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THE NEMATODA FILARIOIDEA: CRITICAL ANALYSIS LINKING MOLECULAR AND TRADITIONAL APPROACHES

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Summary:

The molecular analysis of the Filarioidea and the endobacteria *Wolbachia* is no more limited to the agents of human diseases and the diversified sampling permits a synthesis with the morphological and biological results. The validity of the genera with "uncoherent host range", such as *Mansonella*, *Litomosoides* and *Cercopithifilaria*, is confirmed and, consequently, their evolution by host-switchings. *Dirofilaria* and *Onchocerca*, types of two subfamilies, appear more closely related than with other onchocercids. Waltonellinae from anurans and Oswaldofilariinae from reptiles have a basal position. These filariae, and some others also considered primitive, do not harbour *Wolbachia*. Evidence for transversal transmission of the bacteria and a second acquisition event is given with the supergroup F, identified in *Mansonella*, in one of the *Cercopithifilaria* species and in arthropods.

KEY WORDS : Filarioidea, *Onchocerca*, *Dirofilaria*, *Dipetalonema*, *Wolbachia*, phylogeny.

Filarioids are parasitic nematodes extensively studied because several species are the agents of human diseases. The need to elucidate the epidemiological conditions of transmission and the requirement for relevant experimental models have stimulated a widespread zoological investigation. Our present concern is the systematics of the superfamily Filarioidea with emphasis on its generic composition, the relationships between genera, the validity of the supra-generic divisions, the roots and epoch of emergence of Filarioidea, related to the question of mono- or polyphyly. Two main investigative tools are currently available, the traditional morphological and biological, and the more recent molecular analyses. They are commonly used by independent specialized teams, with the risk of uncontrolled divergent interpretations for the same biological object. On the other hand, the combining of their respective results may foster more appropriate interpretations.

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The traditional tool includes the morphological characters of the adult and larval phenotypes, the biological data provided by vectors and experimental rodent models of filariases, as well as the host range and geographical distribution of worms. Since Wehr (1935), several classifications have been built on these data and the most recent (Anderson & Bain, 1976), seemed to provide a correct synthesis; however new features, such as the discovery of genera or the better knowledge of larval development, have revealed several problems.

Nowadays, molecular analyses are available for a rather important sample of filariae, including also the study of the endosymbionts *Wolbachia sensu lato* (Casiraghi group and collaborators, work in progress). The genes studied are 5S rDNA, 12S rDNA and *coxI*, less often 18S rDNA.

Our aim is to compare the results of both approaches with some examples chosen to distinctly highlight confirmation or contradiction of previous interpretations, and finally to delineate some unresolved problems.

EXAMPLES OF CONGRUENCE BETWEEN MOLECULAR AND TRADITIONAL ANALYSES

Among the filariae, there are several genera of which the host range is "zoologically incoherent" (Chabaud, 1981, see Chabaud & Bain, 1994). They are used to argue a model of evolution through host-switchings (captures), in opposition to a parallel evolution of the hosts and their parasites. However it may be objected that the genus is not well-delineated and, in fact, is a non-natural grouping of species. The molecular analyses should decipher such artefacts.

The congruence of the two methods of analyses appears quite good for the three genera studied *Cercopithifilaria* Eberhard, 1980, *Litomosoides* Chandler, 1931 and *Mansonella* Faust 1929, parasitic in mammals.

Cercopithifilaria is known with 28 species described from monkeys, ruminants, carnivores, lagomorphs, por-

