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Invited review

Molecular approaches to the study of myiasis-causing larvae

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Abstract

Among arthropod diseases affecting animals, larval infections – myiases – of domestic and wild animals have been considered important since ancient times. Besides the significant economic losses to livestock worldwide, myiasis-causing larvae have attracted the attention of scientists because some parasitise humans and are of interest in forensic entomology. In the past two decades, the biology, epidemiology, immunology, immunodiagnosis and control methods of myiasis-causing larvae have been focused on and more recently the number of molecular studies have also begun to increase. The 'new technologies' (i.e. molecular biology) are being used to study taxonomy, phylogenesis, molecular identification, diagnosis (recombinant antigens) and vaccination strategies. In particular, more in depth molecular studies have now been performed on Sarcophagidae, Calliphoridae and flies of the Oestridae sister group. This review discusses the most topical issues and recent studies on myiasis-causing larvae using molecular approaches. In the first part, PCR-based techniques and the genes that have already been analysed, or are potentially useful for the molecular phylogenesis and identification of myiasis-causing larvae, are described. The second section deals with the more recent advances concerning taxonomy, phylogenetics, population studies, molecular identification, diagnosis and vaccination.

Keywords: Myiasis; Oestridae; Calliphoridae; Sarcophagidae; Phylogenetics; Molecular technologies

1. Introduction

Although myiases have been known since very ancient times, at the beginning of the third millennium they still remain an unresolved problem for animal production and are responsible for severe economic losses to the livestock industry in developing and developed countries (through abortion, reduced milk production, losses in weight and fertility, and poor hide quality). An exception to this assumption is constituted by the success achieved in the eradication of screwworm fly in North and Central America (Krafsur et al., 1987) and of bovine hypodermosis in many European countries (reviewed by Tarry, 1998) by use of the sterile insect technique and chemotherapy (see below). Some myiasis-causing larvae, mainly in poor socio-economic contexts, parasitise humans causing dermal, urogenital or internal infections (Hall and Wall, 1995).

Among the arthropods causing diseases to animals, myiases cause a broad range of infections depending on the location of larvae on the body of the host (e.g. dermal/subdermal, nasopharyngeal, internal organs, intestinal and urogenital myiases) or their relationship with the host (e.g. obligatory or facultative myiases).

Blowflies (Calliphoridae) and fleshflies (Sarcophagidae) cause myiases of relatively short duration by both obligate and/or facultative parasitism, infections maturing within 4–7 days, in host body orifices or wounds (e.g. *Lucilia cuprina*, *Lucilia sericata*, *Cochliomyia hominivorax*). Botflies (Oestridae) are obligate parasites that are harboured and feed for several weeks/months in the host's nasopharyngeal tract (e.g. *Oestrus ovis*), gut system (e.g. *Gasterophilus* spp.) and internal organs, as well as in subcutaneous tissues (e.g. *Hypoderma bovis* and *Hypoderma lineatum*, *Przhevalskiana silenus*) (Table 1).

The differences among Calliphoridae, Sarcophagidae and Oestridae larvae in terms of location in the host body – biology, pathogenesis and host immune response to larval infestation – have steered investigations along different lines (see below). The relationship between myiasis-causing larvae and their hosts is for the most part regulated by the degree of host immune response and by the way larvae cope with this. During their evolution, myiasis-causing larvae have produced biological, physiological and biochemical strategies to cope with host non-specific (NK cells, complement)

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Table 1
Species of Oestridae, Calliphoridae and Sarcophagidae larvae ranked within subfamilies: host, site of parasitism and molecular biology fields investigated

Species	Host	Site of parasitism	Interest of molecular biology fields
OESTRIDAE			
Hypodermatinae			
Hypoderma bovis	Cattle, horses, humans	Dermis and internal organs	Biological activities, phylogenesis, identification, diagnosis, vaccination
Hypoderma lineatum	Cattle, horses, humans	Dermis and internal organs	Biological activities, phylogenesis, identification, diagnosis, vaccination
Hypoderma diana	Deer	Dermis and internal organs	Phylogenesis, identification
Hypoderma tarandi	Reindeer	Dermis and internal organs	Phylogenesis, identification
Hypoderma actaeon	Roe deer	Dermis and internal organs	Phylogenesis, identification
Hypoderma sinense	Yaks	Dermis and internal organs	Phylogenesis, identification
Przhevalskiana silenus	Goats	Subcutaneous tissue	Phylogenesis, identification, diagnosis
Oestrinae			
Cephenemyia trompe	Reindeer	Nasopharynx	Phylogenesis
Cephenemyia stimulator	Reindeer	Nasopharynx	Phylogenesis
Cephenemyia ulrichii	Moose	Nasopharynx	Phylogenesis
Rhinoestrus usbekistanicus	Horses, donkeys	Nasopharynx	Phylogenesis
Rhinoestrus phacocoeri	Warthog	Nasopharynx	Phylogenesis
Oestrus ovis	Sheep, goats, humans	Nasopharynx	Phylogenesis, identification, diagnosis
Gasterophilinae			
Gasterophilus intestinalis	Horses, donkeys	Digestive tract	Biological activities, phylogenesis, identification
Gasterophilus haemorrhoidalis	Horses, donkeys	Digestive tract	Phylogenesis, identification
Gasterophilus nasalis	Horses, donkeys	Digestive tract	Phylogenesis, identification
Gasterophilus pecorum	Horses, donkeys	Digestive tract	Phylogenesis, identification
Cuterebrinae			
Alouattamyia baeri	Monkey	Dermis	Phylogenesis
Cuterebra jellisoni	Rabbit	Dermis	Phylogenesis
CALLIPHORIDAE			
Chrysomyinae			
Chrysomya albiceps	Carrions, sheep	Decomposing organic matter, wounds	Phylogenesis, identification
Chrysomya bezziana	Carrions, humans, ruminants, horses	Decomposing organic matter, wounds	Biological activities, identification, vaccination
Chrysomya chloropyga	Carrions, ruminants	Decomposing organic matter, wounds	Identification
Chrysomya megacephala	Carrions, ruminants, donkeys	Decomposing organic matter, wounds	Phylogenesis, identification
Chrysomya norrisi	Carrions	Decomposing organic matter, wounds	Identification
Chrysomya rufifacies	Carrions, sheep, cattle	Decomposing organic matter, wounds	Phylogenesis, identification
Chrysomya semimetallica	Carrions	Decomposing organic matter, wounds	Identification
Chrysomya varipes	Carrions, sheep	Decomposing organic matter, wounds	Identification
Cochliomyia hominivorax	Animals, humans	Wounds	Phylogenesis, identification
Cochliomyia macellaria	Animals, humans	Wounds	Phylogenesis, identification
Compsomyiops callipes	Carrions	Decomposing organic matter	Identification
Phormia regina	Carrions, sheep	Decomposing organic matter, wounds	Phylogenesis, identification
Protophormia terraenovae	Carrions, sheep	Decomposing organic matter, wounds	Biological activities, phylogenesis, identification
Protophormia atriceps	Carrions, sheep	Decomposing organic matter, wounds	Identification
Protocalliphora sialia	Carrions	Decomposing organic matter	Identification
Calliphorinae			
Calliphora albifrontalis	Carrions, sheep	Decomposing organic matter, wounds	Identification

Table 1 (*continued*)

