Origins of *Wohlfahrtia magnifica* in Italy based on the identification of mitochondrial *cytochrome b* gene haplotypes

Marianna Marangi¹ • Martin J.R. Hall² • Alex Aitken² • Paul D. Ready² • Annunziata Giangaspero^{1*}

¹Department of Science of Agriculture, Food and Environment (SAFE), University of Foggia, Foggia, Italy

²Department of Life Sciences, Natural History Museum, London SW7 5BD, UK

*** Corresponding author**

Tel + 39 0881 589227

E-mail address: annunziata.giangaspero@unifg.it (Annunziata Giangaspero)

Abstract

To identify the geographical origins of larvae of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae) causing myiasis of sheep in Italy, comparative DNA sequence analysis of the mitochondrial *cytochrome b* gene was performed, based on gene fragments amplified by PCR from genomic DNA isolated from individual specimens. DNA extractions of 19 larvae from Lazio, Molise, Puglia and Sicilia generated 17 readable sequences homologous to two haplotypes, either CB_magn01 or CB_magn02; DNA extracts from four adult flies from Calabria (reared from larvae) produced four readable sequences belonging to the haplotype CB_magn01.

The two haplotypes found represent both the East and West phylogenetic lineages of *W. magnifica*, which is consistent with the species' arrival from central/southeast Europe (East lineage) and/or from southwest Europe/northwest Africa (West lineage). This is the first report of the sympatric occurrence of the two lineages, which could have resulted from natural or human-assisted dispersal. Polymorphic nuclear loci will have to be characterised in order to explain the origins and lack of mitochondrial haplotype diversity of this pest in Italy, where it poses increasing veterinary problems.

Keywords

Livestock myiasis; *Wohlfahrtia magnifica*; mitochondrial haplotypes; *cytochrome b* gene; phylogeography; Italy

Introduction

Wohlfahrtia magnifica (Diptera: Sarcophagidae) is one of the three obligate agents of traumatic myiasis of medical and veterinary importance (Szpila *et al.*, 2014). Myiasis caused by the fly *W. magnifica* appears to be confined to the central and southern parts of Italy, where it has been discovered with increasing frequency in sheep flocks during the last decade (Bonacci *et al.* 2013; Giangaspero *et al.* 2014) (Figure 1). Mitochondrial DNA markers have the potential to identify the origins of these infestations, which might be caused by local flies or those invading from other regions and countries. Unique DNA sequences (or haplotypes) of the mitochondrial gene *cytochrome b* (*cyt b*) have been shown to form two phylogenetic lineages that are geographically separate. One lineage (East) has been found only in the eastern Mediterranean region (Italy, Greece, Hungary, Israel, Iraq), while the other (West) has been found only in the western Mediterranean region (France, Spain and Morocco) (Hall *et al.* 2009a, b; Ready *et al.* 2014). These markers have already been successfully used to investigate the origins of *W. magnifica* on Crete, where myiasis reports increased significantly in the last decade (Hall *et al.*, 2009b). It was hypothesised that *W. magnifica* might have been introduced to Crete in sheep imported from Spain, but this was disproved by discovering only the East lineage on the island (Hall *et al.* 2009b; Sotiraki *et al.*, 2010).

The current report relates the comparative DNA sequence analysis of *cyt b* fragments amplified by PCR from total genomic DNA extracted from individual larvae of *W. magnifica* removed from sheep and from adults reared from larvae removed from one dog in central/southern Italy in order to identify the geographical origins of these obligate parasites.

Material and methods

Sampling and morphological identification of larvae infesting sheep

Larvae were collected during epidemiological studies on traumatic myiasis carried out in sheep farms from several regions of Italy, namely: Sicilia (Gaglio *et al.* 2011), Lazio and Molise (Giangaspero *et al.* 2011, 2014), and more recently Puglia (A. Giangaspero) and Calabria (Bonnaci *et al.* 2013).

Larval specimens of 2nd and 3rd instars were removed from the wounds using blunt forceps, killed by immersion in hot water (>80° C) for 30', then transferred into tubes

containing 80% ethanol, and labelled with date, farm and animal code. The specimens were taken to the Laboratory of Parasitology, Department of Agriculture Science, Food and Environment, Foggia, Italy (Dept SAFE; larvae from all regions except Calabria) or the Natural History Museum, London, UK (NHM; four adult flies reared from larvae from a case of canine traumatic myiasis in Spezzano della Sila, Calabria, 10/07/2013), where they were identified morphologically (Zumpt, 1965; Spradbery, 1991) under a stereomicroscope at 10-50x magnification. They were then stored at -20 °C prior to molecular characterization.