cupine and murid rodents, marsupials and monotremes. The small subunit of mitochondrial ribosome (12S rRNA) was analysed for nine species, from African porcupine hystricid, Japanese bear, Japanese bovids and cervids. The taxon, which had been created for a parasite of African cercopithecid monkeys, is actually a group of species having a large host-range and a worldwide distribution. As a matter of fact, the 12S rDNA analysis confirms the value of the traditional characters used to define *Cercopithifilaria* (Fig. 1), the adult morphology and the infective larvae with no or minute buccal cavity. In addition adults have dermal or sub-cutaneous localizations, and skin-dwelling microfilariae. The vectors are the hard ticks Rhipicephalidae and it is hypothesized that they had the major role in the spread and diversification of *Cercopithifilaria*. In Australia, *C. johnstoni* Spratt & Varughese, 1975, type host-Muridae, is found in marsupials and monotremes as well (communication by D. Spratt) and this might represent the first step of future speciations. Interestingly, *Wolbachia* is absent from species of the genus, except *C. japonica* (Uni, 1983) from bears, which harbours the form F of the endosymbiont (work in progress), a *Wolbachia* type shared with filariae of the genus *Mansonella* and with some arthropod hosts (Casiraghi *et al.*, 2005).

32 species have been described in *Litomosoides*, from microchiropterans, marsupials, murids and a few other rodents. The 12S rRNA was analysed for five species, three from bats and two from murids. The monophyly is also proved in this case. A particular morphological character of the cluster is the large segmented buccal capsule, about 20 µm long in the adult worm and third stage larva (Fig. 1). The vectors are macronyssid acarians. *Litomosoides* is confined to the New World and has intensely diversified in the Neotropical region, probably by means of the mites. In the Old World, another genus has a large segmented buccal capsule and is parasitic in microchiropterans, and a common origin is supposed for the two genera. Indeed, a species parasitc in North American rodents Geomyioidea, *Litomosa westi* (Gardner & Schmidt, 1986), which was initially placed in *Litomosoides*, would represent a link. This hypothesis is supported by the 12S rDNA analysis (Casiraghi *et al.*, 2004; work in progress). *Wolbachia* is present in *Litomosoides*, with one exception (*L. yutajensis* Guerrero, Martin & Bain, 2003) and belongs to the supergroup D; *Wolbachia* of *Litomosa westi* appears to belong to the same supergroup (work in progress). *Mansonella* Faust, 1929 contains 29 species, among which three are human parasites: one in South America, *M. (M.) ozzardi* (Manson, 1897), one in Africa, *M. (Esslingeria) streptocerca* (Macfie & Corson, 1922), and one with an African origin subsequently introduced to South America since a few centuries, *M. (E.) perstans* (Manson, 1891). Two important diversifications are observed in anthropoid and platyrhinian monkeys. Other hosts are insectivores, including tupaids, carnivores and sciurid and caviomorph rodents (Eberhard & Orihel, 1984). Recently, ruminant ungulates have joined this large host-range with species initially assigned to *Cutifilaria* Bain & Schulz-Key, 1974, now a subgenus of *Mansonella*. The 5S rDNA and/or 12S rDNA analyses (Xie *et al.*, 1994; work in progress) have been performed with one species of *Cutifilaria* and three species from primates belonging to the subgenera *Mansonella*, *Esslingeria* Chabaud & Bain, 1976 and *Tetrapetalonema* Faust, 1935, respectively. They cluster together and this correlates with distinctive morphological characters in adult worms: the oesophagus is thread-like, its blurred anterior end fused with the muscular body layer, and no buccal capsule is identifiable (Fig. 1). *Wolbachia* was not detected in *M. (E.) perstans* (Grobusch *et al.*, 2003) but was detected in the other three species; it belongs to the supergroup F of the endosymbiont (work in progress).

IS THE *DIPETALOMEMA* LINEAGE A REALITY?

During the past 30 years, *Dipetalonema* Diesing, 1861 has been split into several subgenera, many elevated to the generic rank, and the whole encompassed in the "*Dipetalonema* line". A common history from a Gondwanian ancestor during the late Second era was proposed for all of them, followed by divergences in the different regions separated by the continental drift (Chabaud & Bain, 1976, *in* Chabaud & Bain, 1994). In the "*Dipetalonema* line" males display a basic arrangement of the caudal sensory system (four precloacal pairs, pairs 5 and 6 post-cloacal, and a posterior group of pairs 7 to 10); the *area rugosa*, a cuticular anti-slit apparatus used during mating, is generally present. However the lineage is composed of plesiomorph and derived species (such as *Acanthocheilonema* and *Cercopithifilaria,* respectively), and a clear definition was difficult. The present question is: do the molecular data support the *Dipetalonema* lineage?