Species	Host	Site of parasitism	Interest of molecular biology fields
Calliphora augur	Carrions, humans, sheep, birds	Decomposing organic matter, wounds	Identification
Calliphora dubia	Carrions	Decomposing organic matter	Identification
Calliphora hilli	Carrions, sheep	Decomposing organic matter, wounds	Identification
Calliphora livida	Carrions	Decomposing organic matter	Identification
Calliphora maritima	Carrions	Decomposing organic matter	Identification
Calliphora quadrimaculata	Carrions, sheep	Decomposing organic matter, wounds	Phylogenesis
Calliphora stygia	Carrions, sheep	Decomposing organic matter, wounds	Identification
Calliphora vicina	Carrions, humans, dogs, monkeys, sheep	Decomposing organic matter, wounds	Biological activities, phylogenesis, identification
Calliphora vomitoria	Carrions, sheep	Decomposing organic matter, wounds	Phylogenesis, identification
Cynomyia cadaverina	Carrions	Decomposing organic matter	Identification
Cynomyia mortuorum	Carrions	Decomposing organic matter	Phylogenesis
Eucalliphora latifrons	Carrions	Decomposing organic matter	Identification
Onesia tibialis	Carrions	Decomposing organic matter	Identification
Lucilinae			
Lucilia cuprina	Carrions, humans, sheep, goats, cattle, dogs	Decomposing organic matter, wound, soiled and wet fleece	Biological activities, phylogenesis, insecticide resistance, vaccination
Lucilia sericata	Carrions, humans, sheep, cattle, horses	Decomposing organic matter, wound, soiled and wet fleece	Biological activities, phylogenesis, identification
Lucilia ampullacea	Carrions	Decomposing organic matter	Phylogenesis
Lucilia cesar	Carrions, humans, sheep	Decomposing organic matter, wound, soiled and wet fleece	Insecticide resistance
Lucilia eximia	Carrions	Decomposing organic matter	Phylogenesis
Lucilia illustris	Carrions, sheep	Decomposing organic matter, wound, soiled and wet fleece	Phylogenesis, identification
SARCOPHAGIDAE			
Sarcophaginae			
Sarcophaga africa	Carrions	Decomposing organic matter	Phylogenesis, identification
Sarcophaga peregrina	Carrions	Decomposing organic matter	Biological activities, phylogenesis, identification
Sarcophaga argyrostoma	Carrions, humans, sheep	Decomposing organic matter, wounds	Phylogenesis, identification
Sarcophaga crassipalpis	Carrions	Decomposing organic matter	Biological activities, phylogenesis, identification
Sarcophaga bullata	Carrions	Decomposing organic matter	Biological activities, phylogenesis, identification
Sarcophaga ruficornis	Carrions, horses	Decomposing organic matter, wounds	Phylogenesis, identification
Sarcophaga cooleyi	Carrions	Decomposing organic matter	Phylogenesis, identification
Blaesoxipha plinthopyga	Carrions	Decomposing organic matter	Phylogenesis, identification
Peckia chrysostoma	Carrions	Decomposing organic matter	Phylogenesis, identification
Ravinia lherminieri	Carrions	Decomposing organic matter	Phylogenesis, identification
Paramacronychiinae			
Brachicoma devia	Carrions	Decomposing organic matter	Phylogenesis, identification
Wohlfahrtia vigil	Carrions	Decomposing organic matter	Phylogenesis, identification

and/or specific (antibodies and T lymphocytes) immune responses. The complexity of the mechanisms by which larvae down-regulate the immunological functions of their hosts indicates that they are specifically adapted to a parasitic existence in 'their' hosts and are not only a passive means of injury. For their part, hosts respond to myiasis-causing larvae infestation according to their state of health as well as to larval biology, site of parasitism, nature of antigens and specific larval immunological defence mechanisms.

This great interest around the host–parasite relationships can explain why, in the past 30 years, the 'new approaches' for the study of myiasis-causing larvae have focused mainly on understanding the host immunological response by means of different methods to study the structure and function of proteins and/or target genes.

The advent of molecular biological techniques has changed the approach of parasitologists to the study of parasites of human and animal importance, largely by supplementing

and enhancing knowledge in many fields. This 'molecular revolution' has been made possible not only by new techniques (e.g. the PCR and automated sequencing), but also by a better understanding of the many target genes (i.e. mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA)) useful in evolutionary investigations and by the characterisation of parasite proteins important for diagnostic and vaccination studies. Numerous applications of molecular biology have been investigated also in medical and veterinary entomology, leading to new insights into systematics (taxonomy and phylogeny) and population genetics, together with more practical aspects such as pathogenetic implications, molecular identification, diagnosis and control of some ectoparasite infestations (e.g. ticks).

On the whole about 62 genes of Oestridae, Calliphoridae and Sarcophagidae have been studied (e.g. COI, COII, tRNA-Leu, tRNA-Ile and 12S mtDNA, 16S rRNA and 18S rRNA (rDNA)) and various enzymatic and structural proteins, i.e. alfa-esterase (organophosphate resistance), syntrophin, lectin, catepsin and collagenase (hypodermin C) and *Hypoderma* spp. serine proteases hypodermins A and B and 378 nucleotidic sequences have been registered since 1993 (GenBank, 2002).

The evidence for a host immune response against myiasis-causing larvae has spurred molecular studies for Oestridae and Calliphoridae focussing mainly on their control (see Section 7) and diagnosis (see Section 6) (Pruett, 1999; Otranto, 2001). The topical interest of Calliphoridae and Sarcophagidae larvae in the field of forensic entomology has also greatly attracted the attention of scientists towards their molecular identification (see Section 5). Phylogenetic relationships have been investigated with different approaches for Calliphoridae (e.g. Stevens and Wall, 1996, 1997a,b, 2001; Rognes, 1997; Wells and Sperling, 1999, 2001) and for Oestridae (Otranto and Puccini, 2000; Pape, 2001; Otranto and Stevens, 2002).

The aim of this review is to discuss the current issues and recent molecular investigations on myiasis-causing larvae. In the first section, the most common PCR-based techniques and the genes that have already been analysed, or are potentially useful for molecular phylogenesis and identification of myiasis-causing larvae, are described. The second section deals with the taxonomical, phylogenetic and population studies, while the last section reports the more practical approaches (molecular identification, diagnostic and vaccination tools) to their study.

2. Molecular techniques

2.1. PCR

Since the advent of PCR (Saiki et al., 1985), nearly all fields of the life sciences have been significantly affected in both their theoretical and practical approaches, thereby engendering a real 'revolution' in molecular, evolutionary and systematic thinking. One of the main advantages PCR offers in the field of entomology is that it requires only very small amounts of DNA template for amplification and it is able to rapidly sequence novel genes and other informative DNA markers. By virtue of its high sensitivity and specificity, PCR has met with broad applicability in parasitological and entomological studies (e.g. taxonomic, phylogenetic), also including those with more practical implications (e.g. diagnosis). In particular, the advent of PCR has made it possible to study damaged and incomplete specimens which were hard to identify morphologically, museum specimens, individual species of small size and possibly fossilised insect material.

2.2. Polymerase chain reaction-restriction fragment length polymorphism

Diagnostic polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns have been widely utilised for the identification of closely related taxa of forensic relevance (i.e. Calliphoridae and Sarcophagidae families – see Section 5).

2.3. Single-strand conformation polymorphism

If the goal of molecular analyses is to identify strains and/or species, then single-strand conformation polymorphism analysis (SSCP), which is a PCR-based mutation scanning method based on the different electrophoretic mobility of ssDNA in a non-denaturing gel, may be appropriate. In molecular entomology, the SSCP analysis has been used to assess genetic distance and to identify haplotypes existing in insect populations from different geographic areas belonging to the same species (e.g. flies or mosquitoes) (Gorrochotegui-Escalante et al., 2000; Krafsur et al., 2000) or to identify morphologically indistinguishable species (e.g. mosquitoes) (Koekemoer et al., 1999; Sharpe et al., 1999).

2.4. Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) analysis consists of the amplification of DNA sequences using a single arbitrary primer under low temperature conditions of annealing, allowing rapid screening of variation across a genome. Although RAPD has been used to study various myiasis-causing ectoparasites (e.g. *L. sericata*/*L. cuprina*, Stevens and Wall, 1995, 1996; *C. hominivorax*, Infante-Malachias et al., 1999), the often poor reproducibility of RAPD fingerprints and the variation in fingerprint patterns between specimens and even laboratories have created significant limitations in using this technique.