Molecular characterization of larvae and adults

DNA was extracted from abdominal tissue (from body segments 6-9) of individual larvae using the Nucleospin Tissue kit (Macherey-Nagel, The Netherlands) in Dept. SAFE and from the thoracic flight musculature of individual adults using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) in NHM, according to the manufacturers' instructions. The extracted DNA was eluted in 50 µl of distilled water (Dept. SAFE) or 200 µl of buffer AE (NHM), and all the samples were stored at -20°C.

In SAFE Dept., three pairs of primers were used to amplify DNA sequences from all but the Calabria samples. One pair, CB3-PDR with N1N-PDR, was used to amplify the terminal c. 300 bp of the *cyt b* gene together with c. 250 bp of adjacent sequence (CB3-N1N fragment); or, for poorly preserved specimens, the same *cyt b* region was amplified as two overlapping fragments of 196 bp (primer pair PDR-WF01 and PDR-WR02) and 132 bp (primer pair PDR-WR03 and PDR-WR04) (Ready et al. 2009, 2014). The PCR reaction mix contained: 5x Phire Reaction Buffer (Thermo Scientific, USA), 200 µM of dNTPs (Qiagen, USA), 10 µl of each primers, 1U Phire Hot Start II DNA Polymerase (Thermo Scientific, USA) and 1 µl of genomic DNA in a final volume of 20 µl. The PCR protocol was denaturation at 94°C for 5 min, followed by 94° C for 30s, 62°C for 30 s and 72 °C for 60 s for 35 cycles, and finally 72°C for 7 min. A negative control (PCR water) was included in each PCR run. The PCR products were fractionated on 1.2% agarose gels and positive samples were purified with Exonuclease I (EXO I) and Thermosensitive Alkaline Phosphatase (FAST AP) (Fermentas) enzymes.

In the NHM, the primer pair CB-CAL-F07 with PDR-WR04 was used to amplify 784 bp of the *cyt b* gene from the Calabria samples, using described methods (Ready et al. 2014).

PCR products were directly sequenced in both directions using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and each of the PCR primers according to the

manufacturer's instructions. Sequences were determined on ABI PRISM 3130 and ABI 3730 Genetic Analysers (Applied Biosystems).

Editing DNA sequences and their alignment with reference mitochondrial sequences

Sequences on both DNA strands were edited and aligned using the program Sequencher v 4.8 (Gene Codes Corp) by reference to 19 known *cyt b* haplotypes (Hall et al. 2009b; Ready et al. 2014). These haplotypes consist of all 715 nucleotides at the 3' end of the *cyt b* gene. The 19 haplotypes (CB_magn1-CB_magn19) are distinguished by 21 diagnostic nucleotide sites: 13 are upstream of the CB3-N1N fragment; and only 8 are within the CB3-N1N fragment. Therefore, Table 1 identifies the Italian sequences according to the absence of differences with CB_magn haplotypes (i.e. consistency), although they might differ from the known haplotypes upstream.

Results

CB3 fragments from Lazio, Molise, Puglia and Sicilia

Of the 48 sequences obtained, 43 had moderate-good sequence quality after editing (74.0-95.0%, according to the Sequencher software) and showed homology to *cyt b*, and therefore they were used for comparative sequence analysis. Readable sequences containing 3-8 of the nucleotide sites diagnostic for CB_magn haplotypes were obtained for 17 out of 19 specimens (not 07C, 19C) (Table 1).

Only two haplotypes of the 313-base pair CB3-N1N fragment were found, and these are likely to be homologous to: CB_magn01 (in three larvae), which is the 715-base pair haplotype most frequently found in the East of the Mediterranean region; and CB_magn02 (in 14 larvae), which is the 715-base pair haplotype most frequently found in the West of the Mediterranean region. However, five other 715-base pair haplotypes of the East region (CB_magn10, CB_magn11, CB_magn16, CB_magn17 and CB_magn18) are indistinguishable from CB_magn01 in the CB3-N1N fragment; and, four other 715-base pair haplotypes of the West region (CB_magn05, CB_magn06, CB_magn09 and CB_magn20) are indistinguishable from CB_magn02 in the CB3-N1N fragment.

No polymorphisms were identified in the *tRNA^{Ser}^{TCN}* and *N1N* genes downstream of *cyt b*.