Among its 17 genera and sub-genera, half of them have been submitted to molecular analyses: *Acanthocheilonema* Cobbold 1870, *Dipetalonema, Cercopithifilaria,* and *Mansonella* with four of its sub-genera, *Mansonella, Esslingeria* Chabaud & Bain, 1976, *Tetrapetalonema* Faust, 1935, and *Cutifilaria*.

Acanthocheilonema and *Dipetalonema sensu stricto* are generally viewed as a cluster. The first genus contains parasites of carnivores, macroscelid insectivores and rodents, the second is currently restricted to parasites of South American platyrhinian monkeys. The studied species are *A. reconditum* (Grassi, 1889) from dogs,

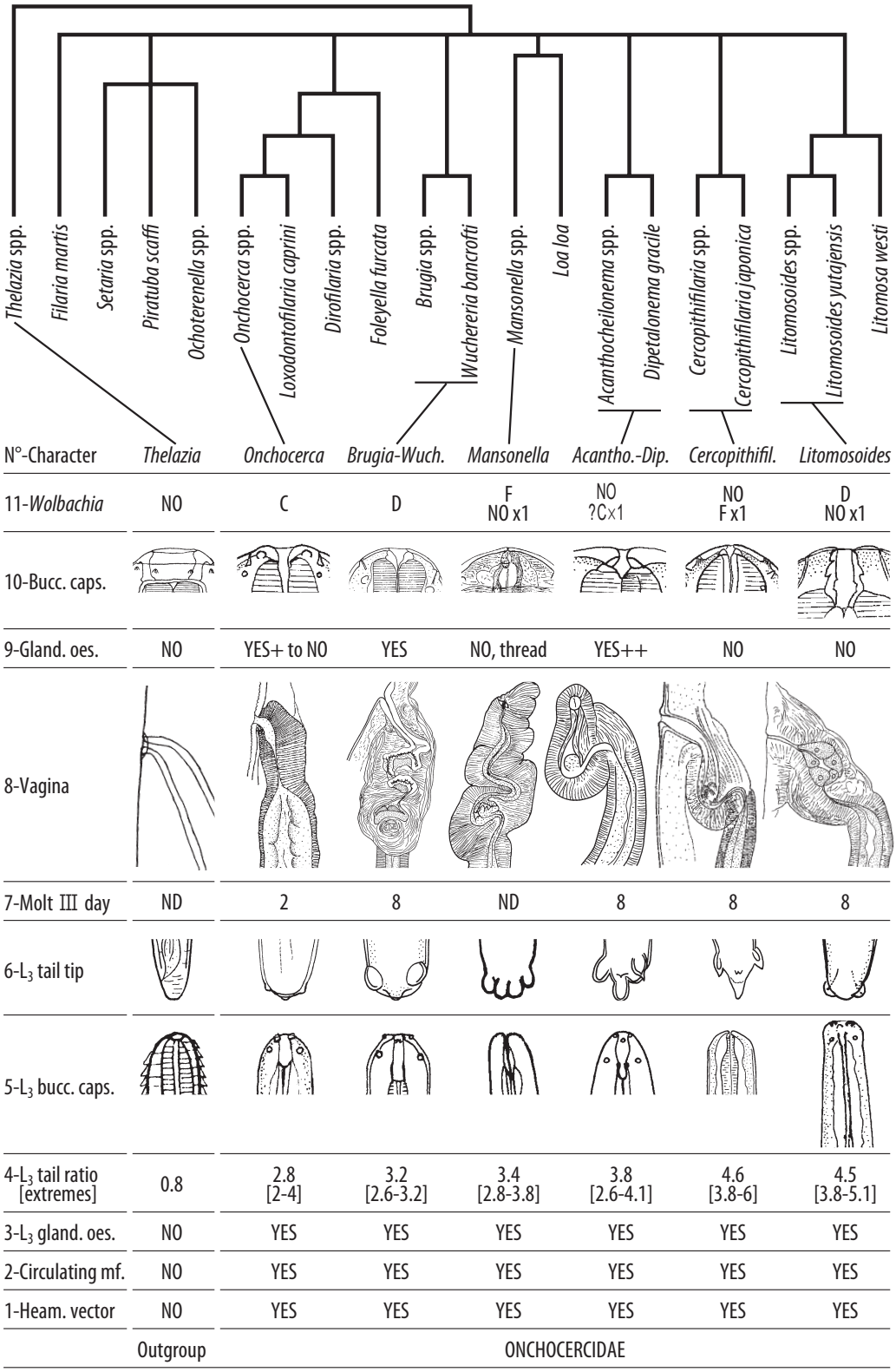

Fig. 1. – Filarioidea and the outgroup *Thelazia*: schematic illustration of molecular analysis and traditional data for some important genera. Distinctive characters selected are 1. Arthropod vector, Heamatophagus/not; 2. Biology of microfilariae, circulating/not; 3. L3 oesophagus, glandular part present/not; 4. L3, mean tail ratio length/width at anus [extremes]; 5. L3, buccal capsule, conspicuous/absent/very long; 6. L3, tail extremity; 7. Molt III, day 2 p. i./day 8 p. i.; 8. Vagina: no bend & no chamber/bends/bends and conspicuous chamber; 9. Adult filaria, oesophagus, glandular part present (Yes to ++)/no; 10. Adult filaria, buccal capsule, 2 rings (or segments)/1 ring/absent/4 rings; 11. *Wolbachia*: absent (NOx1 means 1 species without *Wolbachia*) or a supergroup. The molecular tree presented is a "consensus" of the reconstruction generated in this and previous works. Larval characters mainly from Bain & Chabaud (1986).

