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A preliminary assessment of the occurrence and diversity of mitochondrial COI haplotypes in adventive populations of the biocontrol agent, *Neodryinus typhlocybae*, in Hungary

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Abstract

The parasitoid *Neodryinus typhlocybae* (Ashmead) (Hymenoptera Dryiinidae) was originally introduced from the USA to Italy in 1987 as a classical biological control agent for *Metcalfa pruinosa* (Say) (Hemiptera Flatidae). Additional releases and intentional redistribution of *N. typhlocybae* throughout Italy, as well as several European countries took place over a period of two decades. In 2014, this parasitoid was found in Hungary for the first time, despite the fact that, intentional releases in this country are not known to have occurred. To investigate the haplotype occurrence and diversity of *N. typhlocybae* in Hungary, the COI barcode region was characterized from established populations collected in Hungary. To determine whether the Hungarian populations of *N. typhlocybae* share haplotypes with populations released in classical biological control efforts for *M. pruinosa* in Italy, samples from three additional locations were obtained for comparison. This includes two locations in Italy where *N. typhlocybae* from Connecticut was released in the 1990's: Lazio, where samples were obtained from collections made in 2003 (i.e., shortly after successful parasitoid establishment), and more recent collections from Piedmont; and one location in Texas, USA where additional *N. typhlocybae* were collected and released in Italy in the late 1990's. Among the 250 samples collected, a total of 5 COI haplotypes were observed, with haplotype H2 accounting for 51% of the samples. Four haplotypes (H1, H2, H4, H5) were found in Hungary, with 71% belonging to haplotype H2, which was also the dominant haplotype from samples collected in Texas. Two haplotypes (H1 and H5) observed among Hungarian *N. typhlocybae* samples were also observed from samples collected in Lazio and Texas; H1 was also observed in the Piedmont samples. Haplotype H4 was observed (albeit at very low proportions) from collections in Hungary and Piedmont, but was not present among samples from Texas or Lazio. Although the results are preliminary, haplotypes H1, H2, and H5 in Hungary are consistent with populations associated with biological control releases in Italy that originated from Connecticut and Texas. Additional investigation is needed to determine whether releases in France from other source populations may have contributed to additional diversity, in particular in relation to the occurrence of H4. A more extensive collection and analysis of samples from across the geographic range of *N. typhlocybae* in both the area of origin (North America) and the area of introduction (across Europe) would be necessary to determine the origin(s) of the populations in Hungary, and to capture the full diversity of COI haplotypes in both the area of introduction and the area of origin.

Key words: classical biological control, DNA barcode, dispersal, natural enemy, invasive insect species, haplotype analysis.

Introduction

The flatid planthopper, *Metcalfa pruinosa* (Say) (Hemiptera Flatidae) was accidentally introduced from North America into Italy in the late 1970's (Zangheri and Donadini, 1980), and has already been reported from at least 17 countries in Eurasia (EPPO, 2020). As a highly polyphagous pest, *M. pruinosa* feeds on a variety of trees and shrubs (>300 plant species from >70 different families), including a number of economically important crops such as citrus and fruit trees, as well as grapevines (Bagnoli and Lucchi, 2000). Damage results from phloem-feeding by *M. pruinosa* nymphs, which can cause stunting and wilting of shoots; in addition, the production of honeydew and waxy secretions by the nymphs results in sooty moulds that can reduce fruit quality and make the plant aesthetically unpleasant, which can be problematic in the ornamental plant industry (Della Giustina and Navarro, 1993; Bagnoli and Lucchi, 2000; Strauss, 2010). Economic damage due to *M. pruinosa* is rarely observed in North America, and typically only occurs when other conditions exist that place stress on the plant (e.g., freeze damage) (Mead, 2004). In contrast, the rapid spread and economic damage in Europe is likely due, at least in part, to a lack of natural enemies in the area where *M. pruinosa* has established (Alma *et al.*, 2005). For this reason, a classical biological control approach was initiated using the Nearctic parasitoid *Neodryinus typhlocybae* (Ashmead) (Hymenoptera Dryinidae) in Europe (Girolami and Camporese, 1994). There has been a long history of foreign exploration and mass release of different *N. typhlocybae* populations in several European countries in an effort to control this pest and reduce its spread (table 1).

Original releases of *N. typhlocybae* occurred in 1987 in the Veneto region of northern Italy, and were derived from material originally collected in Fairfield, Connecticut (USA), and establishment was considered successful by 1990 (Girolami and Camporese, 1994). These established, Connecticut-derived populations were subsequently used to initiate colonies at the Institute of Entomology of Padua and BIOPLANET, Martorano di Cesena, Forli-Cesena Province, Italy (Girolami and Mazzon, 1999; Alma *et al.*, 2005). *N. typhlocybae* from these

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Table 1. History of intentional biological control release and redistribution of *N. typhlocybae* in Europe.

