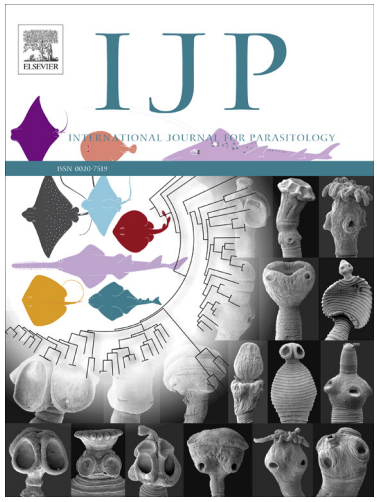

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PII: S0020-7519(17)30083-8
DOI: http://dx.doi.org/10.1016/j.ijpara.2016.12.009
Reference: PARA 3946

To appear in: *International Journal for Parasitology*

Received Date: 26 September 2016
Revised Date: 19 December 2016
Accepted Date: 22 December 2016

Please cite this article as: Lefoulon, E., Giannelli, A., Makepeace, B.L., Mutafchiev, Y., Townson, S., Uni, S., Verocai, G.G., Otranto, D., Martin, C., Whence river blindness? The domestication of mammals and host-parasite co-evolution in the nematode genus *Onchocerca*, *International Journal for Parasitology* (2017), doi: http://dx.doi.org/10.1016/j.ijpara.2016.12.009

Whence river blindness? The domestication of mammals and host-parasite co-evolution in the nematode genus *Onchocerca*★

Emilie Lefoulon[a], Alessio Giannelli[b], Benjamin L. Makepeace[c], Yasen Mutafchiev[d], Simon Townson[e], Shigehiko Uni[f], Guilherme G. Verocai[g], Domenico Otranto[b], Coralie Martin[a,*]

[a] *Unité Molécules de Communication et Adaptation des Microorganismes (MCAM, UMR 7245), Sorbonne Universités, Muséum national d'Histoire naturelle, CNRS, Paris, France*

[b] *Department of Veterinary Medicine, Università degli Studi di Bari, Valenzano, Italy*

[c] *Institute of Infection and Global Health, The University of Liverpool, Liverpool, United Kingdom*

[d] *Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria*

[e] *Tropical Parasitic Diseases Unit, Northwick Park Institute for Medical Research, Harrow, Middlesex, United Kingdom*

[f] *Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia*

[g] *Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA, USA*

*Corresponding author. Coralie Martin, UMR 7245 CNRS, Muséum National d'Histoire Naturelle, 75231 Paris Cedex 05, France. Tel.: +33 1 40793496 ; fax: +33 1 40793499.
E-mail address: cmartin@mnhn.fr

★Note: Nucleotide sequence data reported in this paper are available in GenBank under the accession numbers **KX853314** to **KX853435**i (see Supplementary Table S1).

Note: Supplementary data associated with this article

Abstract

The genus *Onchocerca* includes 34 described species and represents one of the largest genera of the filarial nematodes within the family Onchocercidae. Representative members of this genus are mainly parasites of ungulates, with some exceptions such as *Onchocerca lupi* and *Onchocerca volvulus*, infecting carnivores and/or humans. For a long time, the evolutionary relationships amongst onchocercids remained poorly studied, as the systematics of this genus was impaired by the high morphological variability of species included in the taxon. Although some molecular phylogenies were developed, these studies were mainly focused on bovine *Onchocerca* spp. and *O. volvulus*, including assessments of *Wolbachia* endosymbionts. In the present study, we analysed 13 *Onchocerca* spp. from a larger host spectrum using a panel of seven different genes. Analysis of the *cox*I marker supports its usefulness for the identification of species within the genus. The evolutionary history of the genus has been herein revised by multi-gene phylogenies, presenting three strongly supported clades of *Onchocerca* spp. Analyses of co-evolutionary scenarios between *Onchocerca* and their vertebrate hosts underline the effect of domestication on *Onchocerca* speciation. Our study indicates that a host switch event occurred between Bovidae, Canidae and humans. Cophylogenetic analyses between *Onchocerca* and the endosymbiotic bacterium *Wolbachia* indicate the strongest co-evolutionary pattern ever registered within the filarial nematodes. Finally, this dataset indicates that the clade composed by *O. lupi*, *Onchocerca gutturosa*, *Onchocerca lienalis*, *Onchocerca ochengi* and *O. volvulus* derived from recent speciation.

