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Molecular identification and distribution of leatherjackets (Diptera:Tipulidae) in UK
agricultural grassland.

Running title: Identification of leatherjackets in grassland

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Abstract

1. DNA barcoding is useful for the identification of morphologically cryptic invertebrates. An important application is for pest species, for which it is critical to determine the distribution, biology and ecology of damaging life-stages in order to target management effectively.

2. *Tipula paludosa* (Meigen) and *Tipula oleracea* (Linnaeus) leatherjackets, the larvae of crane flies (Diptera: Tipulidae), are pests of agricultural grassland in Europe and the USA, and difficult to distinguish morphologically.

3. We used cytochrome c oxidase subunit I (COI) barcoding to identify leatherjackets from 19 permanent grassland fields over 2 sampling seasons on the Rothamsted Research North Wyke Farm Platform, south west UK, to assess species-level distribution and genetic diversity.

4. Most larvae (94%) were found to be *T. paludosa*, comprising 18 haplotypes which were spread across the sampling site in a panmictic population. However, *T. oleracea* were found in low abundance (3% of larval samples) and only in the second year of sampling. Other morphologically similar Dipteran larvae (3%) were also found.

5. This dominance of one species suggests there may be underlying differences in species biology, such as choice of oviposition site and dispersal ability, in agreement with other studies, and has implications for monitoring and management.

Keywords: DNA sequencing, COI barcoding, soil insects, soil pests, belowground, grassland, haplotype analysis.

1. Introduction

Tipula paludosa (Meigen) and *Tipula oleracea* (Linnaeus) are polyphagous agricultural pests, mainly inhabiting permanent pasture but also causing damage to a variety of crops. They are known to cause significant damage in the UK (Blackshaw and Coll 1999; Peck and Olmstead 2009) but have also recently become a problem in the USA following their accidental introduction and spread, causing cosmetic and economic damage; larvae feed on the roots and stems of plants as well as decaying vegetation (Rao et al. 2006). *Tipula paludosa* mainly damages pastures and spring cereals, causing characteristic bare patches due to feeding on roots, and sometimes leaves (Blackshaw and Coll 1999; White and French 1968). In contrast, *T. oleracea* have been important pests of winter cereals planted after oilseed rape (Blackshaw and Coll 1999). Other crops including lettuce, celery, sugar beet, sweet corn, berries, tobacco, various vegetables and ornamentals are also targeted (Blackshaw and Coll 1999; Campbell 1975; Emmet 1992).

Adults can be easily separated by morphology, but larvae are more difficult to distinguish and as such identification is challenging. The pattern of microscopic hairs on the abdomen as well as sclerotisation on the final abdominal segment have been suggested as key morphological features for separating these species (Brindle 1959), but this has been disputed as a reliable character (Gelhaus 2005). Isoelectric focusing, a protein based method, has also been used (Humphreys et al. 1993), but it is not able to distinguish between these and other tipulid species. Rao et al. (2006) used sequences of the mitochondrial Cytb gene to identify *T. paludosa* and *T. oleracea* as well as other native North American crane flies, but there is a lack of data at this DNA region for many tipulid species and so it does not have universal applicability which is important in community studies. This lack of a reliable, widely

relevant and cost effective technique often means that larvae are not identified, despite that this is important for applying pest management strategies; there are distinct differences in life cycle, feeding behaviour and the damage period throughout the year (Rao et al. 2006), which can result in potentially costly misuse of biological control applications. It also means that at present we have limited knowledge of populations in areas with a high proportion of grassland (e.g. the south west UK). Since tipulids are also an important food source for birds (Pearce-Higgins et al. 2005, Rhymer et al. 2012), understanding their distribution is critical for predicting their population dynamics.

Molecular genetic methods offer a new approach to this problem. DNA barcoding (Hebert et al. 2003), sequencing a section of the cytochrome c oxidase subunit I (COI) mitochondrial protein coding gene, has become a standard for identification of closely related taxa across a range of taxonomic groups, and as such has been used to distinguish morphologically cryptic species in many taxa. However, to date, the application of molecular genetic techniques to soil insects has lagged behind their aboveground counterparts (Benefer and Blackshaw 2013). Where they have been used, at the COI region and other diagnostic regions of DNA, it has often proven to be invaluable in elucidating previously unclear aspects of the biology and ecology of a range of species (e.g. in wireworms; Benefer et al. 2010; Benefer et al. 2012; Benefer et al. 2013; Staudacher et al. 2011).

In this study, we used DNA barcoding to identify larvae of *T. paludosa* and *T. oleracea* collected using a systematic sampling approach in agricultural grassland on the Rothamsted Research North Wyke Farm Platform (NWFP) in south west UK. The samples collected here were used as a baseline survey to assess the distribution of soil insects across the site (described in Benefer et al. 2016). Using

DNA barcoding to identify leatherjackets collected to the species level, our specific objectives were to:

1. Determine the relative proportion of leatherjacket species present
2. Investigate the intra- and inter-specific genetic variation of the species identified
3. Assess the spatial relationships between tipulid haplotypes.