2.5. Sequencing

However, the identification of strains and/or species increasingly needs to be supported by an accurate and detailed analysis of the genetic variation in DNA sequences

not detected by PCR or RFLP analysis, i.e. primary sequence data. Automation of sequencing has reduced the costs of these procedures and the time required to perform them, making their use ever more widespread.

2.6. Data editing and phylogenetic approaches

Prior to commencing any form of comparative analysis – numerical or phylogenetic – sequences should be compared along their length on a pairwise basis. In straightforward characterisation studies where the presence or absence of a particular sequence motif or even a single nucleotide is sufficient to provide a definitive result (e.g. sequence-based species identification), a simple comparison of sequences – often against a database of existing data – may be all that is required. However, where multiple sequence comparisons are required, e.g. for evolutionary studies, the often complex process of multiple sequence alignment must be undertaken, and the associated problem of identifying true homology between variable sites and portions of sequences must be addressed; this remains one of the most problematic areas of molecular phylogenetic analysis.

Alignment can be performed by one or more approaches: (i) on the basis of secondary structure and functional domains, e.g. secondary structure in ribosomal sequences (Neefs et al., 1990); (ii) using one of a range of specialist alignment programs with various weighting options and gap penalties, e.g. ClustalX (Thompson et al., 1997); (iii) by eye, often in relation to previously aligned sequences.

There are three main categories of phylogenetic analysis in widespread use with molecular data: distance methods (including some essentially phenetic methods), cladistics/parsimony and maximum-likelihood analysis. The relative merits of a variety of methods within each category have been explored by a range of simulation studies (e.g. Nei, 1991; Huelsenbeck, 1995; Wiens and Servedio, 1998). See Swofford et al. (1996) and Kitching et al. (1998) for details of the relative merits of each methodology.

The 'correctness' of a phylogenetic tree cannot be reliably interpreted without some form of statistical support for the evolutionary relationships presented. Bootstrap analysis is perhaps the most commonly employed method for providing such support (Felsenstein, 1985) and involves comparison of the observed phylogenetic tree with trees based on pseudo-replicate datasets resampled from the original data. Debate surrounding the non-linear nature of bootstrap support is ongoing (Hillis and Bull, 1993; Efron et al., 1996).

3. Target genes for molecular studies of myiasis-causing larvae

Besides the genes encoding for structural or enzymatic proteins of importance for the study of myiasis-causing larvae (i.e. hypodermins, or blowfly peritrophic membrane, etc.), the most common target regions in insect systematics and phylogenesis are the mtDNA and the nuclear rDNA. It is well-known that nuclear and mitochondrial genes and inter-gene regions accumulate mutations over the course of time at different rates, dependent on function and mode of inheritance. Generally, introns and non-coding regions, such as the internal transcribed spacer (ITS) of rDNA, exhibit a high mutational rate compared, for example, with the (13) coding genes of mtDNA (with specific translational products) that evolve in accordance to their structure and function. Generically, if the aim of research is differentiation at species level, then a target gene or region with a low intraspecific variability has to be preferred to a gene with a high degree of variation. Conversely, if two larval populations of the same species (e.g. *H. lineatum*) originating from two well-distinguished geographic areas are to be compared, a target gene with a high level of intraspecific variation would be preferred.

rDNA genes are generally useful targets to identify strains and/or species of parasites (Arnheim, 1983; Gasser, 1999); rDNA consists of arrays of tandemly repeated units containing spacers and associated rRNA genes (18S, 5.8S and 28S) with a sequence evolution rate varying across a repeating unit and even within a gene (Hillis and Dixon, 1991). The level of divergence in the rDNA of spacer regions has been widely applied to molecular phylogenetic and identification studies of protozoa (e.g. *Toxoplasma gondii*, Brindley et al., 1993), trematoda (e.g. Fasciolidae, Adlard et al., 1993), cestoda (e.g. *Echinococcus* spp., Bowles and McManus, 1993), nematodes (e.g. Trichostrongylidae, Gasser et al., 1994) and arthropods (e.g. ticks, Zahler et al., 1997; mosquitoes, Marcilla et al., 2001).

Numerous high-level phylogenetic studies have focused on the analysis of the 28S rRNA gene, although ITS and 18S have been more studied for Diptera and Hymenoptera; the 18S rRNA gene yielded results that were both congruent with those of morphologically-based phylogenetic analyses (Caterino et al., 2000) and incongruent (Nirmala et al., 2001). The first and second internal transcribed spacers (ITS-1 and ITS-2) of rDNA have proven useful for the identification of arthropods because of the low level of intraspecific sequence variation combined with higher levels of interspecific differences. Nevertheless, some arthropods exhibit significant heterogeneity even in the ITS-1 and ITS-2 regions, which is appropriate for detecting differences between co-specific individuals. Tandemly repeated sequences or microsatellites (unit of repetition between one and five) of ITSs have proved to be good polymorphic markers for the study of population dynamics (Vogler and DeSalle, 1994; Onyabe and Conn, 1999; De Barro et al., 2000; Marcilla et al., 2001). Microsatellites have been widely used in phylogenetic analyses of insects and for population genetic studies, e.g. for some tsetse flies and mosquitoes (Solano et al., 1998; Ravel et al., 2001). They also proved to be useful for the resolution of interspecific relationships in *Drosophila melanogaster* (Harr et al., 1998) as they are characterised by a high level of poly-

morphism and mutational rate (Queller et al., 1993). Introns, non-coding regions within single-copy nuclear coding loci, have been also used in systematic studies and in interspecific and population investigations in molecular entomology (Adamczyk et al., 1996; Leebens-Mack et al., 1998).

In recent years, mtDNA has been widely used for taxonomic, population and evolutionary investigations in mammals as well as in arthropods because it is easy to isolate, has a high copy number and contains conserved sequences that make it possible to use universal primers (Kocher and Xiong, 1991). mtDNA includes two rRNA genes, 13 protein coding genes and 22 transfer RNA (tRNA) genes which, with the exception of tRNAs, are highly conserved within vertebrates and insects (Wolstenholme, 1992). A useful review on the evolution, weighting and phylogenetic utility of mitochondrial gene sequences has been provided by Simon et al. (1994) together with a dataset of conserved primers; nevertheless, the existence of mitochondrial pseudogenes integrated into a nuclear genome may sometimes affect the reliability of PCR-based mitochondrial studies (Zhang and Hewitt, 1996a). These paralogous nuclear copies of mitochondrial genes or nuclear mitochondrial pseudogenes have recently been demonstrated to be potentially useful in evolutionary studies or for the study of spontaneous mutation in nuclear genomes (Bensasson et al., 2001).

The mitochondrial gene encoding subunit I of cytochrome oxidase (COI) is the terminal catalyst in the respiratory mitochondrial chain and has proven to be particularly suitable as a molecular marker for the taxonomic differentiation and evolutionary studies of insects. It is used as a target gene for a number of molecular phylogenetic objectives because it is large in size, and presents highly conserved and variable regions, with a different range of closely associated mutational rates (Lunt et al., 1996). Studies on the insect COI gene have revealed heterogeneity in sequences and nucleotide variability, making some regions useful for low- or high-level phylogenetic investigations (Zhang and Hewitt, 1996b).