Keywords: *Onchocerca*, Filariae, Phylogeny, Diagnostic marker, Host-switching, Domestication, *Wolbachia*, Co-evolution

1. Introduction

Onchocerca is one of the largest genera within the family Onchocercidae (Nematoda; Spirurida; Filarioidea), as it includes 34 described species which display a worldwide distribution (Anderson, 2000; Bain et al., 2013; Uni et al., 2015a). Species of *Onchocerca* are mainly associated with various ungulate hosts: *Onchocerca fasciata* was described in the Camelidae, four species were reported in the Suidae, eight species in Cervidae, 15 species in the Bovidae (Cetartiodactyls) and four species in the Equidae (Perissodactyls) (Anderson, 2000; Uni et al., 2001, 2015a). Two exceptions are notable: *Onchocerca lupi* in carnivores and the well-known *Onchocerca volvulus* in humans (Bain, 2002; Bain et al., 2013). This is the agent of onchocerciasis or river blindness, a debilitating human disease that causes cutaneous and ocular clinical manifestations (Anderson, 2000). According to the World Health Organization (WHO), more than 110 million people underwent specific treatment for onchocerciasis in 24 tropical countries in 2014 (WHO, 2015). In addition, during the last 10 years increased attention has been paid to zoonotic *Onchocerca* cases, as the number of such reports has shown a strong upward trend. Currently, more than two dozen zoonotic cases have been documented (Orihel and Eberhard, 1998; Otranto et al., 2015b; Uni et al., 2015b); most of them have been associated with *O. lupi* (Sreter et al., 2002; Otranto et al., 2011, 2012; Eberhard et al., 2012, 2013; Biswas and Yassin, 2013; Ilhan et al., 2013; Mowlavi et al., 2014) or *Onchocerca dewittei japonica* (Beaver et al., 1989; Takaoka et al., 1996, 2001, 2004, 2005; Fukuda et al., 2011; Uni et al., 2010, 2015a), whereas the remaining portion has been linked to the occurrence of *Onchocerca gutturosa* of cattle (Azarova et al., 1965; Siegenthaler and Gubler, 1965; Beaver et al., 1974; Ali-Khan, 1977), *Onchocerca cervicalis* of horses (Burr et al., 1998) and *Onchocerca jakutensis* of cervids (Koehsler et al., 2007).

For a long time, the systematics of the *Onchocerca* genus was muddled, mainly due to the high variability of morphological features of both male and female specimens, such as the

size of spicules or the pattern of caudal papillae (Bain, 1975). In addition, the coexistence of different *Onchocerca* spp. in the same host can impair their identification (Bain, 1975). In 1981, Bain proposed a phylogenetic framework for the genus *Onchocerca* based on morphological traits, host range and geographical distribution (Bain, 1981). Since then, some molecular phylogenies of *Onchocerca* have been proposed, but most of those included a low number of *Onchocerca* spp. (Xie et al., 1994; Casiraghi et al., 2001; McNulty et al., 2012) or had a weak phylogenetic resolution, which did not allow improved definition of the relationships between *Onchocerca* spp. (Sreter-Lancz et al., 2007; Fukuda et al., 2010; Ferri et al., 2011; Lefoulon et al., 2012; Otranto et al., 2015a). More recently, some mitochondrial markers (i.e., NADH dehydrogenase subunit 5 (ND5), 16S and 12S rDNA) were developed for phylogenetic purposes (Krueger et al., 2007; McFrederick et al., 2013). However, the study using these markers only involved *Onchocerca* spp. from the Bovidae and *O. volvulus*. The existence of a close relationship between *O. volvulus* and *Onchocerca* spp. of the Bovidae, particularly with *Onchocerca ochengi*, was nevertheless suggested (Krueger et al., 2007; Eisenbarth et al., 2013; McFrederick et al., 2013). However, the evolutionary relationships of *Onchocerca* spp. from a larger host spectrum still remain poorly known, in particular with regard to the diversity of *Onchocerca* spp. recently described in Japan (Yagi et al., 1994; Uni et al., 2001, 2007, 2015a).