CB fragment from Calabria

Good quality sequences (88.5-96.3%, according to the Sequencher software) were obtained from three adult male flies (specimens MHa40, 42, 43) and one adult female fly (MHa41), and they were aligned with the 19 available 715-base pair haplotypes of *cyt b* (CB_magn01-CB_magn19). Each of the sequences from the four flies showed 100% homology to haplotype CB_magn01.

Discussion

Only two haplotypes of *cyt b* fragment were found. One, indistinguishable from haplotype CB_magn01 of the East lineage of *W. magnifica*, and the other indistinguishable from CB_magn02 of the West lineage of *W. magnifica*. Both lineages were found in Italy, sometimes together in the same area. The East lineage was found only in the two more northerly provinces of Lazio and Molise in central Italy and in the province of Calabria in southwest Italy, but the West lineage was found in four out of the five provinces, including Lazio and Molise as well as the more southerly Puglia and Sicilia. This is the first report of the sympatric occurrence of the two lineages in any country.

The results suggest, with caveats, that *cyt b* haplotype diversity might be limited in Italy, because only one haplotype of each of the two lineages was found. However, greater haplotype diversity might have been missed in the 17 larvae typed using only the CB3-N1N fragment, because only five were characterized at all 8 diagnostic sites: and four out of the 15 specimens characterized at 7-8 diagnostic sites had only moderate sequence quality (<90% readable nucleotides). The moderate sequence quality probably resulted from the relatively poor state of preservation of the larvae, and not the presence of chemical inhibitors, because both the PCR products and the sequencing reactions were purified by standard methods.

We can only speculate whether the overlap of the ranges of the two lineages in Italy is natural or assisted by human activities such as rapid transport of sheep. Within other species of insects and mammals, Italy is often occupied by a unique or eastern Mediterranean lineage, but it can be a zone of secondary contact after allopatric divergence (Esseghir et al. 2000; Schmitt 2007). The distributions of the East and West lineages of *W. magnifica* in southern

Italy are consistent with an arrival of the East lineage from central/southeast Europe, but it might also match the arrival of the West lineage from southwest Europe or northwest Africa. Conclusions should not be influenced by the earlier results for Italian flies: only two specimens were typed by Hall et al. (2009a), who reported only the East lineage.

Although analysis is constrained by having only small population sizes and short sequences from Italy, we can compare *cyt b* haplotype diversity in Italy with that already recorded in individual regions of Crete, Hungary, Spain and Morocco (Table 2; Hall et al. 2009b). This shows that Italy has low haplotype diversity, similar to all the other regions sampled except Morocco. Lack of haplotype diversity is consistent with the “recent” arrival of a population in an area, but it can be difficult to distinguish between natural spread in the last c. 10,000 years (by populations reaching the edge of their climate range after the last glacial period) and very recent introductions by humans. It is often very difficult to distinguish these two types of spread if only mitochondrial haplotypes are used in the analysis (Esseghir et al. 2000; Wardhana et al. 2012). Greater sampling effort and the characterization of polymorphic nuclear loci (microsatellites and SNPs) are usually required to distinguish the two spread scenarios, which are both recent on a geological time scale. The characterization of polymorphic nuclear loci might best be achieved by high-throughput Whole Genome Sequencing (Campo et al. 2013).

Acknowledgements We thank Dr. Teresa Bonacci for the adult flies from Calabria region and the DNA Sequencing Facility, Natural History Museum, London, managed by Julia Llewellyn-Hughes.

Conflict of interest The authors declare that they have no conflict of interests.

Authors’ contributions AG and MJRH designed and supervised the study and identified the species morphologically. MM and AA produced the DNA sequences which were analysed by MM and PDR. PDR, MJRH and AG wrote the first draft of the manuscript. All authors revised and approved the final version of the manuscript.

References

- Bonacci T, Greco S, Whitmore D, Curcio U (2013) First data on myiasis caused by *Wohlfahrtia magnifica* (Schiner, 1862) (Insecta: Diptera: Sarcophagidae) in Calabria, southern Italy. *Life Excit Biol* 1:197-201.