The COI amino acid sequence is made up of 12 transmembrane helices (M1–M12), six external loops (E1–E6), five internal loops (I1–I5), one carboxyl (COOH) and one amino (NH_2) terminal. In particular the COI gene may have different rates of evolution in different lineages, which largely depend on functional constraints that may occur in specific regions. This variability has to be taken into account when selecting a region with a mutational rate suitable for the purpose of the investigation; highly variable, quickly evolving, regions may not reveal the phylogeny of anciently divergent taxa, while well-conserved sequences are not helpful in establishing intraspecific phylogenetic relationships. The occurrence of different patterns of nucleotide and amino acid variability has been pointed out in insects, with the COOH terminal region being the most variable part, followed by the E1, M3, E2, I2, I4, M9 and M12 regions (Lunt et al., 1996). Similarly, Zhang and Hewitt (1996b) designed a set of 10 conserved COI primers covering the most conserved regions across Insecta and assessed the usefulness of different amplicons for different phylogenetic questions. In particular, the evolutionary patterns and the utility of conserved COI primers have been investigated for the phylogenetic analysis of many insects such as Orthoptera (Harrison et al., 1987; Zhang et al., 1995; Lunt et al., 1996), Diptera (Nigro et al., 1991; Spicer, 1995; Lunt et al., 1996), Hymenoptera (Lunt et al., 1996), Coleoptera (Městrović et al., 2000) and Lepidoptera (Brown et al., 1994; Sperling and Hickey, 1994).

With regard to myiasis-causing larvae, the mitochondrial genomes of the screwworm fly, *C. hominivorax* (Diptera, Calliphoridae) (Lessinger et al., 2000), and the sheep-blowfly, *L. sericata* (Diptera, Calliphoridae) (Stevens et al., unpublished data), have been entirely sequenced and the usefulness of different genes for phylogenesis or identification purposes investigated. Furthermore, mtDNA control regions have also been examined for *C. hominivorax*, *Cochliomyia macellaria*, *Chrysomya megacephala* (Diptera, Calliphoridae) and *Dermatobia hominis* (Diptera, Oestridae) and nucleotide sequences analysed to determine the structural organisation and phylogenetic usefulness of these regions (Lessinger and Azeredo-Espin, 2000). Concerning family Oestridae, a PCR-RFLP assay on the COI gene of *H. bovis* and *H. lineatum* (bovine hypodermosis), *O. ovis* (sheep and goat oestrosis), *P. silenus* (goat subcutaneous myiasis) and *Gasterophilus intestinalis* (equine gasterophilosis) larvae has provided a molecular tool for their differentiation at the generic level (Otranto et al., 2000) and a large fragment of the COI gene of the same species has also been sequenced and phylogenetically analysed (Otranto and Puccini, 2000).

4. Phylogenesis

In the past 20 years advances in molecular biology techniques have provided great insights into nucleotide and amino acid differences within and among species of insects in a phylogenetic context. In turn, this has led to a broader understanding of the structure and function of insect genes and their relationship to the distribution (phylogeography), physiology, development, evolution and ecology of insects, although the efforts of scientists in this field still need to be better concentrated (Caterino et al., 2000).

The oestrids include 18 genera and 151 species of flies, and can be ranked in four subfamilies: Cuterebrinae, Gasterophilinae, Hypodermatinae and Oestrinae (Wood, D.M., 1987. Are Cuterebridae, Gasterophilidae, Hypodermatidae and Oestridae a monophyletic group? In: Darvas, B., Papp, L. (Eds.), Abstracts of the First International Congress of Dipterology, Budapest, 17th–24th August 1996; Hall and Wall, 1995). These subfamilies have an enormous diversity in terms of lifestyle, geographic distribution and parasite behaviour within the host. Up until now, relatively few

studies have been carried out on the phylogenesis of Oestridae and, of these, many have suffered from inadequate data and methodologies. Recently, however, an exhaustive cladistic analysis has been presented by Pape (2001), who has performed phylogenetic analysis at the generic level, analysing 118 characters (morphological, ontological, physiological and behavioural) of which 15 were considered especially reliable because they were autapomorphic. Pape (2001) provides new information on the monophyly of the Oestridae family within the Oestroidea and confirms that this taxon is subordinate to the sister group Calliphoridae (Rognes, 1997).

Complementary to the morphologically-based cladistic analysis of Pape (2001), the aim of our work has been to analyse Oestridae at the genus, subgenus and lower taxonomical levels using a molecular phylogenetic approach based on a variable region of the COI gene (Otranto et al., unpublished data). In a previous study, a large fragment of the COI gene (1,300 bp spanning from M3 to the terminal carboxylic region) of *H. bovis*, *H. lineatum*, *O. ovis*, *P. silenus* and *G. intestinalis* was sequenced, characterised and compared with analogous sequences of some Calliphoridae larvae available from a database (Otranto and Puccini, 2000). Matrices were calculated with several distance measures and a number of different tree building methods were used (e.g. neighbour-joining, Saitou and Nei, 1987); analysis was performed on a variety of sub-regions and on the entire 1,300 bp sequence. The most robust trees were obtained by analysis of the whole fragment, probably because, when analysing long sequences, the problem of variance in estimating distances is minimised. Although few species of Oestridae were analysed, the major aim of that study was not to find the best tree, but to evaluate the consistency of the morphological classification in comparison with a molecular approach. On the whole, the phylogenetic information we obtained was consistent with the morphology-based taxonomic classification: within family Oestridae two branches were revealed – a branch for Gasterophilinae (*G. intestinalis*) and a branch including Hypodermatinae (*H. bovis*, *H. lineatum* and *P. silenus*) and Oestrinae (*O. ovis*). The most obviously conflicting result was the grouping of *O. ovis* (subfamily Oestrinae) with *H. bovis* and *H. lineatum* (subfamily Hypodermatinae). Conversely, within the Calliphoridae, the split observed between the Chrysomyinae (*Chrysomya rufifacies*, *Chrysomya albiceps*) and Luciliinae (*L. sericata*, *Lucilia illustris*) subfamilies was consistent with the morphological classification; interestingly, however, *Protophormia terraenovae*, belonging to subfamily Chrysomyinae, clustered together with subfamily Luciliinae (Fig. 1). This paper represented a first approach to the molecular characterisation of mtDNA of Oestridae and some inconsistencies observed were probably due to the low number of larval species examined and to the better resolution of the COI gene for low-level phylogenetic investigation.

Following this approach, a large project on the molecular characterisation of the COI of 18 species of larvae belonging to family Oestridae was undertaken and is still under-

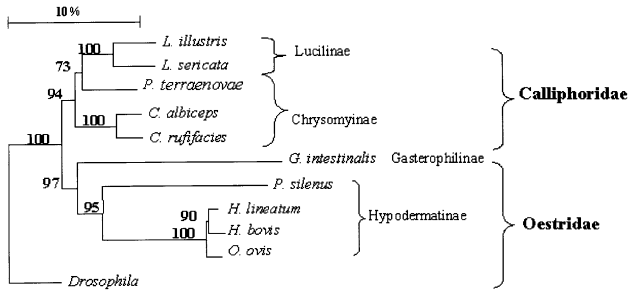

Fig. 1. Neighbour-joining tree based on the whole COI sequence. Distances calculated according to the Kimura two-parameter model. Percentage bootstrap support values indicated at the nodes. The corresponding morphological classification is in bold letters (Otranto and Puccini, 2000).

way. The aim of this project was to define intra- and interspecific relationships and the phylogenesis of Oestridae species belonging to the four subfamilies at a molecular level. Unlike the first study described, a smaller target region, spanning from E4 to the COOH sequence (688 bp), was analysed. This region is known to be more variable among insects and thus suitable to address phylogenetic questions concerning closely related species in low-level analyses (Lunt et al., 1996).

Phylogenetic analysis was performed with minimum evolution methods, inferring the distances with the Kimura two-parameter model (Fig. 2). Overall, the phylogeny was consistent with classical taxonomy. A strong divergence was observed among the four subfamilies, despite the low number of species examined in the Cuterebrinae subfamily. This clear splitting of the four subfamilies confirms their differentiation, as postulated by Wood (1987) (see reference above) and Pape (2001). Although our phylogenetic approach differed from Pape's analysis in terms of aims (inter-genus differentiation) and methods used (morphological/cladistic), our results are in general agreement with his. The phylogenetic signal of the COI gene confirms the fact that the Calliphoridae and Oestridae families are monophylic, as had been previously established on the basis of morphological differences (Otranto et al., unpublished data).