2. Methods

2.1 Sample collection

Adult *T. paludosa* and *T. oleracea* were collected randomly from agricultural grassland on the North Wyke Farm Platform (NWFP), Okehampton, Devon, UK (Lat: 50.73237; Long: -3.99635; Fig. 1) in May 2013 using hand nets and light traps. The species were separated according to morphological characters (Blackshaw and Coll 1999; Hoebeke and Klass 2005), and samples were stored at -20°C before use in DNA extraction. Leatherjackets recovered in the surveys described in Benefer et al. (2016) were used as source material. Briefly, larvae were heat extracted from soil cores that were collected using a systematic sampling approach (a 25m grid across 19 fields) in two sampling periods over two years. Larvae collected in April-May 2012 (106 samples) were stored in 70% ethanol, whilst those collected in April-May 2013 (117 samples) were stored at -20°C before subsequent processing (it was intended to use stable isotope analysis on the 2013 samples and this required that no alcohol be present, hence a different preservation method was used in 2012; no difference in DNA quality or quantity was noticed between these preservation techniques). A subset of these larvae was subsequently analysed.

2.2 DNA extraction, amplification and sequencing

DNA was extracted using a DNeasy® Tissue Kit (Qiagen, Hilden, Germany) following the spin-column protocol for purification of total DNA from animal tissue. For both adult tipulids and larvae, one of the central abdominal segments was cut into small pieces using a sterilized blade and an approximately 1mm³ section used for extraction. All 117 larvae collected in 2013 were processed, but due to time and resource limitations only 59 of the 106 larvae (i.e. 53%) collected in 2012 were processed. Extracted DNA was preserved at 4°C for later PCR.

PCR was performed in individual 0.2mL PCR tubes using a Qiagen Taq PCR Core Kit (Qiagen). Each 25uL PCR contained (final concentrations given in brackets): 10X PCR buffer (1X), MgCl₂ (3mM), dNTPs (200µM of each), forward primer (1uM), reverse primer (1uM), BSA (500ng; larval amplification was unsuccessful without this additive), Taq (0.5 units), DNA grade water and 1uL DNA. Original Folmer primers (Folmer et al. 1994) gave variable results, so we used modified Folmer primers (Ramirez-Gonzalez et al. 2013), which resulted in a greater success rate. The tubes were subjected to the following PCR cycle: initial denaturation at 94°C (3 min), 35 cycles of denaturation at 94°C (1 min), annealing at 52°C (30s) and extension at 72°C (1 min), followed by a final extension at 72°C (10 min). PCR products were stored at 4°C in the short-term (<1 month) before subsequent processing.

PCR success and specificity was determined by running 5uL of PCR product on a 2% agarose gel stained with SYBR® Safe DNA gel stain (Thermo Fisher Scientific, Paisley, UK), and the remaining 20uL cleaned to remove excess primers and dNTPs using Exonuclease I (Exo, 10 units; New England Biolabs, Hitchin, UK) and Shrimp Alkaline Phosphatase (SAP, 1 unit; New England Biolabs, Hitchin, UK). The cleaned

PCR products were sent to Macrogen Inc. (Europe) for sequencing in the forward direction only. 157 larvae out of the original 176 processed (plus 25 adults) were sequenced in total; six 2012 larvae and thirteen 2013 larvae, i.e. 11% of the samples processed, failed to amplify even when PCR was repeated and so were not sent for sequencing.

2.3 Sequence analysis

The sequence trace files were visually checked and sequences manually edited where applicable (i.e. in cases of ambiguous base calling) using BioEdit (Version 7.1.9.0; Hall 1999). Each sequence was translated to protein using Expasy (<http://web.expasy.org/translate/>) to ensure that they complied with an open reading frame (Buhay 2009). In order to achieve larval identification, known adult and larval sequences were aligned and compared using ClustalW in MEGA 5.1 (Tamura et al. 2011), and all sequences were compared to the Barcode of Life Database (BOLD) (BOLD Systems V3; <http://www.boldsystems.org/>).

Intra- and inter-specific genetic characteristics were assessed for each year separately and combined using *P*-distance in MEGA 5.1 (Tamura et al. 2011) and by calculating the number of haplotypes, nucleotide diversity and haplotype diversity in DNAsp. v.5 (Librado and Rozas 2009) for an aligned region of 456bp for *T. paludosa* only (*T. oleracea* contained <5 larval individuals, which can produce variable and potentially inaccurate results; Goodall-Copestake et al. 2012).

A locus-by-locus analysis of molecular variance (AMOVA) with 1000 permutations was carried out in Arlequin ver. 3.5.2 (Excoffier and Lischer 2010) to test for

significant differentiation within and between fields, for both years combined, and only for the 14 fields with >5 individuals (N=14), and a Minimum Spanning Network (MSN) was calculated in Network (Fluxus Technology Ltd. 2010; Bandelt et al. 1999), using median joining (MJ) to assess the relationships between haplotypes and their distribution across the study site.