Country	Region	Year	Source of material	Reference
Italy	Veneto	1987	Connecticut (Fairfield)	Girolami and Camporese, 1994; Girolami and Mazzon, 1999
	Piedmont, Lombardy, Friuli-Venezia Giulia, Emilia-Romagna, Tuscany, Marche, Umbria, Abruzzo, Lazio, Campania, Basilicata, Calabria	1990's - mid-2000's	University of Padua / BIOPLANET, Cesena, Italy (Connecticut)	Tommasini *et al.,* 1998; Sala and Foschi, 2000; Sacchetti *et al*., 2000; Frilli *et al*., 2001; Alma *et al*., 2005; De Leo, 2006
	Tuscany (Pisa)	1997	Texas (College Station)	Lucchi *et al*., 2002
	Sicily	2004	University of Padua (Connecticut) & University of Pisa (Texas)	Zappalà *et al*., 2008
France	Southern France	1996 - 2000	University of Padua (Connecticut)	Malausa *et al*., 2003
	Southern France, Corsica	2000 - 2001	New York State, Delaware	
Croatia	Istria	1998	University of Padua (Connecticut)	Ciglar *et al*., 1998
Switzerland	Ticino	1999	University of Padua (Connecticut)	Jermini *et al*., 2000
Slovenia	Nova Gorica	1999	University of Padua (Connecticut)	Žežlina *et al*., 2001
Greece Netherlands Spain	Information not available	2007	Information not available	Strauss, 2009

colonies were used in subsequent inoculative releases across northern, central, and southern Italy throughout the 1990's and early 2000's (table 1) (Tommasini *et al*., 1998; Sacchetti *et al*., 2000; Sala and Foschi, 2000; Frilli *et al*., 2001; Di Bucchianico *et al*., 2004; Alma *et al*., 2005; De Leo, 2006; Zappalà *et al*., 2008), as well as in southern France from 1996-2000 (Malausa *et al*., 2003), Croatia (Ciglar *et al*., 1998), Switzerland in 1999 (Jermini *et al*., 2000), and Slovenia in 2000 (Žežlina *et al*., 2001) (table 1).

In an effort to increase the establishment and spread following the initial releases, additional populations of *N. typhlocybae* were collected in College Station, Texas by one of the co-authors (M. Olmi) and released in Tuscany at the University of Pisa in 1997 and 1998 (Lucchi *et al*., 2002). Although only a small number of individuals were released, by 2001 the parasitoid was successfully established within a range of 5 km from the release site in all directions (Lucchi *et al*., 2002). Subsequently, both the Connecticut-derived populations (from laboratory colonies at University of Padua) and Texas-derived populations (from laboratory colonies at University of Pisa) were released in Sicily in 2004, with confirmed establishment of populations as of 2006 (Zappalà *et al*., 2008). Similarly, in southern France and Corsica, *N. typhlocybae* populations collected in New York and Delaware (USA) were released between 2000-2001 to supplement the Connecticut-derived releases previously made (Malausa *et al*., 2003).

Releases of *N. typhlocybae* have also been made in Greece, the Netherlands, and Spain in 2007 with material supplied from Bioplanet (reported as personal communication of A. Sala in Strauss, 2009). However, the details of the releases in these countries were not indicated, and it is unclear whether establishment was successful. Although *N. typhlocybae* is known to occur broadly in Corfu, Greece (Burn, 2011). Adventive establishment of *N. typhlocybae* has been reported in several additional countries, indicating natural spread of one or more populations from the original classical biological control releases, e.g.: Montenegro, Serbia, Gibraltar, Austria, Hungary, Bulgaria, Slovakia (Vétek *et al*., 2019). In addition, following the discovery of *N. typhlocybae* in Bulgaria in 2016, additional releases of the parasitoid were carried out from material reared in Greece, which originated from the established Italian populations (Tomov and Vasileva, 2018). *N. typhlocybae* was recorded for the first time in Hungary in 2014 (Szöllősi-Tóth *et al*., 2017), although no intentional releases are known to have taken place. The origin of the established parasitoid population is likely due to the spread of populations released in neighbouring countries, and/or the movement of plant material containing parasitized *M. pruinosa* (Vétek *et al*., 2019). Although the majority of releases in Europe over the last 25 years have been derived from material collected in Connecticut that was mass-reared, released, and redistributed throughout Italy, additional releases from different areas of origin, e.g.: Texas (Lucchi *et al*., 2002); New York, Delaware (Malausa *et al*., 2003), have also taken place in Europe since 1997 (table 1), and the movement and spread of these different populations in Europe is currently unknown.

Mitochondrial DNA sequence data has frequently been used to trace the origins and spread of non-native species (Grapputo *et al*., 2005; Corin *et al*., 2007; Auger-Rozenberg *et al*., 2012; Chapman *et al*., 2015; Gariepy *et al*., 2015), including introduced parasitoids (Roderick and Navajas, 2003; Hufbauer *et al*., 2004). The mitochondrial Cytochrome Oxidase I (COI) gene has shown utility in species identification and separation of genetic lineages (Bucklin *et al*., 2011; Stephens *et al*., 2011), in particular as it relates to tracing the source of introduction of non-native species (Auger-Rozenberg *et al*., 2012; Chapman *et al*., 2015; Gariepy *et al*., 2015). Understanding the diversity, movement, and spread of adventive parasitoids may be useful to inform current and future biological control programs, based on the identification of populations that have successfully established and spread following release (Abram and Moffat, 2018).