The Calliphoridae include more than 1,000 species worldwide in around 150 genera, ranked in anything from two to 12 subfamilies (e.g. Shewell, 1987; Rognes, 1991; see Rognes, 1997). It is the most generalised family of the Oestroidea (McAlpine, 1989) and the definition of Calliphoridae continues to be debated and constantly revised (Rognes, 1997). Their larvae exhibit a broad range of life history strategies: many are saprophagous, the larvae developing in dung or animal carcasses, others are facultative or obligate ectoparasites of both vertebrates and invertebrates, while at least one species is haematophagous. They are found in nearly all regions of all continents, except Antarctica, and in many parts of the world they are of major economic and welfare importance as pests of humans and livestock.

Until recently, rigorous phylogenetic studies of Calliphoridae have been largely based on morphological analysis

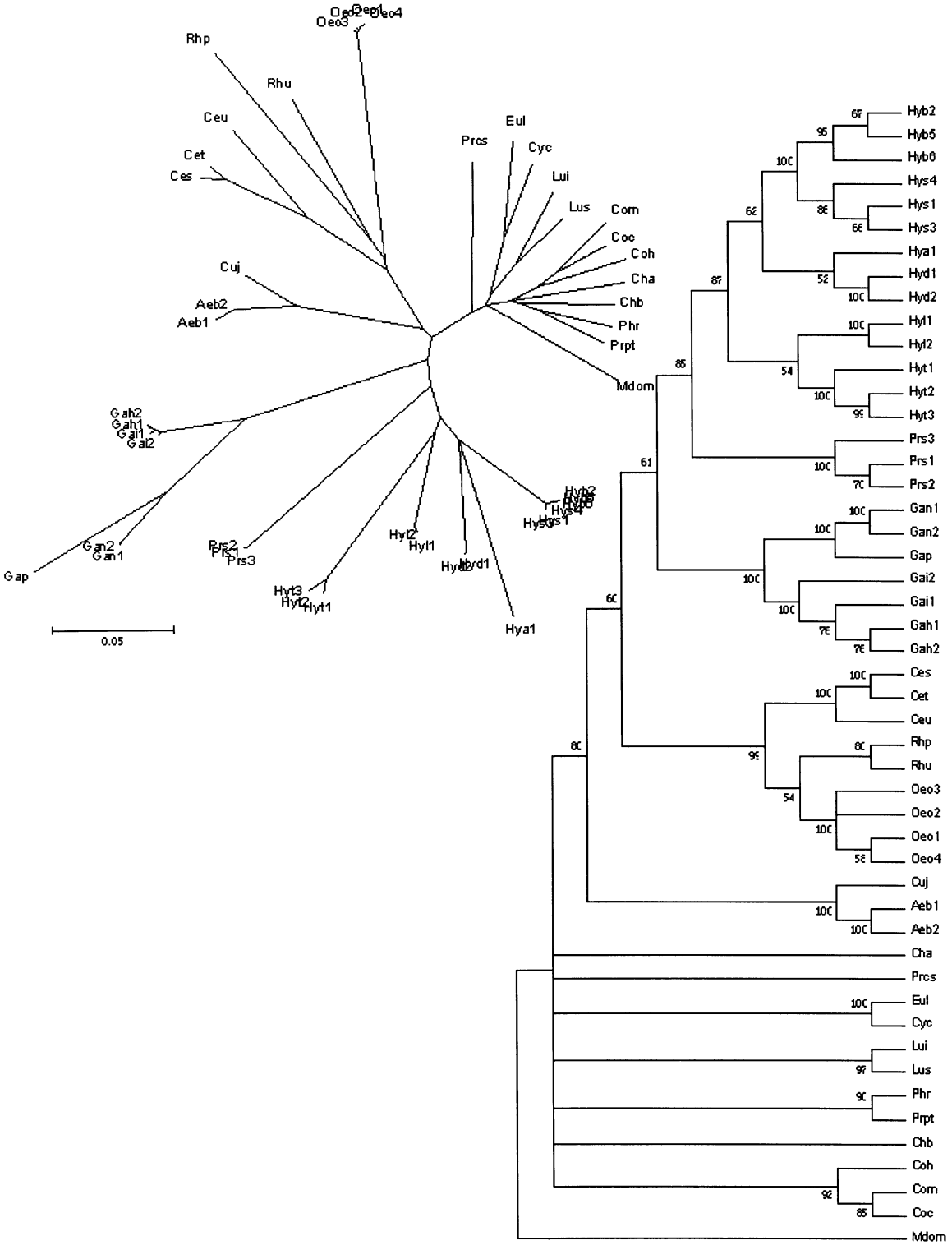

Fig. 2. Minimum evolution tree constructed on the basis of all the nucleotide sequences using the Kimura two-parameter model. ***Oestridae***: *Hypoderma bovis* (Hyb), *Hypoderma lineatum* (Hyl), *Hypoderma diana* (Hyd), *Hypoderma tarandi* (Hyt), *Hypoderma actaeon* (Hya), *Hypoderma sinense* (Hys), *Przhevalskiana silenus* (Prs), *Cephenemyia stimulator* (Ces), *Cephenemyia ulrichii* (Ceu), *Cephenemyia trompe* (Cet), *Oestrus ovis* (Oeo), *Rhinoestrus usbekistanicus* (Rhu), *Rhinoestrus phacocoeri* (Rhp), *Gasterophilus intestinalis* (Gai), *Gasterophilus haemorrhoidalis* (Gah), *Gasterophilus nasalis* (Gan), *Gasterophilus pecorum* (Gap), *Alouattamyia baeri* (Alb), *Cuterebra jellisoni* (Cuj). ***Calliphoridae***: *Cynomyia cadaverina* (Cyc), *Eucalliphora latifrons* (Eul), *Chrysomyia albiceps* (Cha), *Chrysomyia bezziana* (Chb), *Cochliomyia macellaria* (Com), *Cochliomyia hominivorax* (Coh), *Compsomyiops callipes* (Coc), *Phormia regina* (Phr), *Protocalliphora sialia* (Prcs), *Protophormia terraenovae* (Prpt), *Lucilia illustris* (Lui), *Lucilia sericata* (Lus) (Otranto and Stevens, 2002).

of which the most recent and comprehensive is that of Rognes (1997). Since the study by Sperling et al. (1994), however, molecular phylogenetic analysis of Calliphoridae species has progressed steadily. Phylogenetic studies of Calliphoridae tend to fall within one of three categories: (i) forensic; (ii) in relation to myiasis in livestock; (iii) as part of a broader study.

Since Sperling et al. (1994), phylogenetic studies have been performed for several blowfly groups of major forensic importance (see also Section 4). Flies of potential importance in North America have been covered by Wells and Sperling (1999, 2001) using mtDNA COI + II, in Australia by Wallman and Donnellan (2001) also using mtDNA COI + II and in Britain by Stevens and Wall (2001) using 28S rRNA.

Phylogenetic studies of Calliphoridae causing economic myiasis have focused largely on *L. cuprina* and *L. sericata* – the species primarily responsible for sheep strike – in Europe (Stevens and Wall, 1997a) and worldwide (Stevens and Wall, 1996, 1997b; Stevens et al., 2002), using a combination of nuclear and mitochondrial markers. Results suggest the existence of distinct haplotypes from northeast Australia, as described previously by Norris (1990) on the basis of morphology, and a previously undescribed hybrid population from Hawaii (Stevens and Wall, 1996; Stevens et al., 2002). More recently, the Old World screwworm, *Chrysomya bezziana*, has been studied throughout its range from sub-Saharan Africa eastwards to the pacific islands of Papua New Guinea (Hall et al., 2001); phylogenetic analysis of partial mtDNA cytochrome *b* sequences indicates that within its range this fly occurs as two genetically distinct races.