3. Results

3.1 Species identification

In total, good quality sequences were obtained for 151 larval samples (49 from 2012, 102 from 2013) and all 25 of the adult specimens, which matched the original morphological identification (GenBank accession numbers KX698438 - KX698604). Sequences ranged from 590bp – 662bp in length, reflecting the differing quality of the sequence reads at either end.

Eighteen adults and 142 larvae (all 49 samples in 2012 and 93 samples in 2013; 94% of the total larvae processed) were identified as *T. paludosa*. Seven adults and five larvae (3% of the total larvae processed), all of which were found in 2013, were identified as *T. oleracea* in BOLD, with a 92-100% match (eight of these samples had a <97% match). There were seven larval sequences, which, with the exception of one, were a high match to either *T. paludosa* or *T. oleracea* (99-100%), but for which the protein sequence was incorrect (it did not adhere to a reading frame); although these are included in the identification data above since they contain a high match, they were excluded from further genetic analyses to avoid analysis and interpretation of potentially incorrect haplotypic/genetic diversity data.

There were four larval sequences which did not match either of these species in 2013 (3% of the total larvae processed): two matched other related crane fly families, one with >99% match to *Tricyphona immaculate* (Meigen) (Family Pediciidae) and one with >99% match to an unknown species in the Family Limoniidae. The third was a 100% match to *Rhagio scolopaceus* (Linnaeus) (Family Rhagioniidae), and the fourth was >99% match to an unknown Gammaproteobacteria.

3.2 Genetic diversity and haplotype analyses

For *T. paludosa* there were 18 haplotypes, containing between 1 and 79 sequences (Table 1). Six of these haplotypes were shared by both adult and larval samples. Apart from the seven more divergent samples (one was excluded due to having an incorrect protein sequence), which comprised three of these haplotypes with a *p*-distance of between 3.07% and 8.77% to all other non-divergent samples, the intra-specific genetic distance was low – between 0.22% and 1.75% (Table 1). Some of the *T. paludosa* haplotypes found in 2012 were not found in 2013 and vice versa, with six shared between samples collected in different years, but the majority belonged to four haplotypes while the rest were comprised of 1-3 sequences (Fig. 2a,b; Fig. 3). Genetic diversity indices for *T. paludosa* larvae are reported in Table 2.

There were three *T. oleracea* larval haplotypes (one sample was excluded due to having an incorrect protein sequence), and there were an additional three adult haplotypes; intra-specific *p*-distance was 0.19% – 1.1%. Interspecific *p*-distance was between 4.61% - 6.36% (Table 1).

209 **3.3 Haplotype distribution**

210 The AMOVA (Table 3) suggested that most of the variation (>99%) was within rather
211 than between fields, suggesting no population structuring is present at this scale in
212 this study site and that this is a panmictic population. This is further supported by the
213 MSN (Fig. 3), which shows the relationships between the haplotypes and the fields
214 they were collected from. It can be seen here that there is no clear clustering of
215 haplotypes by field. There was also no clear pattern in terms of the distribution of
216 haplotypes between years, with some fields which contained relatively large
217 numbers of larvae one year containing none or reduced numbers the following year
218 (e.g. field 19 and field 10, Fig. 2). However, haplotype 4 in particular was widely
219 distributed in both years.

4. Discussion

This study set out to determine the relative proportions, genetic variation and distribution of two pest tipulid species in a south-west UK grassland using DNA barcoding. We found that a 456bp region of the COI gene is diagnostic for these two species, and the method described is a quick and easy approach to identification of these morphologically cryptic pests. Given the extensive and ever expanding collection of publically available COI sequences stored in databases such as GenBank and BOLD, the method and data presented here will be of use to others investigating the ecology of *T. paludosa* and *T. oleracea* worldwide.

The vast majority (94%) of larvae collected were *T. paludosa*, with *T. oleracea* representing only 3% of the sequences obtained. This is in agreement with Humphreys et al. (1993), who reported 4% of larvae to be this species in a survey of 75 agricultural grass fields in Northern Ireland. Adults were not directly surveyed in this current study, though based on observational data both species were abundant aboveground, which could suggest that there are species-specific oviposition preferences and/or differences in dispersal ability (Blackshaw et al. 1996; Benefer et al. 2016). Although the limited data from surveys in UK agricultural grassland suggest *T. oleracea* may not be present in damaging proportions, in North America this species is becoming more common in turfgrass (Rao et al. 2006) and so it is important to be able to separate damaging species reliably. As for other pests, differences in the biology and ecology of damaging species has implications for the monitoring of populations and targeting of control methods. Our specific observation (concurring with that of Humphreys et al. 1993) that adult *T. oleracea* were widely distributed over this grassland area whereas the larvae were relatively scarce shows the importance of understanding how the presence and abundance of above-ground

adult stages relates to belowground larval populations. A similar dissonance in adult and larval *Agriotes lineatus* (Linnaeus) wireworm distributions has also been reported (Benefer et al. 2012).