As a preliminary step to understand the spread of adventive populations of *N. typhlocybae* into Hungary, we investigated variation in a portion of the mitochondrial COI gene, the DNA barcode region, to document the occurrence and diversity of haplotypes from Hungarian samples. As the majority of biological control releases of *N. typhlocybae* in Europe were the result of redistribution from material originally released in Italy (table 1), we were able to obtain samples from some additional locations for comparison. As such, we also assessed the variation in the DNA barcode region and haplotype diversity from: (1) *N. typhlocybae* used in earlier releases in 2003 in Lazio, Italy (which originated from Connecticut-derived material that was mass-produced in Italy, and likely represents the subset of original haplotypes that established in Italy), (2) recent collections in the Piedmont region in Italy (which likely represent a mixture of successfully established haplotypes decades after the initial releases), and (3) Texas, USA (the source location of material used in releases in the Tuscany region, table 1). The aim was to characterize the occurrence and diversity of COI barcode haplotypes in Hungary to determine whether the established populations in Hungary share haplotypes with *N. typhlocybae* from Italy (where the original releases occurred, and the country with the longest history of releases and re-distributions throughout Europe) and Texas (one of the source locations for material released in Italy), or whether unique haplotypes are present. If the Hungarian samples share haplotypes with the other areas investigated, this may indicate dispersal of successful haplotypes from the original introduction and re-distribution of parasitoid populations in Italy in the late eighties and/or from neighbouring countries where the Italian populations were subsequently redistributed (table 1). In contrast (although not mutually exclusive), if one or more unique haplotypes are observed, *N. typhlocybae* populations in Hungary may represent a separate adventive establishment either directly from North America (i.e., independent of biological control efforts in Europe), or via the release of additional North American populations from biological control efforts outside of Italy.

Materials and methods

Acquisition of material

A total of 250 parasitoid adult specimens were reared from parasitized *M. pruinosa* nymphs collected in the field from different locations in 2003, and 2015-2018 (complete specimen and collection data are available at www.boldsystems.org, project NEODR, *Neodryinus* spp. biological control agents; summary of collection data in table 2). Upon emergence from cocoons, adults were morphologically identified as *N. typhlocybae*, and preserved in 95% ethanol for subsequent molecular analysis.

Hungary

Sampling of established populations of *N. typhlocybae* in Hungary resulted in the collection of 170 parasitoids from 2015-2016, primarily in Budapest (n = 140), but also from Tolna county (n = 29) and Somogy county (n = 1) (figure 1 and table 2).

Table 2. Collection details for *N. typhlocybae*.

Country	Region	City	Year	N	GPS	
					Latitude	Longitude
Italy	Lazio	Latina	2003	43	41.46014	12.8663
	Piedmont	Caramagna, Cuneo	2018	10	44.4737	7.4236
		Carignano, Torino	2015	14	44.5435	7.4035
Hungary	Budapest	Budapest	2015	6	47.3988	19.1580
		Budapest	2015	1	47.4811	19.0385
		Budapest	2015	10	47.4935	19.0939
		Budapest	2015	20	47.4881	19.2542
		Budapest	2016	43	47.4967	19.0868
		Budapest	2016	34	47.4770	19.0326
		Budapest	2016	14	47.4814	19.0369
		Budapest	2016	12	47.4881	19.2542
	Somogy	Balatonszemes	2015	1	46.7945	17.7700
	Tolna	Szekszárd	2016	29	46.3360	18.7022
USA	Texas	Huntsville	2016	13	30.7250	-95.55030

Figure 1. Map of *N. typhlocybae* collection locations in Italy and Hungary.

Italy

Specimens were obtained from two different time points and locations in Italy (figure 1). The first samples (n = 43) were from collections that were done in 2003 in the Lazio region of Italy (figure 1), which was within the time frame of the original releases in Italy, and would have consisted of mass-produced and released material (supplied by Bioplanet) that originated in Connecticut (table 1). These individuals were included in this study, as they should most closely reflect the diversity of *N. typhlocybae* at the time of the original establishment in Italy (however, it is important to note that we cannot verify this, but given the timeline of releases, it is the closest we can get to the original, established populations). After over two decades of releases and successful establishment throughout Italy (table 1), more recent field-collected material (2015 and 2018) was obtained from Piedmont, Italy (figure 1), and consisted of 24 specimens of *N. typhlocybae*. These later collections could contain a mixture of populations of different origins due to multiple releases and redistributions in Europe (table 1).

Texas

As a potential representation of source populations in Italy, field-collected specimens were obtained from Huntsville, Texas (n = 13) in 2016 and 2018. This location neighbours the original source location (Lick Creek Park, College Station, Texas) of the 1997 collections and releases of *N. typhlocybae* in Tuscany, Italy (Lucchi *et al*., 2002). Ideally, more samples from this region would have been collected, however the original source location (Lick Creek Park) was flooded and inaccessible during subsequent collections, and despite repeated collections of *M. pruinosa* in both College Station and Huntsville, very few were parasitized. These were included for a qualitative comparison of haplotypes, given the history of releases in Italy that originated from this general region in Texas. In addition, given the marked difference in voltinism between the populations of *N. typhlocybae* from Connecticut and Texas origin pointed out by Mazzon *et al*. (2001), the inclusion of specimens from this region could be valuable to verify possible differences in the haplotypes.