A. viteae (Krepkogorskaya, 1933), used as a rodent model for filariasis, and *D. gracile* (Rudolphi, 1809). This cluster appears morphologically defined: the adult worms possess an oesophagus with a long thick glandular part, and the infective larvae have a buccal capsule 6-10 µm long (Fig. 1). *Cercopithifilaria* is thus clearly different from the *Acanthocheilonema-Dipetalonema* cluster at the larval stage (no buccal capsule) and *Mansonella* at the adult stage (thread-like oesophagus). The well-developed caudal lappets of the infective larva of the four genera, which was one of their common characters, appears to be a convergence.

Filarioids from elephants, *Loxodontofilaria* Berghe & Gillain, 1939, were not well known and provisionally placed in the *Dipetalonema* line (Bain, Baker, Chabaud, 1982, *in* Chabaud & Bain, 1994). With the recent discovery of a species of *Loxodontofilaria* in a caprine (Uni *et al.*, 2006), a molecular analysis was performed which lead to a different interpretation. It indicates close relationships with *Onchocerca* Diesing, 1841 (work in progress), although the vagina of *Loxodontofilaria* has distinct bend and chamber, contrary to *Onchocerca*. *Loxodontofilaria* also harbours *Wolbachia* of the group C, very similar to the endosymbionts harboured by members of the genus *Onchocerca*. Interestingly, *Wolbachia* is absent in the two *Acanthocheilonema* species studied (Casiraghi *et al.*, 2004). According to the hypothesis proposed by Chabaud & Bain (1994), these would be close to the roots of Gondwanian *Acanthocheilonema*-like filariae from which stemmed *Dipetalonema s. s.* in the Neotropical region, when platyrhinian monkeys arrived after their transoceanic migration (late Eocene). *D. gracile,* the single species studied in this derived branch, harbours a *Wolbachia* related to the C supergroup, even though its real assignation is still under discussion (see below and Casiraghi *et al.*, 2004; 2005).