Broad molecular phylogenetic studies which include a number of calliphorid taxa have been undertaken for Muscoidea (Bernasconi et al., 2000) and Calyptratae (Nirmala et al., 2001) including three and four calliphorid taxa, respectively. While such low numbers of taxa do not (and nor is it their aim to) provide meaningful resolution of relationships within family Calliphoridae, nevertheless, such studies are invaluable for setting the group in evolutionary context, especially in terms of confirming relationships with other closely related families, e.g. Sarcophagidae.

Finally, in the first broad molecular phylogenetic analysis to focus specifically on evolutionary relationships within family Calliphoridae, Stevens (Stevens, 2002. Molecular methods for the study of myiasis-causing Calliphoridae: a phylogenetic approach. Proceedings of the 4th EU-COST 833 meeting: mange and myiasis in livestock) analysed 28S rRNA sequences to confirm the monophyly of subfamily Chrysomyinae and the sister group status of subfamilies Luciliinae and Calliphorinae.

5. Larval identification

Within the Oestrodea superfamily, the molecular identification of larvae or adult flies has played a key role. This technique has been used more frequently in forensic entomology for Sarcophagidae and Calliphoridae (Stevens and Wall, 2001; Wells and Sperling, 2001; Wells et al., 2001a,b) than for the study of Oestridae larvae which cause obligate myiasis and rarely cross-infect hosts.

Molecular identification may be achieved by two different approaches: restriction enzyme analysis and/or comparison of sequences with those available on the database. While these two methodologies are comparable in terms of time consumption (<48 h) with the speed and efficacy of current sequencing techniques, restriction enzyme analysis requires greater operator skill. Furthermore, a limit to the restriction enzyme approach is that it utilises only a small fraction of available apomorphic enzyme-target sites which may contain nucleotide variations, particularly when studying specimens of different populations coming from different geographical areas.

In the past few years the number of reports on the molecular differentiation of myiasis-causing larvae has increased enormously mainly for phylogeographical or forensic studies.

The phylogeographical differentiation and molecular characterisation of myiasis-causing larvae is of topical interest, in monitoring successful eradication programs – such as for *C. hominivorax* from North and Central America (Krafsur et al., 1987) – and changes of hosts and feeding habits of larvae across different areas and habitats.

Concerning genus *Cochliomyia*, it is important to differentiate the larvae of *C. hominivorax* and *C. macellaria* (which are, respectively, primary and secondary screwworms) which share morphological features, hosts and site of infection, in different developmental stages. A PCR-RFLP assay has been developed based on the mtDNA of *C. hominivorax* and *C. macellaria*, with the aim of differentiating larvae originating from different populations at the inter- and intraspecific levels (Taylor et al., 1996). The usefulness of mitochondrial control region sequences as markers in a PCR-RFLP assay for the differentiation of myiasis-causing larvae has been confirmed by examining specimens of *C. hominivorax* and *C. macellaria* from Brazil (Litjens et al., 2001). RAPD fingerprints have also proved to be useful for characterising *C. hominivorax* specimens from segregated populations, i.e. southern Brazil and northern Argentina, by revealing genetic variations which could not be detected using isoenzyme analysis (Infante-Malachias et al., 1999).

Forensic entomology is based on the assumption that larvae of insects, frequently flies belonging to the families Calliphoridae and Sarcophagidae, feed and develop on carrion. The presence of maggots on carrion depends on their biological and ecological characteristics and they are commonly associated with dead or dying humans and animals, even if no direct contact is observed. As such, they are often important in murder investigations (Benecke and Wells, 2000; Wells et al., 2001b). The importance of molecular identification in forensic entomology is based mainly, but not only (Wells et al., 2001b), on the identifica-

tion of carrion fly maggots to species level, in the course of investigations for murders, to elucidate aspects of the time, manner and even place of death (Wallman and Donnellan, 2001). One of the most important benefits molecular identification offers to the forensic entomologist is an accurate species identification since many eggs (Greenberg and Singh, 1995) or larvae (Wallman, 2001) of Calliphoridae and Sarcophagidae are difficult to identify morphologically, even for specialists. Inaccurate larval identification may harm or impede an investigation, potentially leading to a miscarriage of justice (Benecke and Wells, 2000).

Over the past 20 years, different mtDNA genes have been sequenced for the molecular identification of saprophagous larvae (Sperling et al., 1994; Wallman and Donnellan, 2001) and recently regions within the 28S lsrRNA have proven to be a reliable target for the differentiation of some Calliphoridae species (Stevens and Wall, 2001). A database of mtDNA sequences of forensically relevant Calliphoridae (Wells and Sperling, 2001) and Sarcophagidae (Wells et al., 2001a) represents a useful and practical tool by which to compare and identify carrion fly maggots.

Sperling et al. (1994) performed a sequence analysis of the COI gene for 18 different species belonging to family Calliphoridae, facilitating rapid identification of the larvae used to estimate post-mortem intervals. The utility of COI and COII for the identification of the most forensically important species of blowflies from southeastern Australia has recently been demonstrated (Wallman and Donnellan, 2001).

Without the immediate practical application of forensic science, as for species of Sarcophagidae and Calliphoridae, the molecular identification of oestrid flies might erroneously appear to be a less important topic. However, while the morphology of oestrid species at subfamily and genus level may be clear, this may not be the case if one examines the 151 fly species ranked within the genus. For example, a RFLP assay of the most common Italian species of Oestridae (i.e. *H. bovis*, *H. lineatum*, *G. intestinalis*, *P. silenus*, *O. ovis*) demonstrated a clear genetic difference between Oestridae; however, no interspecific variation in RFLPs was detected between two species of *Hypoderma* (Otranto et al., 2000).

The major difficulties occurring in the morphological identification of Oestrids are related to the small number of available collections representing all the developmental stages, bad specimen preservation (damage to many adult fly specimens), variations within specimens of larvae collected from different animals and countries, the absence of unitary morphological keys (beyond the great compendium of Zumpt, 1965), the broad-spanning knowledge an entomologist is required to have, and the usual range of operator-dependent laboratory errors.

The first step toward the assessment of a molecular assay, when analysing similar and taxonomically close species, is the evaluation of the intraspecific variation rate. Generally, a molecular identification assay will be considered reliable when the maximum level of intraspecific variation is below the lowest level of interspecific variation. Wells and Sperling (2001) reported an intraspecific level in the COI + II sequence of Chrysomyinae flies below 1% and greater than 3% between species. For Oestridae our group reported a level of intraspecific variation of 0.35% among the larvae examined, while interspecific variation was 13.1, 13.3, 9.6 and 5.3% in each of the four subfamilies (Hypodermatinae, Oestrinae, Gasterophilinae and Cuterebrinae). Sequence variation in the COI region proved to be appropriate to address different questions concerning Oestrid identification at the subfamily and interspecies level; this analysis also identified degraded larvae and utilised specimens collected from separate geographical locations (Otranto et al., unpublished data).

The morphological differentiation of larvae at a subfamily level is not difficult, but can sometimes be troublesome because Hypodermatinae L3 larvae have somewhat similar ecological, ontological, biological and morphological features. They sometimes share hosts (*H. bovis* and *H. lineatum* in cattle, *Hypoderma diana* and *Hypoderma actaeon* from roe deer) and antigens which are secreted during L1 migration inside host tissues (Boulard et al., 1996a). Their morphological differentiation is based mainly on descriptions by James (1947), Zumpt (1965) and Sugar (1976); an accurate comparative description of four Hypodermatinae under scanning electron microscopy has now been published (Colwell et al., 1998).