DNA extraction, amplification and sequencing

Each parasitoid was placed in an individual well of a 96-well microplate, along with 2 µl of proteinase K (20 mg/ml) and 100 µl of 5% Chelex 100 Molecular Grade Resin (Bio-Rad Laboratories, Hercules, California, USA). A negative extraction control containing the Chelex and Proteinase K solutions, but no insect tissue, was included in each microplate. Sealed microplates were incubated overnight at 55 °C, followed by 10 minutes at 99 °C. Samples were centrifuged at 5800 g for 5 minutes to pellet the Chelex solution, and 50 µl of supernatant (containing DNA) was transferred to wells in a new plate. Microplates containing the extracted DNA were stored at −20 °C until further analysis.

As described by Gariepy *et al.* (2014; 2019), PCRs were performed in a 25 µl volume containing 0.125 µl (= 1.25 units) of Taq Platinum (Invitrogen, Carlsbad, California, USA), 2.5 µl of 10x PCR buffer, 1.25 µl of 50

mM $MgCl_2$, 0.125 µl of 10 µM dNTPs (Invitrogen, Carlsbad, California, USA), 0.25 µl of 10 µM forward and reverse primer (respectively), 19.5 µl ddH_20, and 1 µl of template DNA. A 658-bp sequence of the mitochondrial COI gene was amplified by PCR using primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). Thermocycling conditions included initial denaturation at 94 °C for 1 minute, followed by five cycles of 94 °C for 30 seconds, annealing at 45 °C for 40 seconds, extension at 72 °C for 1 minute, followed by another 35 cycles of 94 °C for 30 seconds, 51 °C for 40 seconds and 72 °C for 1 minute and a final extension period of 5 minutes at 72 °C.

PCR products were visualized with a QIAxcel Advanced automated capillary electrophoresis system (Qiagen, Germantown, MD, USA) using the DNA screening cartridge and method AL320. Results were scored with QIAxcel ScreenGel software (version 1.2.0), and only those samples of the expected fragment size with a signal strength exceeding 0.1 relative fluorescent units were scored as positive.

Samples scored as positive were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. Purified PCR products were bidirectionally sequenced on an ABI 3730 DNA Analyser at the Robarts Research Institute (London Regional Genomics Centre, ON, Canada). Forward and reverse sequences were assembled and edited using CODONCODE ALIGNER program, version 4.2.7 (Codon-Code Corporation, Centreville, MA, USA). Sequence data and trace files were uploaded to the Barcode of Life Datasystems (BOLD; www.boldsystems.org) in the project *Neodryinus* biological control agents (NEODR). All sequences were trimmed to 606-bp to ensure that all samples were the same length of high-quality sequence for subsequent analysis and comparisons (Genbank accession no. MW770462-MW770711).

Distribution and diversity of haplotypes of *N. typhlocybae*

A statistical parsimony cladogram network of the *N. typhlocybae* COI haplotypes (haplotype network) was generated using the software package TCS v1.21 (Clement *et al.*, 2000). Mitochondrial COI sequences from *N. typhlocybae* were grouped based on their area of collection (1) Hungary; (2) Lazio, Italy (the 'original' 2003 collections); (3) Piedmont, Italy; and (4) Texas, USA. The proportion of specimens from each area that belong to a given haplotype was calculated; this was combined with the haplotype network to demonstrate the geographic occurrence and prevalence of each haplotype. In addition, the proportion of each haplotype within a given area was also calculated in order to obtain a representation of the haplotype composition within each geographic collection location.

Standard measures of diversity were calculated overall and for each group using DnaSP v5.10.01 (Librado and Rozas, 2009), including number of haplotypes, haplotype diversity (*h*, the probability that two randomly selected haplotypes are different; Nei, 1987), and nucleotide diversity (π, the average number of nucleotide differences per site between two randomly selected DNA sequences; Nei and Li, 1979).