- Campo D, Lehmann K, Fjeldsted C, Souaiaia T, Kao K, Nuzhdin SV (2013) Whole-genome sequencing of two North American *Drosophila melanogaster* populations reveals genetic differentiation and positive selection. *Mol Ecol* 22:5084-5097.
- Esseghir S, Ready PD, Ben-Ismail R (2000) Speciation of *Phlebotomus* sandflies of the subgenus *Larroussius* coincided with the late Miocene-Pliocene aridification of the Mediterranean subregion. *Biol J Linn Soc* 70:189-219.
- Gaglio G, Brianti E, Abbene S, Giannetto S (2011) Genital myiasis by *Wohlfahrtia magnifica* (Diptera, Sarcophagidae) in Sicily (Italy). *Parasitol Res* 109:1471-1474.
- Giangaspero A, Brianti E, Traversa D, Hall MJR (2014) A retrospective and geographical epidemiological survey of traumatic myiasis in southern Italy. *Med Vet Entomol* 28:391-397.
- Giangaspero A, Traversa D, Trentini R, Scala A, Otranto D (2011) Traumatic myiasis by *Wohlfahrtia magnifica* in Italy. *Vet Parasitol* 175:109-112.
- Hall MJR, Adams ZJO, Wyatt NP, Testa JM, Edge W, Nikolausz M, Farkas R, Ready, PD (2009a) Morphological and mitochondrial DNA characters for identification and phylogenetic analysis of the myiasis-causing fleshfly *Wohlfahrtia magnifica* and its relatives, with a description of *Wohlfahrtia monegrosensis* sp.n. Wyatt & Hall. *Med Vet Entomol* 23(S1):59-71.
- Hall MJR, Testa JM, Smith L, Adams ZJO, Khallaayoune K, Sotiraki S, Stefanakis A, Farkas R, Ready, PD (2009b) Molecular genetic analysis of populations of Wohlfahrt's wound myiasis fly, *Wohlfahrtia magnifica*, in outbreak populations from Greece and Morocco. *Med Vet Entomol* 23(S1):72-79.
- Ready PD, Testa JM, Wardhana AH, Al-Izzi M, Khalaj M, Hall MJR (2009) Phylogeography and recent emergence of the Old World screwworm fly, *Chrysomya bezziana*, based on mitochondrial and nuclear gene sequences. *Med Vet Entomol* 23(S1):43-50.
- Ready PD, Wardhana AH, Adams ZJO, Sotiraki S, Hall MJR (2014) Improved method for screening mitochondrial *cytochrome b* markers to identify regional populations of the Old World screwworm fly and other myiasis agents. *Acta Trop* 138S:S42-48.
- Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Front Zool* 4:11.
- Sotiraki S, Farkas R, Hall MJR (2010) Fleshflies in the flesh: epidemiology, population genetics and control of outbreaks of traumatic myiasis in the Mediterranean Basin. *Vet Parasitol* 174:12-18.
- Spradbery JP (1991) A Manual for the Diagnosis of Screw-worm Fly. Commonwealth Scientific

and Industrial Research Organization (CSIRO) Division of Entomology, Canberra, Australia, 64 pp. Wardhana AH, Hall MJR, Mahamdallie SS, Muharsini S, Cameron MM, Ready PD (2012) Phylogenetics of the Old World screwworm fly and its significance for planning control and monitoring invasions in Asia. *Int J Parasitol* 42:729-738.

Szpila K, Hall MJR, Wardhana AH, Pape T. 2014. Morphology of the first instar larva of obligatory traumatic myiasis agents (Diptera: Calliphoridae, Sarcophagidae). *Parasitol Res* 113:1629-1640.

Zumpt F (1965) *Myiasis in Man and Animals in the Old World: A Textbook for Physicians, Veterinarians and Zoologists*. Butterworth, London.

FIGURE'S LEGEND

Figure 1. Vulvar infestation of a sheep by third-instar larvae of *Wohlfahrtia magnifica* (A. Giangaspero).

Table 1. Results for 26 sequences of the CB3-N1N DNA fragment (313 base pairs: nucleotides 403-715) or the internal WF01-WR02 DNA fragment (162 bp; nt 443-604) amplified by PCR from individual Italian *Wohlfahrtia magnifica*, with the nt positions being those of the aligned 715-nt haplotypes (CB_magnNN) of the 3' terminal of the mitochondrial *cytochrome b* gene (Hall et al., unpubl.) [Only 9 of the 20 haplotypes have diagnostic nts in the CB3-N1N fragment]