Recently, the molecular differentiation of larvae of five species of genus *Hypoderma* (i.e. *H. bovis*, *H. lineatum*, *Hypoderma tarandi*, *H. diana* and *H. actaeon*) has been achieved by the characterisation of a 688 bp COI region spanning from E4 to the COOH terminal region. PCR procedures and sequencing analyses were performed as previously described (Otranto et al., 2000; Otranto and Puccini, 2000). Primer combinations yielded amplicons of the expected size as described by Zhang and Hewitt (1996b). The rate of intraspecific variation within species was 0.30%. The restriction analysis was performed with the support of a computer analysis and among all the enzymes cutting the sequences analysed, enzyme *Bfa*I produced restriction patterns which clearly differentiated the five species (Fig. 3) (Otranto et al., unpublished data).

Besides these molecular approaches for the identification of myiasis-causing larvae, an mAb-based ELISA has been recently developed for the rapid identification (approximately 4 h) of eggs, larvae, pupae and adult flies of *C. hominivorax* and is able to differentiate them from those of closely related secondary screwworms (i.e. *C. macellaria*, *Phormia regina*, *L. sericata*, *Calliphora vicina*, *C. rufifacies*); this mAb-ELISA showed a specificity and a sensitivity of 99 and 92%, respectively (Figarola et al., 2001).

6. Diagnosis

Immunodiagnostic methods are instrumental in the detection of many myiases, thus replacing the need for clinical

parasitological (e.g. warble palpation in cattle hypodermosis) and post-mortem examinations (e.g. for equine gasterophilosis, nasal botflies). They are a simple and economic means to perform diagnoses on living animals (even when larvae are still migrating or are otherwise undetectable in the animal body), allowing the planning of timely treatments *before* larvae have caused economic losses, and the monitoring of eradication programs across broad areas (Otranto, 2001). Over the past 30 years the immunodiagnosis of bovine hypodermosis has been widely explored using excretory secretory (ES) products produced by first instar larvae (L1) during their migration within the host tissues as antigens (Sandeman, 1996). The most important ES products produced by *Hypoderma* spp. larvae have been biochemically characterised as serine proteases, namely collagenolytic chymotrypsin, hypodermin C (Boulard, 1970; Boulard and Garrone, 1978; Lecroisey et al., 1987) and trypsins, hypodermin A and hypodermin B (Lecroisey et al., 1979; Tong et al., 1981). The three-dimensional structure of hypodermin C has also been examined in depth, demonstrating the presence of several disulphide bridges responsible for the correct folding of collagenase (Broutin et al., 1996). Hypodermin C is involved in larval tissue migration and stimulation of the host antibody-dependent response, while hypodermin B and hypodermin A are responsible for the way larvae cope with specific and non-specific host immune systems (Boulard and Bencharif, 1984; Chabaudie and Boulard, 1992; Nicolas-Gaulard et al., 1995).

An ELISA prepared with hypodermin C antigen extracted from *H. lineatum* L1 (Boulard, 1970) is currently used in many countries for the serodiagnosis of hypodermosis. In Europe, peak titres of anti-*Hypoderma* antibodies in infected cattle have been registered between November and March; these are the best months for sampling sera to perform an early diagnosis when larvae have not yet caused warbles on the animal back (Boulard and Villejoubert, 1991). ELISAs have been applied to pooled serum or milk samples (Boulard and Villejoubert, 1991) and, more recently, on commercial milk samples (Otranto et al., 2001) for epidemiological surveillance and in large eradication programs, even in the presence of low levels of infestation (Boulard et al., 1996b).

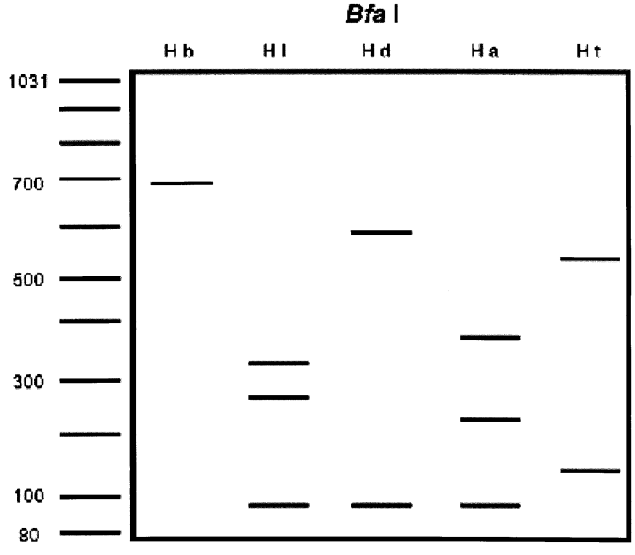

Fig. 3. Restriction site map for the COI terminal region of five larvae belonging to the *Hypoderma* genus (H.b., *Hypoderma bovis*; H.l., *Hypoderma lineatum*; H.d., *Hypoderma diana*; H.a., *Hypoderma actaeon*; H.t., *Hypoderma tarandi*). Location of restriction sites for *Bfa*I enzyme across an entire sequence.

When developing ELISAs for the diagnosis of hypodermosis some problems may arise such as difficulties in preparing enough antigen from larval ES products, in purifying and identifying antigens and in optimising the procedures.

The characterisation and the isolation of cDNA encoding the entire translational products of hypodermin C make it possible to produce a great amount of specific recombinant antigen for extensive immunological surveys. This avoids the use of crude antigens or ES products, which are sometimes responsible for the low specificity of the ELISA techniques. It has been calculated that the amount of recombinant hypodermin C obtained from 1 l of recombinant *Escherichia coli* culture may be sufficient to perform tests on more than 3,000 cattle in duplicate (Casais et al., 1998).

The cDNAs of hypodermins have been sequenced and cloned and their analysis at different larval stages indicated that protein overexpression is regulated transcriptionally for hypodermin A and hypodermin B, and by transcriptional and DNA amplification for hypodermin C; it also highlighted that the mature hypodermin C protein lacked the first 30 amino acid residues encoded by cDNA (Moiré et al., 1994).

The cDNA encoding the entire hypodermin C of *H. lineatum* was cloned by reverse transcription (RT) PCR (Casais et al., 1998) and expressed in *E. coli* as a glutathione S-tranferase fusion protein (Casais et al., 1998; Boldbaatar et al., 2001). Purified recombinant hypodermin C is enzymatically active in azocoll substrate and in gelatin polyacrylamide gels and has been used in ELISA (Casais et al., 1998) and Western blot tests (Boldbaatar et al., 2001), showing good sensitivity and specificity for the detection of anti-*Hypoderma* antibodies in naturally infected cattle (Boldbaatar et al., 2001).

The detection of epitopes shared by hypodermin C from *H. lineatum* and *H. bovis*, with antigens of other larvae belonging to subfamily Hypodermatinae (*H. diana*, *H. tarandi* and *P. silenus*) (Boulard et al., 1996a), emphasises the importance of producing recombinant hypodermin C for the immunodiagnosis of all myiases caused by these larvae. This importance of this finding is also supported by a study demonstrating the efficacy of hypodermin C in testing sera and milk samples by ELISA, for the immunodiagnosis of the goat warble fly, *P. silenus* (Otranto et al., 1998, 1999).

Concerning oestrosis, an ELISA using L1 crude somatic antigen showed 97.4% sensitivity and 97.6% specificity compared with clinical post-mortem examination (Goddard et al., 1999). Recently, Western blot analyses have been used

to screen *O. ovis* larval ES products from salivary gland and digestive tube contents to evaluate their diagnostic and protective role (Tabouret et al., 2001). A 1 kDa protein complex (pc28) from the salivary gland contents has been demonstrated to be the main antigenic component of ES products. The diagnostic value of this protein was estimated by an ELISA test performed on sheep sera which showed higher anti-*O. ovis* antibody titres in summer and winter due to high L1 metabolic activity (Tabouret et al., 2001).

No immunodiagnostic tools have been developed for Calliphoridae and Sarcophagidae myiasis-causing larvae, mainly because larvae are usually detectable on skin wounds. However, mAb against ovine IgE anti-blowfly in sheep have been produced (MacDiarmid et al., 1995) and used in a number of studies (i.e. to examine sera and wound exudates from sheep affected by *Lucilia* spp.) to monitor vaccination projects using concealed antigens (see below).