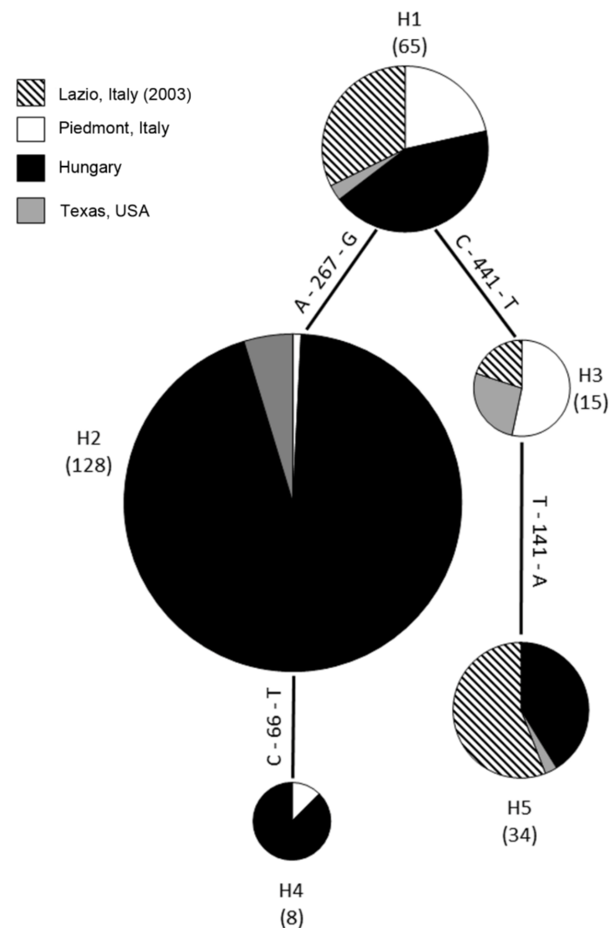

Figure 2. Statistical parsimony cladogram network representing the relationships among haplotypes for a 606-bp fragment of the COI gene of *N. typhlocybae* collected in Lazio, Italy (2003), Piedmont, Italy (2015 and 2018), Hungary (Budapest, Tolna, and Somogy counties; 2015-2016), and Texas, USA (2016 and 2018). Each circle is labelled by haplotype number, and the frequency of each haplotype is in brackets. The size of each circle is proportional to the frequency of each haplotype in the total number of samples collected.

Results

Haplotype network and haplotype distribution

A haplotype network was constructed based on the 250 samples analysed, consisting of five COI haplotypes, with four polymorphic sites and a single nucleotide base substitution between each haplotype (i.e., no intermediate haplotypes) (figure 2). Collectively, haplotype H2 was dominant and represented 51% of the specimens. Haplotypes H1 and H5 were also common, representing 26% and 14% of samples, respectively, whereas H3 and H4 were represented only occasionally (6% and 3%). Within each haplotype in the network, the proportion of samples from each of the four collection areas is presented in figure 2. Haplotype H1 was the most widespread geographically, as it was found in all four of the locations sampled, including: Hungary (Budapest, Tolna, Somogy); Lazio, Italy; Piedmont, Italy; and Huntsville, Texas. Haplotype H2 was detected in samples collected

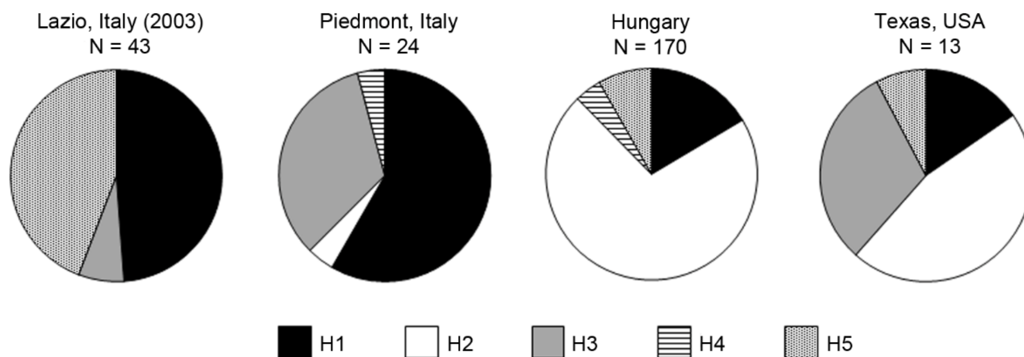

Figure 3. Haplotype frequency and composition of *N. typhlocybae* samples collected from each geographic location.

from three regions: Hungary (Budapest, Tolna County), Texas (Huntsville), and Piedmont, Italy. H3 was also detected from samples collected in three regions, including the recent collections in Piedmont and Texas, as well as the 'original' 2003 collections in Lazio; however, H3 was not observed among samples collected in Hungary. Haplotype H4 was only found in samples from Hungary (Budapest) and Piedmont, Italy. Finally, H5 was collected from three locations: Lazio, Hungary (Budapest, Tolna), and Huntsville, Texas.

The occurrence of haplotypes within each region is shown in figure 3. The earlier 'original' collection in Lazio in 2003 consisted of three of the five known haplotypes, whereas the locations with recent collections (Texas, Piedmont, and Hungary) had specimens representing four of the five haplotypes. Each region differed in the proportion and composition of haplotypes (figure 3). The haplotype composition in Lazio was dominated by H1 (49%) and H5 (44%), with a minor contribution from H3 (7%), whereas H2 and H4 were absent. Piedmont was similarly dominated by H1 (58.3%), but showed a high proportion of H3 (33.3%), and minor contributions of H2 and H4 (4.2% each), whereas H5 was not detected. In contrast, Hungarian samples were predominantly H2 (71.2%), followed by H1 (16.5%), H5 (8.2%), and H4 (4.1%), whereas H3 was not present. Samples from Texas were also predominantly H2 (46%), but H3 was also prevalent (31%), whereas H1 and H5 were minor contributors to the haplotype composition of at 15% and 8%, respectively, and H4 was not present.