That other related crane fly species in the Tipuloidea superfamily were found here (albeit in very low abundance suggesting they are unlikely to be pests) and that they were initially identified as tipulid larvae indicates how difficult it is to identify some soil-dwelling larval species and emphasises the role of molecular methods for species identification. Validated barcoding approaches, such as that described here, which improve the efficiency and reliability of species identification for cryptic species are therefore vital for future studies.

The level of genetic variation between *T. oleracea* and *T. paludosa* found in this study (4.61% - 6.36%) is in accordance with that described by Rao et al. (2006), who reported an average inter-specific *p*-distance of ~7% at the mitochondrial *cytb* region and suggested that these species are sister groups. Intra-specific variation was generally less than 2% (excluding the five more divergent samples), meaning individuals could be unambiguously assigned to the correct species. Although we did not screen all of the samples collected at our study site using this molecular technique, we did assess a substantial proportion (67%) of them, comprising a large collection of individuals from spatially separated locations as well as morphologically identified adults. We also validated our sequences against those available in GenBank/BOLD, which provided a >97% match for most individuals. While we can therefore be relatively confident that we have accounted for variation adequately and that this approach would be suitable for other populations, we did also find four larval individuals that had an intra-specific *p*-distance of 4% and one that had a *p*-distance of 8%. This could indicate that intra-specific variation is higher than the standard 3%

sequence divergence threshold used in many COI barcoding studies (e.g. Hebert et al. 2004a) and/or that cryptic species are present (e.g. Hebert et al. 2004b). Alternatively, there may be issues with contamination through the use of universal COI primers that amplify across many species (e.g. we did find one individual with a >99% match to a Gammaproteobacteria), or nuclear copies of mitochondrial DNA (numts) may be present, increasingly found to be an issue in barcoding studies that use mitochondrial genes for species identification (Song et al. 2008; Buhay 2009; Benefer et al. 2012). Here, 11% of larvae failed to amplify and six of the sequences (4%) contained additional underlying sequence leading to ambiguous base calling. However, by using the validation steps here (manually checking trace files carefully for errors, checking the protein sequence for stop codons, comparing sequences to public sequence databases) we were able to highlight and omit low quality and potentially erroneous sequences, leaving us with a high quality dataset. The use of specific tipulid primers, more than one region of DNA and sequencing of morphologically identified adults from grassland may aid in improving the efficacy of the method and the identification of cryptic species.

Across both years, 17 larval *T. paludosa* haplotypes were found, with greater nucleotide and haplotype diversity and haplotype abundance in the second year (which may be somewhat expected as there was a bigger sample size this year). Differences between haplotypes were generally small, but there were five more divergent haplotypes, four of which were found in a single field each (Fig. 3). Interestingly, haplotypes 3 and 9, two of the more divergent haplotypes that were more closely related to each other (Table 1) than all other haplotypes (apart from an adult; haplotype 18), were also found in neighbouring fields. This might suggest that

there are limitations to dispersal in this area, which can isolate and maintain local populations through which little gene flow takes place (Schowalter 2011). Depending on the temporal scale of isolation, this may eventually lead to divergent groups with different behavioural phenotypes (Edelaar et al. 2008), reducing interaction with other individuals even further. Given that some haplotypes (e.g. haplotype 4) are common throughout the study site, and that AMOVA results suggest a panmictic population with little evidence of spatial structuring (in accordance with Benefer et al. (2016) who in a broader assessment found little evidence of scale or space affecting tipulid larvae presence/absence in general), lack of dispersal ability in *T. paludosa* in general does not seem to be the case, at this scale of study at least. For example, field 1 and field 19 are separated by a distance of approximately 1.6km but share haplotype 1. Bearup et al. (2013) have suggested that directional, wind-assisted dispersal may account for synchronisation in *T. paludosa* dynamics over landscape distances, and so a lack of genetic spatial structure at this scale is not unexpected. The present study, along with those of Bearup et al. (2013) and Blackshaw and Petrovskii (2007), suggest that *T. paludosa* would be a useful model organism for investigating the relationship between spatial and genetic variation with respect to adult dispersal, and interactions with the landscape and environment.

In conclusion, DNA barcoding of the COI region has proven to be reliable in separating *T. paludosa* and *T. oleracea*, with the former being by far the most prevalent species in this study site. The data suggest a relatively high level of genetic variation and dispersal at this study scale, as well as the potential presence of cryptic species, and future more extensive studies might use the method

319 described here to address whether tipulid species exist as a complex and the
320 implications of this for damage, monitoring and control.