NEW LIGHT ON THE ONCHOCERCINAE AND DIROFILARINAE

Within the past 15 years, several molecular analyses of *Onchocerca* Diesing, 1841 and *Dirofilaria* Railliet & Henry, 1910 were conducted, with ribosomal genes 5S (Xie *et al.*, 1994), *cox1* (Casiraghi *et al.*, 2001), 12S rDNA (Casiraghi *et al.*, 2004). All these molecular analyses placed the two genera close together. However each genus is presently the type of a subfamily (Anderson & Bain, 1976). Dirofilariinae are distinguished from Onchocercinae by the shorter male tail, well-developed lateral alae and pedunculated caudal papillae.

A first disturbance to the classical systematics was provoked by *Loa* Stiles, 1905, Dirofilariinae: 5S rDNA analysis placed *Loa* close to *Mansonella,* Onchocercinae (Xie *et al.*, 1994). Reconsideration of the morphological characters of *Loa* revealed that its position close to *Dirofilaria* was no longer sustainable: the infective stage of *Loa* is distinguished from that of *Dirofilaria* by possession of a long tail with well-developed caudal lappets, like *Acanthocheilonema, Cercopithifilaria*, and other onchocercids. Later, biological evidence strengthened the new interpretation. Larval development in *Loa*, *Onchocerca*, *Dirofilaria*, as well as in *Brugia* Buckley, 1958, *Acanthocheilonema*, *Litomosoides* and *Monanema* Anteson, 1968 were compared and demonstrated two strategies of worm establishment in the definitive host (Bain *et al.*, 1997): the third moult occurs as soon as day 2 post-inoculation in *Onchocerca* and *Dirofilaria* but not until the end of the first week in the other genera, *Loa* included.

Molecular analysis has been carried out with two species of *Dirofilaria*, *D. immitis* (Leidy, 1856) and *D. repens* Railliet, 1911 from dogs, and many species of *Onchocerca* (Krueger *et al.*, 2007; Sreter *et al.*, 2007). However, only two studies encompassed several other genera of filarioids (Casiraghi *et al.*, 2004; work in progress). The cluster *Dirofilaria* and *Onchocerca* is composed of two main branches, each corresponding to a genus. The cluster and its dichotomy concur with the morphological characters: the buccal capsule is reduced to an inconspicuous lamina in the adults, and is normally developed in the infective larvae (4 to 9 µm long); these have a cylindrical tail with rounded extremity and tiny caudal lappets in both genera. However the tail of *Dirofilaria* larvae is short (ratio length/width less than 1) in contrast to that of *Onchocerca*, and the females of the 33 *Onchocerca* species (Uni *et al.*, 2007) possess a simple vagina, without bend and chamber (Fig. 1) in contrast to *Dirofilaria*.

Dirofilaria harbours the group C of *Wolbachia*. *Onchocerca* harbours the same supergroup, when present. *O. flexuosa,* the single species without *Wolbachia*, displays a particular morphology and was assumed to be an "ancient" representative of the genus; this is congruent with the molecular datasets (Krueger *et al.*, 2007). The other Onchocercinae listed above harbour the supergroups D (most of the cases) or F (in the case of *Mansonella* spp. and *Cercopithifilaria japonica*).

POSITION OF THE SPECIES PARASITIC IN ANURANS AND REPTILES IN THE EVOLUTION OF FILARIOIDEA

Icosiellinae Anderson, 1958 and Waltonellinae, Bain & Prod'Hon, 1974, with one and four genera respectively, are parasites of anurans. They are classically considered ancient, the first subfamily because the infective larva possesses cephalic spines, the second because

