3	1	NA	NA	NA	NA
	Total	261	11	0.546 ± 0.0123	0.003 ± 0.002	−0.406	−1.138
Europe	Norway1	44	15	0.777 ± 0.061	0.004 ± 0.002	**−1.504**	**−8.049**
	Norway2	2	1	NA	NA	NA	NA
	Germany	3	2	NA	NA	NA	NA
	U.K.	1	1	NA	NA	NA	NA
	Total	50	16	0.738 ± 0.064	0.004 ± 0.002	**−1.674**	**−9.686**
New Zealand	New Zealand	5	1	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	NA
Overall		316	25	0.589 ± 0.016	0.003 ± 0.002	**−1.437**	**−13.496**

H, number of haplotypes; NA, not available. The use of bold indicates statistical significance ($P < 0.05$). ONQU, Ontario and Quebec; BC, British Columbia; NSNB, Nova Scotia and New Brunswick.

Table 4 Analysis of molecular variance for *Trioxys sunnysidensis* haplotypes partitioned by geography (groups = North America, Europe and New Zealand)

Source of variation	d.f.	Percentage variation	Fixation indices	*P*
Among groups	2	6.68	$\Phi_{CT} = 0.067$	0.07
Among populations within groups	6	5.76	$\Phi_{SC} = 0.067$	0.015
Within populations	307	87.57	$\Phi_{ST} = 0.124$	0.00000

Table 5 Pairwise Φst values between groups

	North America	Europe	New Zealand
North America	–		
Europe	**0.090**	–	
New Zealand	**0.249**	0.0849	–

The use of bold indicates statistical significance ($P < 0.05$).

and U.K.) and New Zealand. This result highlights the importance of databases such as BOLD and GenBank, especially with respect to records that are publicly available, even if they lack a species designation. Comparing the analyzed sequences with those already deposited in such databases facilitates species identification and helps to clarify distribution patterns and biological associations.

It is likely that *T. sunnysidensis* prefers colder climates, considering the lack of records from southern (warmer) parts of America and Europe, despite extensive faunistic surveys, especially in southeastern Europe (Kavallieratos *et al.*, 2004; Žikić *et al.*, 2012). According to Fulbright and Pike (2007), it is not clear whether *R. padi* is the main host of this parasitoid because information about its biology is limited. Most of our specimens lacked host data, although specimens from Germany were reared from *R. padi* on *T. aestivum*. This represents the first record of *T. sunnysidensis* parasitizing *R. padi* in agricultural wheat fields. *Trioxys sunnysidensis* has not been

Table 6 Pairwise Φst values between populations

	ONQU	NSNB	BC	Norway1	New Zealand
ONQU	–				
NSNB	−0.002	–			
BC	**0.092**	**0.106**	–		
Norway1	**0.099**	**0.043**	**0.053**	–	
New Zealand	0.282	**0.276**	0.065	0.109	–

The use of bold indicates statistical significance ($P < 0.05$). ONQU, Ontario and Quebec; BC, British Columbia; NSNB, Nova Scotia and New Brunswick.

reported previously from Europe, despite extensive faunistic studies, which suggests that the species is either rare or has been overlooked by misidentification (Elias *et al.*, 2013). If its primary host is *R. padi* on cereal crops, there is competition for this resource from numerous other Aphidiinae species (Powell, 1982; Höller *et al.*, 1993; Tomanović & Brajković, 2001; Traugott *et al.*, 2008). Rare and possibly new Aphidiinae species can easily be overlooked, especially in large scale ecological studies.

Genetic variation and phylogeography of T. sunnysidensis

COI haplotype diversity shows very distinct patterns within species of Aphidiinae. It ranges from almost no diversity in *Aphidius uzbekistanicus*, with only two haplotypes registered throughout the Holarctic (Kos *et al.*, 2011), to high diversity in *Aphidius banksae* (Petrović *et al.*, 2018). Baer *et al.* (2004) recorded 44 different mitochondrial haplotypes in *Diaeretiella rapae* (M'Intosh, 1855), although this was based on several mitochondrial genes. In total, we registered 25 different haplotypes of *T. sunnysidensis*, which represents one of the highest COI haplotype diversities recorded in any species of Aphidiinae. Interestingly, the only record of higher haplotype diversity (30 haplotypes) involved *T. pallidus*, used in the biological control of the walnut aphid *C. juglandicola* and the filbert aphid *M. coryli* in California (Andersen & Mills, 2016). In the case of *T. sunnysidensis*, two haplotypes (H1 and H2) are dominant in most of the populations analyzed. Furthermore, those are the only two shared haplotypes. Haplotype H2 is dominant (> 50% of sequences) and occurred in all localities except Alberta where only three specimens were analyzed. Fulbright *et al.* (2007) noted the Nearctic origin of *T. sunnysidensis*, although alternative scenarios cannot be excluded given the high genetic variation of COI sequences in Canadian and European populations. Shared haplotypes across all analyzed areas (H1 and H2) suggest close relationships between these groups. However, the geographical restriction of certain groups of haplotypes to Canada and Europe indicates that those populations have been evolving independently for some time. The fact that haplotype diversity was highest in Norway suggests that Europe may be the centre of origin of this species and that the introduction to North America happened later. However, based on the results of the present study, *T. sunnysidensis* most likely has a Holarctic origin, where two ancestral haplotypes (H1 and H2) survived the last glaciation, after which the populations expanded rapidly from refugia in both North America and Europe. This conclusion is supported by the haplotype network, which shows differing distribution patterns for European and Canadian haplotypes; almost all European haplotypes are most closely connected to H2, whereas Canadian haplotypes are almost evenly connected with the H1 and H2 haplotypes (Fig. 2). Although the analysis employed all currently available individuals of this species, some populations may appear to be undersampled, and so further investigations could provide better insight into the phylogeography of *T. sunnysidensis*. Its presence in New Zealand likely reflects an accidental introduction because there is no record of an intentional introduction. An accidental introduction is not unlikely, given its small size and association with cereal crops that are traded globally.