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1. <i>T. paludosa</i> hap 1		0.3	0.81	0.22	0.3	0.29	0.36	0.36	0.82	0.3	0.31	0.31	1.22	0.3	0.57	0.32	0.3	0.84	0.97	0.97	1	0.99	0.99	0.99	2.3	2.26	2.29	2.33
2. <i>T. paludosa</i> hap 2	0.44		0.8	0.21	0.29	0.29	0.35	0.35	0.81	0.3	0.3	0.3	1.23	0.29	0.56	0.3	0.3	0.83	0.98	0.98	1.02	1	1	1	2.29	2.26	2.29	2.33
3. <i>T. paludosa</i> hap 3	3.29	3.29		0.78	0.81	0.75	0.78	0.79	0.3	0.81	0.81	0.82	1.3	0.81	0.93	0.81	0.8	0.36	1.1	1.1	1.14	1.07	1.08	1.11	2.33	2.27	2.27	2.34
4. <i>T. paludosa</i> hap 4	0.22	0.22	3.07		0.21	0.2	0.29	0.29	0.79	0.21	0.22	0.22	1.21	0.21	0.53	0.22	0.21	0.81	0.95	0.95	0.98	0.98	0.97	0.97	2.29	2.26	2.29	2.33
5. <i>T. paludosa</i> hap 5	0.44	0.44	3.29	0.22		0.3	0.36	0.35	0.82	0.3	0.31	0.3	1.19	0.29	0.58	0.3	0.3	0.85	0.92	0.92	0.96	0.95	0.95	0.94	2.31	2.27	2.3	2.31
6. <i>T. paludosa</i> hap 6	0.44	0.44	2.85	0.22	0.44		0.21	0.21	0.75	0.28	0.3	0.29	1.2	0.29	0.56	0.31	0.28	0.78	0.94	0.94	0.98	0.97	0.96	0.96	2.29	2.26	2.28	2.33
7. <i>T. paludosa</i> hap 7	0.66	0.66	3.07	0.44	0.66	0.22		0.29	0.78	0.35	0.36	0.36	1.22	0.35	0.6	0.37	0.35	0.8	0.96	0.96	1	0.98	0.98	0.97	2.29	2.26	2.28	2.33
8. <i>T. paludosa</i> hap 8	0.66	0.66	3.07	0.44	0.66	0.22	0.44		0.79	0.34	0.36	0.36	1.22	0.36	0.61	0.37	0.34	0.81	0.96	0.96	0.99	0.99	0.98	0.98	2.29	2.26	2.28	2.33
9. <i>T. paludosa</i> hap 9	3.29	3.29	0.44	3.07	3.29	2.85	3.07	3.07		0.82	0.82	0.83	1.31	0.82	0.93	0.82	0.81	0.21	1.1	1.1	1.14	1.07	1.08	1.11	2.31	2.27	2.26	2.33
10. <i>T. paludosa</i> hap 10	0.44	0.44	3.29	0.22	0.44	0.44	0.66	0.66	3.29		0.3	0.3	1.22	0.3	0.57	0.3	0.29	0.84	0.97	0.97	1	0.99	0.98	0.98	2.3	2.25	2.3	2.34
11. <i>T. paludosa</i> hap 11	0.44	0.44	3.29	0.22	0.44	0.44	0.66	0.66	3.29	0.44		0.3	1.23	0.3	0.57	0.31	0.31	0.84	0.97	0.97	1.01	1	1	0.99	2.3	2.26	2.29	2.32
12. <i>T. paludosa</i> hap 12	0.44	0.44	3.29	0.22	0.44	0.44	0.66	0.66	3.29	0.44	0.44		1.21	0.3	0.58	0.31	0.3	0.85	0.95	0.95	0.98	0.98	0.97	0.97	2.3	2.26	2.29	2.33
13. <i>T. paludosa</i> hap 13	7.68	7.68	8.33	7.46	7.24	7.24	7.46	7.46	8.33	7.68	7.68	7.46		1.21	1.3	1.24	1.2	1.33	0.76	0.81	0.82	0.86	0.77	0.79	2.33	2.25	2.33	2.29
14. <i>T. paludosa</i> hap 14	0.44	0.44	3.29	0.22	0.44	0.44	0.66	0.66	3.29	0.44	0.44	0.44	7.68		0.48	0.3	0.29	0.84	0.96	0.96	1	0.96	0.98	0.98	2.3	2.27	2.29	2.33