of its Gondwanian host-range. The 12S rDNA analysis performed on two *Ochoterenella* species confirms the basal position of the Waltonellinae (work in progress). Onchocercids parasitic in reptiles are dispersed among several genera and subfamilies. Their heterogeneity has been confirmed with the two species analyzed, an Oswaldofilariinae Chabaud & Choquet, 1953 (*Piratuba* Freitas & Lent, 1947), and a Dirofilariinae (*Foleyella* Seurat, 1917). The first has the most basal position and the second clusters with *Dirofilaria* and *Onchocerca*. The Oswaldofilariinae have several morphological characteristics: the infective larvae have longitudinal cuticular body crests; the vulva opens at midbody or posteriorly; however the female genital primordium migrates anteriorly during morphogenesis in the larval stage, as in other onchocercids. No *Wolbachia* has been identified in any of these filarioids from anurans and reptiles (Casiraghi *et al.*, 2004; work in progress).

It is noted that *Setaria* Viborg, 1795 parasitic in ungulates is often placed close to Oswaldofilariinae and Waltonellinae near the base of the molecular phylogenetic trees. *Setaria* does not harbour *Wolbachia*, alike the outgroup genus *Thelazia* Bosc, 1819 (Casiraghi *et al.*, 2004; work in progress).

WOLBACHIA AND THE FILARIOIDEA

Since the identification of the bacteria *Wolbachia* in the tissues of some human filariae, a broader range of filarioid species has been studied resulting in a number of new features (work in progress). The assessment of *Wolbachia* omnipresent in infected species and its exclusive vertical transmission needs to be revised as well as the evolution of *Wolbachia*-filaria relationships.

In the human filariae *O. volvulus*, *B. malayi* and dog filaria *D. immitis*, the tissular distribution of *Wolbachia* includes the hypodermis of the lateral chords but this is not a general feature; in the animal filariae *Loxodontofilaria caprini*, *M. (Cu.) perforata* and *O. dewittei japonica, Wolbachia* is not detected in the hypodermis. Moreover, the prevalence of the bacteria in a given host species does not reach 100 % in all cases; absence/presence is observed in the three last species cited above and in *O. skrjabini*, although *Wolbachia* occurs in the lateral chords in this species and thus is expected to be positive regardless of which part of the worm is used for PCR. These data suggest different life styles of the wolbachiae and mutualistic relationships.

Acquisition of the mutualist through lateral transfer was demonstrated with a species of *Cercopithifilaria*, *C. japonica*, which harbours the supergroup F shared with *Mansonella*, and some insects (Lo *et al.*, 2002; work in progress), whereas all other species of *Cercopithifilaria* have no *Wolbachia*.

A fact that recently has come to light is that the Onchocercidae which are considered primitive on the basis of traditional morphological and biological data have no *Wolbachia*. The list includes the Oswaldofilariinae and Waltonellinae, species of *Foleyella* (Dirofilariinae) parasitic in cold-blooded vertebrates, and the Setariinae. The case of *O. flexuosa* might be interpreted also as a primitive state in *Onchocerca*, or might be explained by a secondary loss of the endosymbiont. This highlights the fact that identification of a loss of *Wolbachia* may be problematic. However such regressive evolution seems well-established with *Litomosoides*: the endosymbiont is present in five of six species studied and *L. yutajensis*, which lacks the endosymbiont, has no morphological particularity.

The genus *Wolbachia* was divided into supergroups encompassing symbionts of arthropods (A, B and E) or nematodes (C and D). This picture changed when a further supergroup was described (F), which encompasses *Wolbachia* from both arthropods (in particular termites) and filarial nematodes. Additional molecular diversity in *Wolbachia* has been found in Australian spiders (supergroup G), other termites (supergroup H), fleas and potentially in the filarial nematode *Dipetalonema gracile* (Casiraghi *et al.*, 2004).

Wolbachia is patchily distributed, leading to a complicated picture (Fig. 2). At the beginning of the evolutionary radiation of *Wolbachia*, around 100 mya (Bandi *et al.*, 1998; Fenn *et al.*, 2006) transfer of this endosymbiont from arthropods to nematodes (or *vice versa*) must have occurred. It is coherent with an ancestral absence of the endosymbiont in deep branches of Filarioidea, followed by at least one acquisition in the lineage leading to the derived species *Onchocerca* and *Dirofilaria,* the same or another acquisition leading to many other Onchocercinae. The strict relationship of *Wolbachia* from arthropods and nematodes in the F supergroup shows that a similar transfer might also have occurred more recently and independently from the ancestral host switch (work in press).