Haplotype and nucleotide diversity

Haplotype and nucleotide diversity measures for collections from all four regions are presented in table 3. Haplotype diversity measures ranged from 0.460 ± 0.041 in Hungary to 0.718 ± 0.089 in Texas, and nucleotide diversity ranged from 0.00117 ± 0.00026 (Piedmont) to 0.00199 ± 0.00030 (Texas). The overall haplotype and nucleotide diversity (all samples combined) was 0.650 ± 0.022 and 0.00184 ± 0.00011, respectively.

Discussion

Introduced species, including classical biological control agents, typically experience reduced genetic variation in founding populations (Fauvergue *et al.*, 2012). Although this may decrease the fitness of established population, some species have become very successful colonizers despite founder effects (Sax and Brown 2000), particularly when founding populations exhibit some level of intraspecific genetic variation (Fauvergue *et al.*, 2012). Classical biological control relies on these successful colonizers to establish and spread following release (Hopper *et al.*, 1993); however, retrospective post-release studies examining the population genetic consequences of the introduction and subsequent spread of successful parasitoid haplotypes or genotypes are few and far between (Hufbauer *et al.*, 2004). Although a thorough examination of population genetic consequences of parasitoid introductions is beyond the scope of this study (and would require a more in-depth multilocus analysis, e.g., variability of microsatellite loci, SNPs; Roderick and Navajas 2003; Hufbauer *et al.*, 2004), understanding the similarities and differences between populations of *N. typhlocybae* from different localities using mitochondrial DNA haplotype analysis may verify the source of the parasitoid population recently found in Hungary. *N. typhlocybae* provides an excellent case study for examining the establishment and spread of a classical biological control agent in Europe: initiated in the late 1980's, populations from Connecticut were continually released and re-distributed throughout Italy and later to several additional countries (table 1). The history of parasitoid releases in Europe is

Table 3. Mean haplotype and nucleotide diversity (± SD) measures for *N. typhlocybae*.

Collection locations	Specimens	Haplotypes	Mean haplotype diversity	Mean nucleotide diversity
Lazio, Italy	43	3	0.575 ± 0.035	0.00168 ± 0.00006
Piedmont, Italy	24	4	0.569 ± 0.074	0.00117 ± 0.00026
Texas, USA	13	4	0.718 ± 0.089	0.00199 ± 0.00030
Hungary	170	4	0.460 ± 0.041	0.00125 ± 0.00016
Total	250	5	0.650 ± 0.022	0.00184 ± 0.00011

well-documented, and in addition to the Connecticut-derived populations released and redistributed throughout Italy, also include specimens collected from Texas (released in Italy; table 1), as well as New York and Delaware (released in France; table1; Malausa *et al*., 2003). Decades later, the parasitoid continues to spread in Europe, having most recently shown up in Hungary despite the absence of an intentional release program. This provided the opportunity to investigate the haplotype diversity of *N. typhlocybae* in Hungary as a preliminary step to understanding the movement of *N. typhlocybae* into Hungary, and to determine whether haplotypes consistent with the original, intended releases conducted in Italy have been retained decades following their establishment.

As the majority of samples were collected in Hungary, it is not currently possible to compare the results in a global context, as other areas were represented to a lesser extent based on the availability of specimens and resources for collection. However, we are able to present quantitative and qualitative observations regarding the occurrence and diversity of haplotypes in Hungary, as well the occurrence of haplotypes in additional (although limited) locations in Italy and Texas, as a preliminary step for describing the diversity and possible sources of *N. typhlocybae* populations found in Hungary.

Haplotype network and haplotype distribution

As *N. typhlocybae* was intentionally released in Europe from a few small, introduced populations from North America, we expect to see limited number of successfully-established haplotypes in Europe, and these likely represent only a subset of the haplotypes that exist in the area of origin. Unfortunately, we did not have access to samples of the parasitoid from across its native geographic range with which to compare; however, we have evidence of five haplotypes, which were successful in the establishment and spread from biological control releases in Europe. Of the five haplotypes described, H2 (50%) and H1 (26%) were dominant, and H1 was the only haplotype found in all four collection locations, whereas all other haplotypes were found in two (H4) or three (H2, H3, H5) of the collection locations. Additional research on the haplotype diversity in additional locations in North America and Europe will be necessary to explore the full range of haplotypes in both native and introduced ranges and confirm our speculation that the five haplotypes detected in the present study are a representative of subset of those released in biological control programs.

Considering the different haplotypes that occur within each region (figure 3), the ‘original’ collections in Lazio, Italy (2003) had the fewest haplotypes (three out of the five), and our assumption is that these populations would be most similar to the original established material in Italy, which was primarily of Connecticut-origin. This remains an assumption, as the 2003 samples are just a ‘snap-shot’ from one of the release locations shortly following establishment. These ‘original’ established populations in Lazio are predominantly H1 and H5, with a minor contribution from H3. How this relates to the occurrence and prevalence of these haplotypes in Connecticut (the presumed area of origin for original releases) needs to be explored in future studies by collecting and analysing samples from this area.