15. <i>T. paludosa</i> hap 15	1.54	1.54	4.39	1.32	1.54	1.54	1.75	1.75	4.39	1.54	1.54	1.54	8.77	1.1		0.57	0.56	0.95	1.07	1.07	1.1	1.06	1.09	1.08	2.28	2.27	2.28	2.32
16. <i>T. paludosa</i> hap 16	0.44	0.44	3.29	0.22	0.44	0.44	0.66	0.66	3.29	0.44	0.44	0.44	7.68	0.44	1.54		0.3	0.84	0.98	0.98	1.02	1.01	1	1	2.3	2.25	2.3	2.35
17. <i>T. paludosa</i> hap 17	0.44	0.44	3.29	0.22	0.44	0.44	0.66	0.66	3.29	0.44	0.44	0.44	7.68	0.44	1.54	0.44		0.83	0.95	0.95	0.99	0.98	0.98	0.97	2.29	2.25	2.29	2.32
18. <i>T. paludosa</i> hap 18	3.51	3.51	0.66	3.29	3.51	3.07	3.29	3.29	0.22	3.51	3.51	3.51	8.55	3.51	4.61	3.51	3.51		1.11	1.11	1.16	1.08	1.1	1.13	2.31	2.27	2.26	2.33
19. <i>T. oleracea</i> hap 1	5.04	5.04	6.14	4.82	4.61	4.61	4.82	4.82	6.14	5.04	5.04	4.82	3.07	5.04	6.14	5.04	5.04	6.36		0.21	0.29	0.37	0.22	0.2	2.32	2.29	2.3	2.3
20. <i>T. oleracea</i> hap 2	5.04	5.04	6.14	4.82	4.61	4.61	4.82	4.82	6.14	5.04	5.04	4.82	3.29	5.04	6.14	5.04	5.04	6.36	0.22		0.35	0.42	0.31	0.29	2.32	2.3	2.31	2.3
21. <i>T. oleracea</i> hap 3	5.48	5.48	6.58	5.26	5.04	5.04	5.26	5.26	6.58	5.48	5.48	5.26	3.51	5.48	6.58	5.48	5.48	6.8	0.44	0.66		0.46	0.37	0.36	2.32	2.29	2.3	2.3
22. <i>T. oleracea</i> hap 4	5.26	5.26	5.92	5.04	4.82	4.82	5.04	5.04	5.92	5.26	5.26	5.04	3.73	4.82	5.92	5.26	5.26	6.14	0.66	0.88	1.1		0.43	0.41	2.33	2.31	2.3	2.3
23. <i>T. oleracea</i> hap 5	5.26	5.26	5.92	5.04	4.82	4.82	5.04	5.04	5.92	5.26	5.26	5.04	3.29	5.26	6.36	5.26	5.26	6.14	0.22	0.44	0.66	0.88		0.31	2.32	2.29	2.31	2.29
24. <i>T. oleracea</i> hap 6	5.26	5.26	6.36	5.04	4.82	4.82	5.04	5.04	6.36	5.26	5.26	5.04	3.29	5.26	6.36	5.26	5.26	6.58	0.22	0.44	0.66	0.88	0.44		2.31	2.29	2.29	2.3
25. <i>Tricyphona immaculata</i>	47.4	47.4	46.3	47.1	47.4	47.1	47.1	47.4	46.7	47.4	47.4	46.9	49.6	46.9	48	47.4	46.9	46.7	46.9	47.1	47.1	46.5	46.7	46.7		2.25	1.68	1.61
26. <i>Gammaproteobacteria</i>	57.2	57	56.8	57	57.2	56.8	56.8	56.8	57	57.2	57	57.2	58.6	56.8	57.2	57.2	56.8	57	56.4	56.6	56.6	55.9	56.4	56.4		43.6		2.3
27. <i>Limoniidae</i>	44.1	44.1	44.3	44.1	44.3	43.9	43.9	43.9	44.7	44.3	44.3	43.9	46.7	43.9	45	44.3	44.3	44.7	43.9	43.6	44.1	44.1	43.6	43.6	15.6	43.9		1.62
28. <i>Rhagio scolopaceus</i>	47.1	46.9	45.8	46.9	46.7	46.9	46.9	46.9	46.3	47.1	46.7	47.1	49.1	46.9	48	47.1	46.7	46.3	46.7	46.5	46.9	46.7	46.5	46.7	14.3	43.2	16.2	