UNRESOLVED QUESTIONS

The present critical analysis stresses the general congruence between traditional and molecular approaches at the generic rank in the Filarioidea. This validates the morphological and biological characters selected for diagnosis (Fig. 1). The major unresolved questions are the delineation of the Filarioidea, their origin and evolution.

Larval characters are reliable for the suprageneric relationships since ontogeny reflects phylogeny in Spirurida. The character “glandular part of eosophagus present in the infective stage” encompasses the Filarioidea

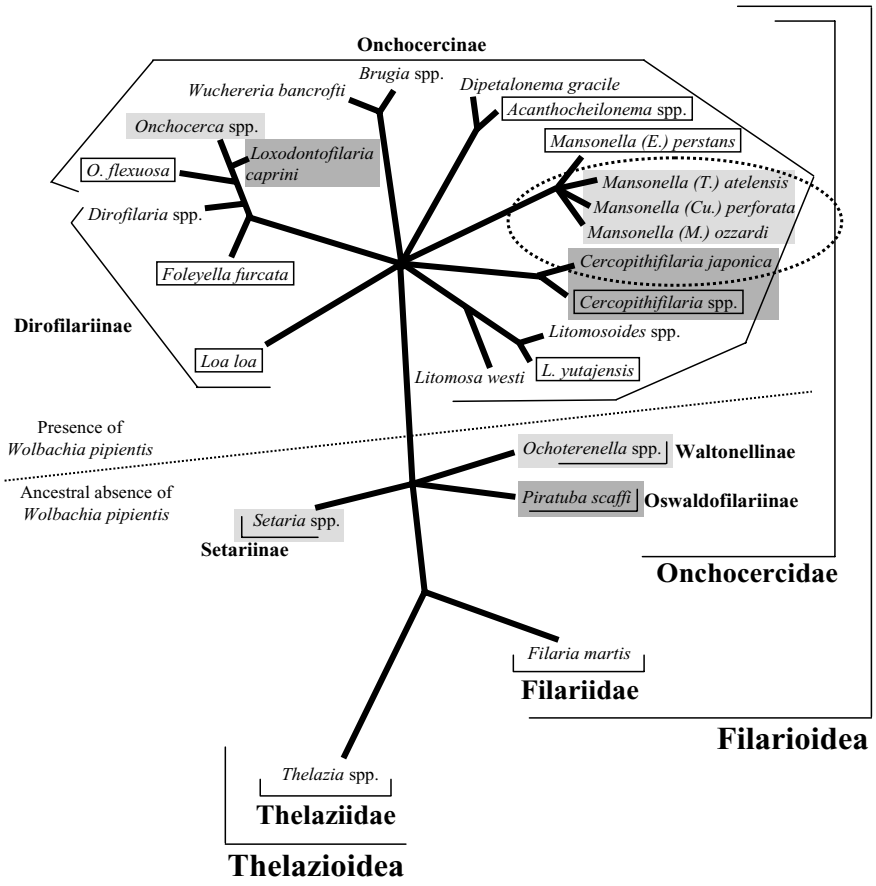

Fig. 2. – Hypothetical evolution of *Wolbachia* infection mapped on the phylogenetic tree of filariae and related nematodes. The present data suggest that *Wolbachia* was ancestrally absent from the lineages leading to Thelaziidae, Filariidae, Setariinae, Oswaldofilariinae and Waltonellinae, these two last parasitic in cold blooded vertebrates. *Wolbachia* was acquired on the lineage leading to the Onchocercinae + Dirofilariinae, diverging into supergroups C and D. *C. japonica* and *Mansonella* spp. (outlined in dashed ellipse) share a common supergroup F *Wolbachia*, due to a secondary acquisition. Losses along some lineages presented here (outlined in boxes) are more controversial and, except for *Litomosoides yutajensis* and *Loa loa*, could also be explained by a remote lineage birth, as for *Foleyella* from saurians, *Acanthocheilonema* spp., *Cercopithifilaria* spp., *Onchocerca flexuosa,* and *Mansonella (Esslingeria) perstans*. Grey areas are for species or genera studied since Casiraghi *et al.*, 2004; half grey are for species included in previously studied genus (example *Onchocerca*). Subgenera of *Mansonella*: (*Cu.*) *Cutifilaria*; (*M.*) *Mansonella*; (*T.*) *Tetrapetalonema*.

and negatively defines the outgroup (Fig. 1). *Thelazia* is well chosen because the oesophagus is not divided (*e.g.* no divisions of the three primordial glandular cells). *Parafilaria* Yorke & Maplestone, 1926 and *Stephanofilaria* Ilhe & Ilhe-Landenberg, 1933, which also have a short undivided oesophagus in adult and infective larva, are not Filarioidea but Thelazioidea (Bain, 2002). The persistence of such primitive oesophageal anatomy places the three genera close to some Seuratoidea Chabaud, Campana-Rouget & Brygoo, 1959, a complex superfamily in need of molecular analysis.