More recent collections in Piedmont, Italy are also dominated by H1, suggesting that the H1 haplotype from the original releases of *N. typhlocybae* has been retained in the population over time. However, in contrast to the Lazio collections, H3 is more prevalent in Piedmont and its frequency of occurrence in the haplotype composition is ~5x higher than that observed in the Lazio 2003 collections. This could be due to differences in the success of different haplotypes in terms of establishment over time and/or in different locations, or due to movement and spread of successfully-established haplotypes from additional releases. To resolve this, widespread haplotype analysis of *N. typhlocybae* populations are necessary and recommended to determine the current frequency of haplotypes across Italy to characterize the haplotype composition in different regions. Two additional haplotypes (H2, H4) occur in Piedmont that were absent from Lazio. The occurrence of these two haplotypes in the recent Piedmont samples could be explained in one (or more) of the following ways: (1) they may have been present in the original releases, but were not represented in the Lazio samples from 2003; (2) they could be the result of movement and spread of haplotypes from additional releases of specimens in Italy that originated from Texas (Lucchi *et al*., 2002), in particular, H2, which is dominant among our samples from Texas; (3) they could be the result of the movement and spread of additional populations of *N. typhlocybae* from New York and Delaware which were released in France (Malausa *et al*., 2003), and have not been subject to haplotype analysis (and could be relevant for H4). In all of the above scenarios, collection and analysis of samples from the area of origin (in particular Texas, Connecticut, New York, and Delaware) and the areas of initial classical biological control releases (i.e., Italy and France) should clarify the origin of haplotype H4, and provide a more complete understanding of the distribution of H2 in the native and introduced ranges.

The Hungarian samples, which were the focus of the present study, represent four of the five haplotypes, and only H3 is not represented. There are no unique haplotypes in Hungary, which supports the idea that the occurrence of *N. typhlocybae* in Hungary is from the spread of successfully-established populations (in particular H1, H2, and H5) associated with biological control efforts for *M. pruinosa* in Europe, as opposed to the independent, adventive establishment of other North American populations outside of the context of biological control (but see discussion of the H4 haplotype in the next paragraph). The dominant haplotype (approximately 70% of the haplotype composition) is H2, which is also the dominant haplotype from the samples collected in Texas, and a minor contributor to the haplotype composition in recent collections in Piedmont, Italy. It is possible that populations originating from Texas and released in Pisa in 1997 consisted of the H2 haplotype and although this is largely speculative, these established populations from Pisa may have spread within Europe (either on their own or through subsequent biological control re-distribution efforts). Future studies from more

broadly distributed populations in Italy and Texas, as well as across the range of *N. typhlocybae* in Europe, will be required to provide evidence to support or refute this. We find some support for the spread of bivoltine populations of *N. typhlocybae* in Europe. Mazzon *et al*. (2000) state that, although the Connecticut-derived population of *N. typhlocybae* has established fairly rapidly in all areas where it has been released in Italy, spread beyond the release sites appears limited, and could be attributed to the fact that the Connecticut population is predominantly univoltine. In contrast, field-collected populations of *N. typhlocybae* collected in Pisa (which is of Texan origin) showed a much higher tendency for bivoltinism (79% of individuals) when compared to field-collected populations of *N. typhlocybae* from the Veneto region (which is of Connecticut origin), which showed lower levels of bivoltinism (21%) (Mazzon *et al*., 2001). Vétek *et al*. (2019) found that *N. typhlocybae* in Hungary were predominantly bivoltine, with 67-85% of the population displaying bivoltinism. This supports the possibility that Texas-derived populations released and established in Italy have spread to neighbouring countries, and are the likely source of this parasitoid in Hungary, based on (1) the predominance of bivoltinism; (2) the predominance of haplotype H2 in both Texan and Hungarian populations of *N. typhlocybae*, and (3) the release history of Texan samples in Italy. This is not to suggest that the parasitoid was introduced directly from Italy to Hungary, but rather that, through the bridgehead effect, a successful haplotype has become the source population for the spread of adventive populations to neighbouring countries (Engelkes and Mills, 2011). Mazzon *et al*. (2001) suggest that bivoltinism of *N. typhlocybae* is of major importance for the successful biological control of *M. pruinosa*, as this second generation decreases the proportion of the pest population that would otherwise overwinter and emerge the following growing season. Further, range expansion is often attributed to bivoltinism, with the establishment of bivoltine populations allowing more rapid colonization and spread due to a higher growth rate than their univoltine counterparts (Fahrner and Aukema, 2018). This could result in successful establishment and spread of bivoltine populations of *N. typhlocybae*, although the genetic basis of voltinism in *N. typhlocybae* would need to be investigated further to corroborate this.