448 Table 1. Genetic distance (p-distance, %) for haplotypes of all species, for adults and larvae combined, identified by DNA barcoding.

449 One sequence haplotype was used in a 452bp alignment (excluding gaps and missing data). Standard error estimates (%) are

450 given above in italics on the diagonal.

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	2012	2013	All
Number of individuals	46	90	136
Number of haplotypes	10	14	17
Haplotype diversity	0.688 (0.071)	0.703 (0.049)	0.695 (0.040)
Nucleotide diversity	0.00478 (0.00171)	0.00607 (0.00192)	0.00562 (0.00141)

Table 2. Genetic diversity indices for a 456bp section of the COI gene of *T. paludosa* larvae collected from the North Wyke Farm Platform, UK, in 2012, 2013, and these years combined. Figures in brackets are \pm standard deviation.

Source of variation	d.f.	Sum of squares	Variance components	% of variation
Among fields	13	18.325	0.00799	0.59
Within fields	114	152.441	1.3372	99.41
Total	127	170.766	1.34519	

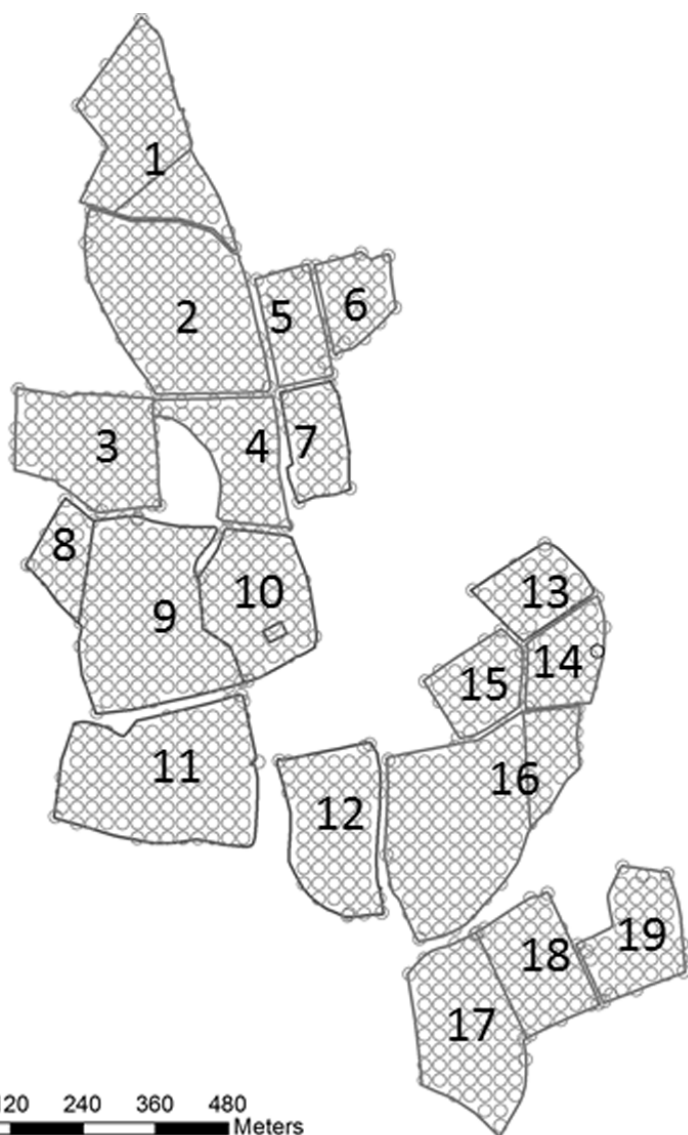
Table 3. Locus-by-locus AMOVA for a 456bp region of the COI gene for *T. paludosa* larvae (both years combined) collected on the North Wyke Farm Platform, UK.

468 **Fig. 1** The study site at the North Wyke Farm Platform, Okehampton, Devon, UK.
469 Fields are numbered and small open circles represent the individual sampling points,
470 which are on a 25m grid.

471 **Fig. 2** The number of sequences per haplotype for *T. paludosa* larvae collected
472 across 19 fields in a) 2012 (N=46) and b) 2013 (N=90) on the North Wyke Farm
473 Platform, UK.

474 **Fig. 3** Minimum spanning network of *T. paludosa* adult and larval COI sequences
475 (456bp; N = 154). The size of the pie charts, which represent the field(s) the
476 haplotype was found in, is proportional to the number of sequences of that haplotype
477 in the population. The length of the connectors is proportional to the number of
478 nucleotide changes between haplotypes (the longest branches were contracted; the
479 number of mutations is indicated next to the connector)