Setaria and *Filaria,* both tissular parasistes like all filarioids, suggest that several stems evolved separately. Setariinae have circulating microfilariae (first stage larva) but their adult cephalic end possesses a salient ring and often pointed papillae; a considerable multiplication of nuclei of the glandular part of the oesophagus occurs during the second larval stage, and the length of the buccal capsule of infective larvae reaches 17 µm in some species. *Filaria* has a "smooth" head, eggs containing first stage-larva and no circulating microfilariae. The definition of Filarioidea should be: tissular, circulating microfilariae and heamatophagous arthropod vector, glandular oesophagus in the infective stage. This applies well only to Onchocercidae. The tendency for several groups of Spirurida to become tissular, such as in dracunculids transmitted by aquatic crustaceans, has long been known and, not surprisingly, is confirmed with the molecular analysis (Nadler *et al.*, 2007).

Dating the origin of the diverse branches of filarioids is not possible with the molecular methods used presently because the mitochondrial 12S rDNA and *coxI* genes are at saturation for deep evolutionary time (see discussion in Casiraghi *et al.*, 2001). At present, the scenario proposed is based on the host-range and geographical distribution. *Oswaldofilaria*, with two very close species parasitic in crocodiles in South America and in South Africa, is proposed as the first filarioid, 140 mya ago (Bain *et al.*, 1984, in Chabaud & Bain, 1994). However this hypothesis will need a congruence with future molecular datasets based on slow evolving genes.

CONCLUSION

The Filarioidea, although zoologically a small group of nematodes, have been intensively studied and are still worthy of further investigation. They cause several tropical diseases and the filariae of non-human animals are potential sources for zoonoses. Filarioids have acquired a unique mode of transmission among nematodes, with haematophagous arthropods acting as intermediate hosts and in which a degree of larval morphogenesis occurs. At present *Wolbachia* is only known

in this group of nematodes and these endosymbionts are shared with another large group of Ecdysozoa, the arthropods. The genome of the human filaria *Brugia malayi* is now known (Ghedin *et al*., 2007) and demonstrates that new genes appeared and others were lost during the long evolution from free-living ancestors, *e. g. Caenorhabditis elegans* genome. It seems thus unlikely that filarioids will return to a free living life style. Field research on filarial biodiversity undertaken by a worldwide network of collaborators, has given access to new specimens which are informative for the evolution of the filarioids and their wolbachiae endosymbionts. Several species (*L. yutajensis/L. sigmodontis; Cercopithifilaria* spp.) have now been identified which may serve as models to investigate the "mutualistic" relationships at gene level.

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