Seq code	Seq primer	Seq quality before edit (%)	Seq quality after edit (%)	% read- able nt 403-715 Or 443-604	nt 418 C magn1 5 T cons	nt 436 C magn1 4 T cons	nt 445 A magn0 3 A magn1 3 G cons	nt 526 A East G West	nt 555 C magn0 4 C magn0 7 T cons	nt 568 A East A magn0 8 G West G magn1 9	nt 610 G magn1 2 T cons	nt 676 C magn0 7 T cons	Consiste nt with 715 nt CB_magn haplotype / lineage
01B	N1N	82.2	94.8	99.7 (1 UR)	T	T	G	A	T	A	T	T	01 / East
01C	WR02	77.4	87.8	87.7 (20)	-	-	G	A	T	A	-	-	01 / East
11A	CB3	82.4	89.7	92.3 (24)	UR	T	G	A	T	A	T	T [T WF03]	01 / East
12A	N1N	71.4	94.2	99.7 (1)	T	T	G	A	T	A	T	T [T WF03]	01 / East
02A	CB3	82.6	91.2	93.0 (22)	UR	T	G	G	T	G	T	T	02 / West
02B	WR02	70.6	81.1	87.7 (20)	-	-	G	G	T	G	-	- [T WF03]	02 / West
03A	CB3	84.8	91.4	90.7 (29)	UR	T	G	G	T	G	T	T	02 / West
03B	WR02	86.0	95.0	87.7 (20)	-	-	G	G	T	G	-	- [T WF03]	02 / West
04A	CB3	85.6	93.9	90.1 (31)	UR	T	G	G	T	G	T	T	02 / West
04B	WR02	72.2	80.9	87.7 (20)	-	-	G	G	T	G	-	- [T WF03]	02 / West
05A	CB3	59.7	78.6	64.2(11 2)	UR	T	G	G	T	G	T	T	02 / West
05B	WR02	78.9	89.7	85.2 (24)	-	-	G	G	T	G	-	- [T WF03]	02 / West
06A	CB3	85.1	91.2	94.5 (17)	T	T	G	G	T	G	T	T	02 / West
06B	WR02	66.8	74.0	91.3 (14)	-	-	G	G	T	G	-	- [UR]	02 / West
08A	CB3	84.6	91.2	94.5 (17)	T	T	G	G	T	G	T	T [T WF03]	02 / West
09B	WR02	76.0	82.2	90.7 (15)	-	-	G	G	T	G	-	- [T WF03]	02 / West
10B	WR02	78.4	87.8	90.7 (15)	-	-	G	G	T	G	-	- [T WF03]	02 / West
13A	CB3	85.6	94.5	92.3 (24)	UR	T	G	G	T	G	T	T	02 / West
13B	WR02	47.8	80.2	83.9 (26)	-	-	G	G	T	G	-	- [T WF03]	02 / West
14A	N1N	73.0	91.9	93.9 (19)	T	T	G	G	T	G	T	T	02 / West
14B	WR02	73.4	89.8	87.7 (20)	-	-	G	G	T	G	-	- [T WF03]	02 / West
15A	CB3	83.4	92.6	88.2 (37)	UR	T	G	G	T	G	T	T	02 / West
15B	WR02	67.6	85.1	80.8 (31)	-	-	G	G	UR	G	-	- [T WF03]	02 / West
16A	CB3	86.4	93.3	92.0 (25)	UR	T	G	G	T	G	T	T [T WF03]	02 / West
17A	CB3	81.3	93.3	86.9 (41)	UR	T	G	G	T	G	T	T [T WF03]	02 / West
18A	CB3	84.1	93.1	86.6 (42)	UR	T	G	G	T	G	T	T [T WF03]	02 / West

Key: cons = consensus; - = external of DNA fragment; UR = unreadable; WF03 = single haplotype-diagnostic nt in WF03-WR04 fragment sequenced with WF03 primer (15 readable WF03 sequences included in penultimate column of the table; identical WF03 sequences also obtained for samples 07C and 19C).

Table 2 *Cytochrome b* haplotype diversity for *W. magnifica* from different geographical regions based only on the eight diagnostic nucleotides in the CB3-N1N region [Data for other regions from Hall et al. (2009b)]

	Total specimens with each CB_magn haplotype number in each <i>cyt b</i> lineage									
	East lineage						West lineage			
Haplotype ->	"01"	12	13	14	15	19	"02"	03	04	07
Region										
Crete	16	0	0	0	0	0	0	0	0	0
Hungary	5	0	0	0	0	0	0	0	0	0
Italy	7	0	0	0	0	0	14	0	0	0
Spain	0	0	0	0	0	0	3	0	0	0
N Morocco	0	0	0	0	0	0	17	0	12	1
C Morocco	0	0	0	0	0	0	24	2	6	0

Key: "01" = CB_magn01 or five other haplotypes indistinguishable in the CB3-N1N fragment; "02" = CB_magn02 or four other haplotypes indistinguishable in the CB3-N1N fragment; some haplotypes occur only in other regions.