Most *Onchocerca* spp. are infected by *Wolbachia* endosymbiotic bacteria (Casiraghi et al., 2001, 2004; Ferri et al., 2011; Lefoulon et al., 2016). Indeed, the first description of bacteria in the lateral chords and in the female germline of a filarial species was made in *O. volvulus* in 1977 (Kozek and Marroquin, 1977). Within the genus *Onchocerca*, only *Onchocerca flexuosa* (among species screened to date) is not infected by *Wolbachia* (Casiraghi et al., 2004), although the identification of extensive horizontal gene transfer from *Wolbachia* in the *O. flexuosa* genome indicates that even this species once harboured the

symbiont (McNulty et al., 2010a, b). The nature of the association has been demonstrated to be mutualistic for *O. ochengi, Onchocerca lienalis*, *O. gutturosa* and *O. volvulus* (Langworthy et al., 2000; Townson et al., 2000; Hoerauf et al., 2001). Regarding phylogenetic analyses, *Wolbachia* from *Onchocerca* spp. are placed within supergroup C (Bandi et al., 1998). A strong pattern of co-evolution between supergroup C and their onchocercid hosts has been recently highlighted, whereas a localized pattern of co-evolution and horizontal transmission events characterized the other supergroups D, J and F (Lefoulon et al., 2016).

In the present study, we revise the evolutionary history of the genus *Onchocerca* using species from a large host range and a multi-gene phylogeny that we recently developed (Lefoulon et al., 2015), aiming to elucidate the relationships among *Onchocerca* spp. and their host associations.

2. Materials and methods

2.1. Specimens

Thirteen different species of *Onchocerca* were analysed together with *Loxodontofilaria caprini* from serow (Caprinae). *Loxodontofilaria caprini* was included in the study due to previous molecular analyses in which it clustered in the same clade as *Onchocerca* spp. (Bain et al., 2008; Lefoulon et al., 2015). A list of all the studied species and their authorities can be found in the Supplementary Data S1. DNA from adult specimens of *Onchocerca boehmi*, *Onchocerca cervipedis* and *O. lupi*, and from two pools of microfilariae of *O. lienalis*, were extracted specifically for this study (Table 1). All procedures were conducted in compliance with the rules and regulations of the respective competent national ethical bodies. *Onchocerca lupi* from dogs and *O. boehmi* from horses were provided by Dr. Dominico Otranto and no permits were necessary (veterinary procedures). An *O. cervipedis*

specimen from a moose was provided by Dr Guilherme G. Verocai] and was previously studied in Verocai et al. (2012). *Onchocerca lienalis* microfilariae from naturally infected cattle in slaughter houses in southern Wales (UK) were provided by Dr. Simon Townson *Loxodontofilaria caprini*, *Onchocerca eberhardi* and *Onchocerca suzukii* DNA were obtained from previous studies (Lefoulon et al., 2012, 2015). *Onchocerca armillata* and *O. lienalis* DNA were provided by Dr Benjamin L. Makepeace (Table 1).

The adult samples were fixed and kept in 70% ethanol or absolute ethanol. DNA from the *Onchocerca* spp. was extracted using the QIAamp kit following the manufacturer's recommendations (Qiagen, France), with a preliminary step of disruption for two cycles of 30 s at 30 Hz using a TissueLyser II (Qiagen, Germany) followed by overnight incubation at 56°C with proteinase K.

2.2. Molecular screening

The PCR screening of the filarial nematodes was based on the partial sequence of seven genes according to Lefoulon et al. (2015): two mitochondrial genes, 12S rDNA and cytochrome oxidase subunit I (*cox*I); two ribosomal genes, 18S rDNA and 28S rDNA; and three nuclear genes, the myosin heavy chain (*MyoHC*), RNA polymerase II large subunit (*rbp1*), and 70 kilodalton heat-shock protein (*hsp70*). The screening of *Wolbachia* was determined by nested PCR screening of the seven genes according to Lefoulon et al. (2016): 16S rDNA gene, *dnaA*, *coxA*, *fbpA*, *gatB*, *ftsZ* and *groEL*. The PCR products were purified using the SV Wizard PCR Purification Kit (Promega, USA) and directly sequenced. One hundred and twenty-two sequences were deposited in the GenBank Data Library, Accession numbers **KX853314** to **KX853435** (Supplementary Table S1).