7. Control strategies

Although chemotherapeutic products (e.g. organophosphates, macrocyclic lactones) have been successful in the control of many myiasis-causing larvae, there are many constraints linked to the use of these drugs due to the risks they engender for animals (toxic effects, development of parasite resistance), for human health (residues in meat and milk) and for the ecosystem (environmental pollution).

Currently a major objective of myiasis-causing larvae control strategies is to improve farm management practices and to integrate them with measures consistent with the principles of environmental sustainability. Hence, in the last few years alternative strategies for the control of myiasis have been investigated such as the sterile insect technique, biological and genetic methods, and vaccines (Hall and Wall, 1995).

Biological methods have been developed against many myiasis-causing larvae of human and animal concern (i.e. *D. hominis*, *O. ovis*, *L. cuprina*, *Cephalopina titillator*) by using entomopathogenic fungi, *Bacillus thuringiensis*, and phoretic mites (Hall and Wall, 1995; Mazyad and Raheem, 2001). However, these methods present some difficulties in terms of field application (selective killing of myiasis-causing larvae, administration to the animals) and production.

In 1992, the release of irradiated sterile screwworm fly males (sterile insect technique) successfully eradicated *C. hominivorax* from Lybia (Cunningham et al., 1992). Despite its success in eradicating screwworms, the use of the sterile insect technique is fraught with problems, such as difficulties in rearing flies, in estimating the amount of treatment needed and the cost linked to the integrated approaches (early insecticide application to reduce fly population, monitoring, restriction of movement of infected animals, massive information campaigns, etc.) (Krafsur et al., 1987).

Vaccination methods against arthropods have been under development for a number of years with different degrees of success; their protective role against ectoparasites in general, and against myiasis-causing larvae in particular, is still controversial since they do not induce the rapid knockdown effect produced by chemical pesticides. Vaccine strategies are, however, considered an important population-reducing tool if used over successive parasite generations (Pruett, 1999).

In the past few years, vaccination strategies against myiasis-causing larvae have focused on two types of antigen vaccines: 'conventional antigens' produced by larvae in an animal's body and eliciting an immune response in the host (e.g. *Hypoderma*), and the parasite's own antigens (hormone, gut structure) defined as 'concealed' antigens (e.g. *L. cuprina*) (Willadsen, 1997).

In hypodermosis, hypodermin A, B and C are involved in a number of immunological mechanisms and are therefore logical targets for use as vaccines. Hypodermosis vaccination trials have recently turned to the use of hypodermins in different combinations of larval 'crude extract' or adjuvant compounds (but have not produced encouraging results) (Baron and Colwell, 1991; Chabaudie et al., 1991). The biochemical characterisation of hypodermins probably opens a new era in vaccination trials, although the protective role of vaccines against *Hypoderma* infestation is still controversial.

Findings related to *L. cuprina* feeding on the sera leaking from animal wounds by means of trypsin encouraged scientists to design different methods for sheep immunisation using natural antigens. IgG degradation caused by tryptic and chymotryptic enzymes produced by L1 larvae (Sandeman et al., 1990) occurs mainly in the acidic middle region of the midgut of *L. cuprina*, where binding to the peritrophic membrane results in reduced permeability and larval death (Eisemann et al., 1993, 1995). Thanks to knowledge of the physiology and biology of *Lucilia*, different vaccination strategies have been developed. The first involves vaccination with the three larval peritrophic membrane antigens isolated (East et al., 1993). The amino acid sequence of peritrophin 95 (p95), purified from peritrophic membrane of *L. cuprina*, has been recently determined and sequenced in cDNA, and is now available for a recombinant antigen vaccine (Casu et al., 1997). A recent study demonstrated that recombinant forms of p95, produced both in bacteria and baculovirus infected insect cells, have a lower inhibitory activity on larval growth than the native p95, probably due to the incorrect folding. The authors concluded that the polypeptide structure of native p95 is essential for inhibition of larval growth in the sera of sheep vaccinated with this antigen (Tellam et al., 2001). Furthermore, intradermal immunisation with recombinant peritrophic membrane antigens (i.e. peritrophin 44, peritrophin 45 and p95) may enhance the immunological control of blowfly larvae by significantly increasing the production of specific antibodies (Colditz et al., 2002). By using molecular approaches (RT-PCR), the p95 has been detected in regurgitated or excreted larval material as soluble monomeric protein, clearly revo-

lutionising the theory of the 'concealed' antigens (Tellam et al., 2000).

Another approach relates to the use of larval ES products and homogenate extracts. The inhibition of larval growth was confirmed to be mediated by ingested ovine antibodies mainly produced against peritrophic membrane and larval cuticle, and less against other internal organs (Tellam and Eisemann, 1998).

Finally, Meeusen and Brandon (1994) isolated antibodies produced by B cells present in the lymph nodes of previously infested sheep. These antibodies recognised four major antigens in Western blots which caused an 85% decrease in infestation when used to immunise sheep compared with a control group (Bowles et al., 1996).

Similarly, immunogenic antigens of *C. bezziana* screwworm have been extracted from the peritrophic membrane and cardia of L1 and L3. Vaccination in vitro and in vivo with both antigens showed a reduction in the weight of recovered larvae of 82 and 45%, respectively (Sukarsih et al., 2000). A peritrophin gene (peritrophin 48) of *C. bezziana* has been identified and biochemically characterised, demonstrating a predicted protein structure (constituted by five domains, each containing six cysteines) that was strictly conserved (Vuocolo et al., 2001).

8. The future

Although myiases have been described since ancient times, many aspects of these infestations are still unknown and their eradication has been obtained in two cases using the sterile insect technique against screwworm in America and the organised programme of chemotherapy against bovine hypodermosis. Over the past century the advent of chemotherapeutic products (i.e. organophosphates and macrocyclic lactones) created a true 'revolution' in the control of arthropod-borne diseases and, in particular, of myiasis-causing larvae, even if this approach has been partially superseded due to environmental safety issues.

In the past 30 years, efforts to enhance our knowledge of myiasis-causing larvae based on molecular biology tools have no doubt provided new insights into many aspects of larval infestations (e.g. aetiology, biology, epidemiology, diffusion and pathogenicity). Nevertheless, many important issues will require investigations by molecular biology in the near future, namely:

(a) the development of a target-specific recombinant vaccine against the most pathogenous myiasis-causing larvae for environmentally sustainable myiasis-control programmes;
(b) in-depth genetic and evolutionary information on myiasis-causing larvae in relation to their hosts, behaviour and ecology, whilst offering insights into their phylogeographic and demographic patterns, and an understanding of the origins of the myiasis habit and the future of myiasis-causing species (preservation of biodiversity);
(c) enlargement of the sequence databases of larvae of forensic concern and the evaluation of genetic differences among the same species originating from different areas and/or countries;
(d) evaluation of larval resistance mechanisms to pharmaceutic compounds.

Indeed, the resistance of myiasis-causing larvae to pharmaceutic compounds still remains a moot issue, although the importance of this subject has been recognised for a number of insects such as *D. melanogaster* (Kane et al., 2000). In this specific field molecular biological investigations have proved to be instrumental in detecting strains of insects resistant to ivermectin, organophosphates, and nodulisporic acid by characterisation of the ligand-gated ion channel protein superfamily. The ability of organisms to develop resistance threatens the efficacy of antibiotic drugs, therefore alternative drugs and other treatment possibilities should be made available (Köhler, 2001).

Such information is of paramount importance to promote environmentally sustainable control of myiasis-causing larvae and to understand the origin of these larvae and their future in a habitat which is constantly changing due to environmental changes, new farm management strategies and the immunological adaptation of hosts to larval infection. Larval preservation and/or control are no doubt issues for considerable discussion among scientists and this review aims to offer them further food for thought.

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