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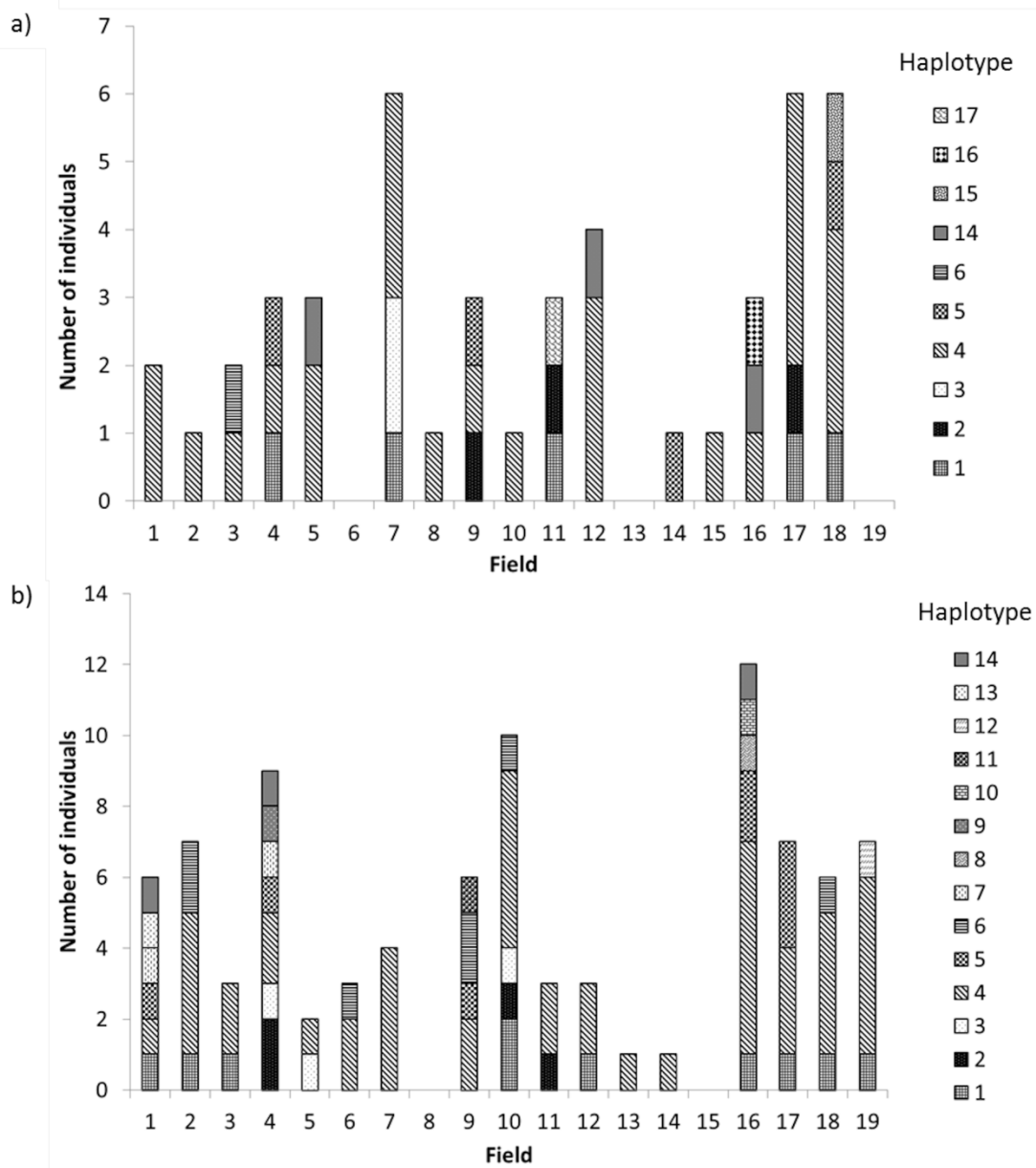
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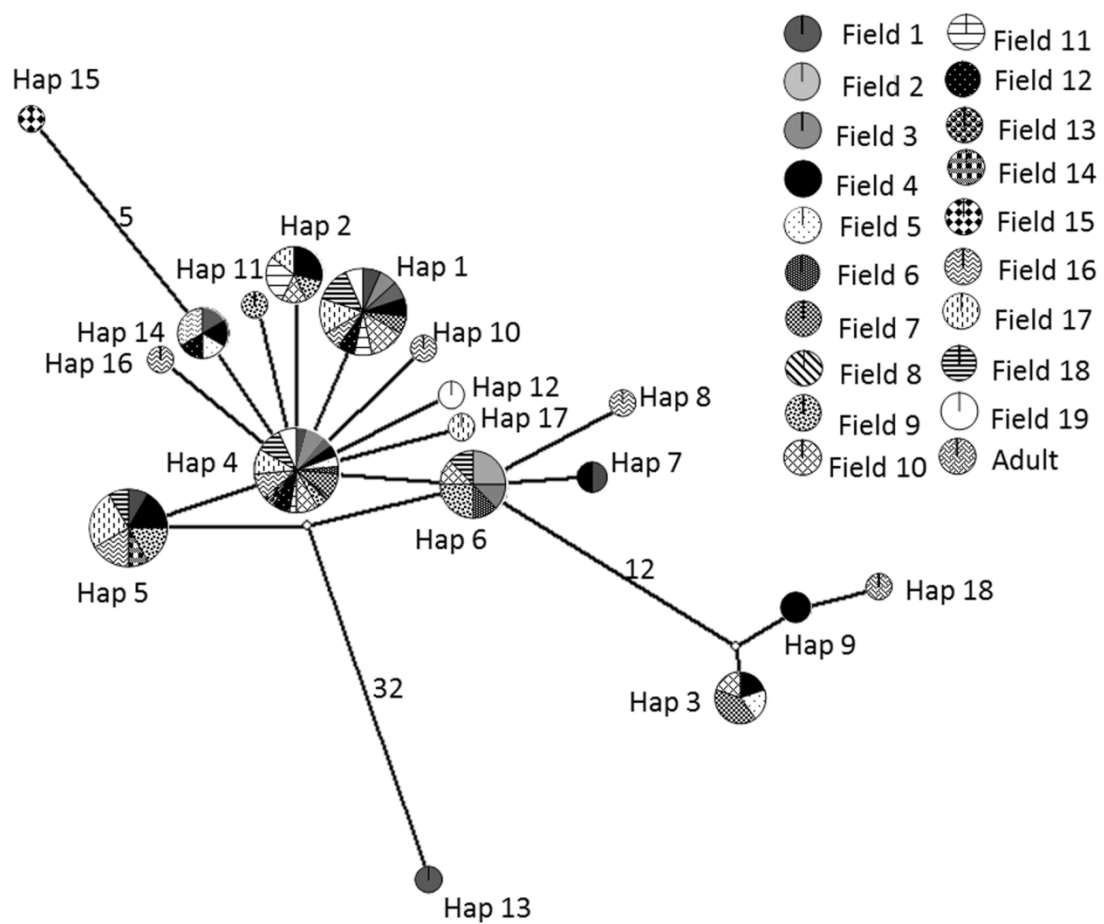
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514

515 **Conflict of interest statement**

516 There are no disputes over the ownership of the data presented in the paper and all
517 contributions have been attributed appropriately.