2.3. Phylogenetic analyses

Sequences generated during the current study and previously published sequences from draft/complete genomes were aligned using MAFFT (Katoh and Toh, 2008). The alignment of coding genes was translated using EMBOSS Transeq (Li et al., 2015) to check for the absence of stop codons. JModelTest analysis (Posada, 2008) was performed to establish the evolutionary model best adapted to the sequence alignment for each individual gene and for the concatenation of all genes, using the corrected version of the Akaike Information Criterion (AICc) (Supplementary Table S2). A partitioned model was implemented to estimate evolutionary parameters separately for each gene. For the Onchocercidae, rooted phylogenetic trees were created both by Bayesian inference and by Maximum Likelihood (ML) inference using, respectively, MrBayes (Ronquist and Huelsenbeck, 2003) and RaxML (Stamatakis, 2014). For *Wolbachia*, unrooted phylogenetic trees were created by ML inference using RaxML (Stamatakis, 2014). Two runs were performed using five million steps with four chains, with tree sampling every 1,000 generations; the first 1,250 points were discarded as burn-in and Posterior Probabilities were calculated from these post-burn-in trees for the Bayesian analyses. Two runs were performed with 1,000 slow bootstrap replicates for the ML analyses. Independent analyses were performed using the alignments, masking with Gblock version 0.91b (Castresana, 2000) to test the effect of ambiguously aligned positions (Supplementary Fig. S1). Different outgroups were included according to the context: *Icosiella neglecta*, *Oswaldofilaria chabaudi* and *Setaria labiatopapillosa* (Spirurida: Onchocercidae) for analyses focused on the genera *Onchocerca* and *Dirofilaria*; *Filaria latala* (Spirurida: Filariidae) and *Protospirura muricola* (Spirurida: Spiruridae) for analyses including all other Onchocercidae (Supplementary Fig. S2).

2.4. Filarial coxI gene analysis

A DNA barcoding approach based on the *cox*I marker was used to discriminate between *Onchocerca* spp. (Ferri et al., 2009; Lefoulon et al., 2012). The *cox*I sequence divergence is estimated by the number of base differences per site between two sequences (p-distance) using MEGA version 6. Pairwise comparisons between 59 *cox*I sequences were processed and classified into two levels: intraspecific (differences between individuals of the same species) and interspecific (differences between individuals of different species).

2.5. Immunohistochemical staining of nematode sections

The presence of *Wolbachia* was determined in an *O. lupi* specimen by immunohistochemical staining according to Kramer et al. (2003). A rabbit polyclonal antiserum raised against the *Wolbachia* surface protein (WSP) of *Wolbachia* from *Brugia pahangi* (Wol-Bp-WSP, dilution 1:2000, designed by Bazzocchi et al. (2000) and provided by Dr. Maurizio Casiraghi, Università degli Studi di Milano Bicocca, Italy) was used to stain 5 µm paraffin sections of filarial species placed on Superfrost Plus slides (Thermo Scientific, United-States) as previously described (Ferri et al., 2011). Sections were counterstained with H&E. Sections of a laboratory strain of *Litomosoides sigmodontis* were used as a positive control.

2.6. Cophylogenetic analysis

Two cophylogenetic analyses were performed: the first one to evaluate co-evolutionary scenarios between *Onchocerca* parasites and their vertebrate hosts (Table 1,

Supplementary Table S3) and the second one to evaluate the global fit between *Onchocerca* spp. and their *Wolbachia* symbionts.

Jane 4.0 (Conow et al., 2010) was used to associate overall costs of co-evolutionary scenarios between *Onchocerca* spp. and their vertebrate hosts. This event-based method was used with the default settings for cost regimes as follows: a "co-speciation" event (two partners speciate simultaneously) is associated with null cost; a "duplication" event (the symbionts speciate in the same host), "loss" event (the symbiont does not speciate while the host does) and a "failure to diverge" (when a host speciates while the parasite does not but remains on both new host species) event are associated with a cost equal to one; and a "duplication then host-switching" event (the symbiont speciates and one switches to another host) is associated with a cost equal to two (Charleston, 1998). All analyses were performed with a number of generations of 5,000 and a population of 500. The Jane program manages topologies and not distance branches, so the hypothetical topology of vertebrate hosts was built on previous analyses (Scientists, 2009; Song et al., 2012; Bibi, 2013). Two different datasets were analysed: the first including associations with sampled vertebrate hosts for this study, and the second including associations with the totality of the known vertebrate host spectrum.