Although parallel evolution between phytophagous insects and their natural enemies is well known (Kraaijeveld *et al.*, 2002), several studies have shown that geographical factors, rather than aphid hosts and their population structuring, have been significant factors in the diversification of Aphidiinae populations (Baer *et al.*, 2004; Lozier *et al.*, 2009; Mitrović *et al.*, 2013). Additional analysis of the patterns of molecular variation in parasitoid populations could help determine whether geographical or ecological factors prevail in promoting population differentiation.

Relationships between genera Trioxys and Binodoxys

Most work on *Trioxys* and *Binodoxys* species conducted after Mackauer (1960, 1961, 1965) and Starý (1981) was performed within the context of the whole subfamily Aphidiinae and so only a few species were included from both genera. Belshaw and Quicke (1997) used elongation factor-1α, cytochrome *b* and the second expansion segment of the 28S ribosomal subunit to infer the phylogeny of the subfamily Aphidiinae. One *Binodoxys* and one *Trioxys* species were used in the analysis and they grouped together on phylogenetic trees, although the amplification of cytochrome *b* for *Trioxys* was unsuccessful. Analysis of the subfamily by Smith *et al.* (1999) based on the NADH1 dehydrogenase gene showed a close relationship between *Binodoxys* and *Trioxys* (two species from each genus were analyzed). The paraphyly of *Trioxys* was also noted, although this was ascribed to limited taxon sampling (Smith *et al.* 1999). Sanchis *et al.* (2000) noted that the subgeneric ranks of *Trioxys* and *Binodoxys* should be retained because the four species used in the analysis based on 18S rDNA gene clustered together with strong support. Kambhampati *et al.* (2000) used three *Binodoxys* and two *Trioxys* species in their analysis of 16S rRNA that also showed close relationships between the two groups, as did Shi and Chen (2005) using one species of each genus and sequences of 16S rRNA, 18S rDNA and ATPase 6 genes. When Derocles *et al.* (2012) employed the barcode region of the COI gene, one *Trioxys* species nested in the middle of the clade formed by four *Binodoxys* species.

Our current analysis of sequence variation in the barcode region of COI suggests there is no clear distinction between the two genera. Genetic distances between the species analyzed are much higher than that considered to be a species boundary within Aphidiinae (Derocles *et al.*, 2012; Tomanović *et al.*, 2014); even *T. sunnysidensis* and *T. auctus*, with a similar morphology and the same aphid host, have a fairly high

distance. Furthermore, the barcode region does not separate the species into two monophyletic groups (*Trioxys* and *Binodoxys*) because some differences between congeneric pairs of species were greater than those between taxa currently assigned to different genera (Fig. 1). Although these findings suggest that *Trioxys* and *Binodoxys* are one genus or that certain species need to be reassigned, more intensive taxonomic sampling is necessary, combined with additional molecular markers and morphological/ecological studies, aiming to clarify relationships between these two groups and their status within the subfamily Aphidiinae.

Implications for biological control

The only currently known host of *T. sunnysidensis* is *R. padi*, which is one of the most important cereal pests with a near cosmopolitan distribution. Viewed from this perspective, more research is needed to determine whether *T. sunnysidensis* could play a significant role in the biological control of this aphid. As with all biological control attempts, this should involve extensive testing because there are recorded cases of parasitoids becoming widespread after their introduction and expanding their host range in non-native areas, regardless of whether the introduction was intentional or not (Roy *et al.*, 2011; Mitrović *et al.*, 2013; Petrović *et al.*, 2013). The importance of understanding the genetic structure of both pest species and their natural enemies in biological control programmes has now been recognized in numerous studies (Roderick & Navajas, 2003; Lozier *et al.*, 2008; Muirhead *et al.*, 2012; Tavares *et al.*, 2015; Andersen & Wagner, 2016). Molecular analyses often uncover cryptic species of natural enemies, showing different relationships with the target pest organism, especially when those species cannot easily be differentiated morphologically, as is often the case with Hymenoptera used in biological control. A high genetic diversity of parasitoids used in biological control provides the possibility of overcoming aphid resistance (Vorburger, 2018). The results obtained in the present study on the haplotype diversity of *T. sunnysidensis* may prove useful to future biological control attempts and research because genetically distinct populations of a natural enemy can often have significantly different effects on the target pest and exhibit varying success with respect to biological control as a result of local adaptations.

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Supporting information

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

Table S1. Specimens used in the population analysis of *Trioxys sunnysidensis*

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