The remaining haplotypes that occur in Hungary include H1, which is fairly widespread in all geographic regions sampled. Given the successful establishment and retention of this haplotype in Italy (demonstrated by its dominance in the 'original' Lazio 2003 collections and the more recent Piedmont collections), we assume that this haplotype was among the successfully-established populations in the initial biological control program for *M. pruinosa*, and was likely among the populations redistributed throughout Europe (table 1), any of which could have served as a source population for adventive *N. typhlocybae* in Hungary (likely via neighbouring countries). As previously mentioned, understanding the frequency of H1 in the haplotype composition in other European countries may clarify the movement and spread of this haplotype. This is also the case for H5, which was observed at a low level in Hungary, but which was also prevalent among the original collections in Lazio in 2003, and despite its absence in the more recent Piedmont collections, could have been retained elsewhere in Italy following the extensive biological control release programs. Understanding the frequency of H5 in the haplotype composition within Italy and in neighbouring countries, may clarify the persistence and spread of H5 within Europe. Finally, H4 was a minor contributor to the haplotype composition in Hungary, and is currently of unknown origin, having only been found in common with recent collections in Piedmont. As previously discussed above for the Piedmont samples, occurrence of H4 could be the result of the movement and spread of additional haplotypes released in France (from New York and Delaware; table 1) that have not been characterized, or they may have been present in the original releases, but were not among the samples collected in Lazio in 2003, or they could be a separate establishment in Europe from North America, independent of the documented biological control releases that took place in Italy and France. In all three scenarios, more widespread collection and analysis of samples from the area of origin and the area of introduction may clarify the origin and spread of H4 in Hungary.

Haplotype and nucleotide diversity

In terms of species diversity measures, Goodall-Copestake *et al*. (2012) recommend that, in order to provide qualitative descriptions of low and high diversity, estimates of haplotype and nucleotide diversity should be compared to the median values ($h = 0.701$; $\pi = 0.00356$) that they report across a variety of groups (including numerous Crustacea, Hexapoda, and Mollusca). In this context, the overall mean values for haplotype ($h = 0.650$) and nucleotide diversity ($\pi = 0.00184$) of *N. typhlocybae* (table 3) fall below this benchmark median, indicating that they would be categorized as low diversity. We are confident in this statement as it pertains to the introduced populations in Europe, as we would expect low diversity in an area of introduction due to a genetic bottleneck and founder effects (Nei *et al*., 1975; DeBach and Rosen, 1991; Dlugosch and Parker, 2008; Fauvergue *et al*., 2012). However, we recognize that a more thorough sampling of *N. typhlocybae* in North America is necessary to obtain a more accurate representation of the species across its native range before such a conclusion regarding the species diversity measures in the area of origin can be drawn. Further, sample sizes of 25 or more individuals per population are preferred in population level diversity studies using the COI gene to ensure a more accurate comparison of diversity measures (Goodall-Copestake *et al*., 2012). Although we included the specimens from Texas in this study, it was primarily to provide a basis of comparison for the haplotype composition, as it is a potential source population for biological control releases in Italy. For a more thorough and accurate investigation of genetic diversity indices in the area of origin, a more thorough collection of specimens in Texas and elsewhere in the USA would be required.

Conclusions

Overall, it is difficult to draw concrete conclusions on the origin of each haplotype occurring in Hungary (or elsewhere in Europe) without further collection and analysis of samples throughout the geographic range in the areas of origin and introduction of *N. typhlocybae*. As such, the conclusions here are mainly speculative based on the haplotype distribution and known release history. Although the study conducted here is preliminary in nature, the results suggest the movement and spread of different *N. typhlocybae* haplotypes, with different populations successfully establishing in Hungary, either related to previous biological control efforts in Italy (e.g., H1, H2, and H5) or of unknown origin (e.g., H4). It also demonstrates that there are a number of gaps that remain to be filled in order to better understand the movement and spread of this parasitoid following approximately two decades of intentional releases in several European countries. Further collection of *N. typhlocybae* across the known geographic range in Europe, coupled with more extensive collections in North America – or at the very least more extensive collection in areas where material originated for biocontrol releases in Europe (e.g., Texas, Connecticut, Delaware, New York) – would help reconstruct possible pathways of movement and spread over the last three decades – not only in countries where the parasitoid was released, but also in areas with adventive establishment. This would also help identify which haplotypes or populations may be more successful, and can therefore inform future biological control decisions and/or redistribution of the parasitoid in an effort to improve control of invasive *M. pruinosa*. In addition, more coordinated sampling efforts with larger sample sizes from across the distribution of *N. typhlocybae* would be useful in conducting statistical analysis of different introduction scenarios. For example, approximate Bayesian computation (ABC) has been used to quantitatively compare different pathway scenarios and reconstruct invasion routes for insect pests (Fraimout *et al.*, 2017). The same approach could be used in future work to reconstruct the movement and spread of *N. typhlocybae* in Europe, and identify the origins of different populations, and when and where genetic bottlenecks occurred. This may provide insight into whether multiple introductions from different areas of origin prevented a loss of molecular diversity and contributed to more successful establishment and spread (Dlugosch and Parker, 2008). And if we consider classical biological control as a "planned invasion" (Abram and Moffat, 2018), then an improved understanding of the factors that led to successful establishment and spread of a classical biological control agent, such as *N. typhlocybae*, over a broad geographic area would be extremely useful to inform biological control theory and practice.

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