The global-fit method was used to study cophylogenetic patterns between filariae and their *Wolbachia* symbionts. The global fit of filarial phylogeny with their bacterial phylogeny was estimated using the PACo application (Balbuena et al., 2013) in the R environment (R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.). The differences between matrices of principal coordinates (PCo) (based on matrices' pairwise patristic distance) of the onchocercid nematodes species and their *Wolbachia* symbionts were minimized by Procrustes analysis using least-squares superimposition. An ordination plot was produced, representing the global

fit. The global fit was evaluated by the residual sum of squares value (m^2_{XY}) of the Procrustean fit calculation, which is inversely proportional to the topological congruence, and its significance was tested by random permutations (100,000,000 permutations). Each host-symbiont association was evaluated by a jack-knife procedure to estimate the square residual of each single association and its 95% confidence interval. A bar chart plot of these jack-knifed squared residuals was produced. Low residuals are interpreted as a low contribution of m^2_{XY} and thus as a strong congruence between the filariae and the bacteria. Two different datasets were analysed: the whole onchocercid nematodes and their symbionts, or a cluster of species belonging to *Dirofilaria*, *Onchocerca* and *Loxondotofilaria* and their symbionts.

3. Results

3.1. Accuracy of coxI for identification of Onchocerca spp.

The *cox*I mean nucleotide distance within *Onchocerca* spp. is 0.53% (S.E. = 0.29%; range = 0-7.06%) and between *Onchocerca* spp. is 9.47% (S.E. = 1.84%; range = 0.78-13.73%). There is an overlap between the distributions of intraspecific and interspecific distances between 2% and 4.5% (Fig. 1). However, some pair comparisons are inconsistent with this threshold. Firstly, pair comparisons of three species exhibit nucleotide distances lower than 2% (Table 2): *O. ochengi* and *O. volvulus*; *O. ochengi* and *Onchocerca* sp. "Siisa"; and *O. volvulus* and *Onchocerca* sp. "Siisa". Secondly, two specimens of *O. lienalis* are characterized by a nucleotide distance higher than 4.5% (Table 2).

Regarding *O. lupi*, two populations were observed with a *cox*I mean nucleotide distance of 1.59% (S.E. = 0.78%; range = 1.57%-1.96%) (Fig. 2). The first population includes adult specimens from the USA (88YTD to 88YTF) and presents *cox*I sequences identical to those of *O. lupi* previously collected in the USA (Labelle et al., 2011, 2013;

Hassan et al., 2015; Otranto et al., 2015a), in Romania (Tudor et al., 2016), in Greece (Sreter-Lancz et al., 2007; Mutafchiev et al., 2013) and in Turkey (Otranto et al., 2011). These specimens are also closely related to specimens collected in Hungary (Egyed et al., 2002) with a *cox*I nucleotide distance of 0.37%. The second population includes specimens collected in Portugal (88YTA and 88YTB) and presents *cox*I sequences identical to those of *O. lupi* previously collected in Portugal (Sreter-Lancz et al., 2007, Mutafchiev et al., 2013).

3.2. Molecular phylogeny of the genus Onchocerca

The phylogenetic trees confirm that the 13 *Onchocerca* spp. including *L. caprini* form a monophyletic group (Fig. 3, Supplementary Figs. S1, S2). The phylogenetic analyses reveal three clades among the *Onchocerca* spp. (Fig. 3, Supplementary Fig. S1). The first clade includes six species: *O. cervipedis*, *O. suzukii*, *L. caprini*, *O. boehmi*, *O. armillata* and *O. dewittei japonica* (Fig. 3). Within this clade, *O. suzukii* is closely related to *O. armillata*, and *L. caprini* to *O. dewittei japonica*. However, the relationships between the different species of this clade are weakly supported if we take into account all of the available onchocercid sequences (Supplementary Fig. S2). The second clade is composed of *O. skrjabini*, *O. eberhardi* and *O. flexuosa*, in which *O. skrjabini* is a sister group of the two others (Fig. 3). Finally, the third clade is composed of *O. lupi* of carnivores, *O. gutturosa*, *O. linealis* and *O. ochengi* of domestic bovids, and *O. volvulus* of humans (Fig. 3). The phylogenetic analyses indicate that *O. ochengi* and *O. volvulus* spp. are derived species in this clade. The relationship between the three clades remains unresolved due to the weak phylogenetic resolution (Fig. 3, Supplementary Figs. S1, S2).

3.3. Onchocerca-host associations

We compared two datasets: first, the parasites with the vertebrate hosts in which they were recovered, and second, the parasites with their known vertebrate host spectrum (Fig. 4). The event-based method estimated 12 co-evolutionary scenarios associated with the lowest cost for the first dataset, grouped into three isomorphic solutions, versus 78 co-evolutionary scenarios, grouped into 15 isomorphic solutions, for the more extensive dataset. For each dataset, the different isomorphic solutions exhibit the same pattern of co-speciation with the exception of the equid parasite *O. boehmi,* likely derived from a host-switch from a different lineage (either the Caprinae or the Bovinae). Our results underline two main groups of *Onchocerca* spp.: on one hand, *Onchocerca* spp. adapted to cervid hosts - and antilocaprid hosts for the larger dataset - (with *Onchocerca skjrabini*, *O. flexuosa*, *O. eberhardi* and *O. cervipedis*); and on the other hand, *Onchocerca* spp. adapted to domestic bovines, humans and carnivore hosts (with *O. lupi*, *O. gutturosa*, *O. lienalis*, *O. ochengi* and *O. volvulus*) (Fig. 4). The two sets of analyses display some disparities. Most of the co-evolutionary scenarios suggest that the common ancestor of *Onchocerca* spp. would be adapted to parasitism in the common ancestor of the Bovidae, the Cervidae and the Antilocapridae (Fig. 4A). However, the analysis based on the larger dataset shows alternative scenarios with the same cost for an older adaptation to the common ancestor of the Bovidae, the Cervidae, the Antilocapridae, the Felidae and the Canidae (Fig. 4B). In addition, the first dataset (Fig. 4A), but not the larger dataset (Fig. 4B), indicates that *O. lupi* could have emerged from a host switch from the ancestor of *Onchocerca* spp., those infecting the Bovinae. Interestingly, the two types of analyses present the following similarities (Fig. 4): i) host association of *O. armillata* would be derived from an independent acquisition, different from the other domestic bovine parasites, probably by host switching from cervids; ii) host association of *O. dewittei japonica* and *O. suzukii* would derive from a more recent host switch with the ancestor of *L. caprini* or

O. armillata; iii) the infection by *O. volvulus* would derive from a more recent host switch with the lineage of domestic bovine parasites into humans.

3.4. Co-evolution between Onchocerca spp. and their endosymbiont, Wolbachia

Sequences of *Wolbachia* symbionts were produced for our specimens of *O. armillata*, *O. suzukii, O. eberhardi*, *L. caprini*, *O. lupi* and *O. cervipedis* in which infection was already documented (Townson et al., 2000; Egyed et al., 2002; Neary et al., 2010; Ferri et al., 2011; McFrederick et al., 2013) and for the newly examined *O. boehmi* specimens. The presence of *Wolbachia* in hypodermal lateral chords and intra-uterine embryos was observed on immunostained sections of *O. lupi* (Supplementary Fig. S3). The phylogenies place *Wolbachia* from *Onchocerca* and *Loxondotofilaria* spp. as a monophyletic group belonging to the supergroup C *Wolbachia* (Supplementary Fig. S4). Comparing bacterial phylogenies with filarial phylogenies using a global-fit analysis reveals a global co-evolution between the two partners (PACo: $m^2_{XY} = 0.239$, $P < 0.001$). The cophylogenetic global-fit between the three genera *Dirofilaria*, *Onchocerca* and *Loxodontofilaria* and their *Wolbachia* symbionts (supergroup C) is even stronger than for the other associations (PACo: $m^2_{XY} = 0.005$, $P < 0.001$; ParaFit: ParaFitGlobal=0.00129, $P = 0.0007$) (Figs. 5A, 6A). The associations between *Dipetalonema* spp. and their *Wolbachia* symbionts (supergroup J) are the only ones to show a similar co-evolutionary pattern. The global-fit analysis performed on the cluster *Dirofilaria*, *Onchocerca* and *Loxodontofilaria* shows three different subgroups: one with the associations between *Dirofilaria* spp. and their symbionts; one with *O. dewittei japonica*, *O. boehmi*, *O. cervipedis*, *O. suzukii*, *O. armillata, O. skrjabini, O. eberhardi* and *L. caprini* and their symbionts, and one with *O. gutturosa*, *O. lupi*, *O. lienalis*, *O. ochengi* and *O. volvulus*

(Fig. 5B) and their symbionts; these associations in this last subgroup are characterized by the lowest squared residual values, which reflect strong co-evolution (Fig. 6B).

4. Discussion

Identification of *Onchocerca* spp. using *cox*I as a molecular marker is accurate as previously indicated for other filarial species (Ferri et al., 2009). More specifically, intraspecific distances between most of the studied species are lower than 2% and interspecific distances are higher than 4.5%. In the case of *O. lupi*, specimens from different isolates exhibited the strongest genetic intraspecific variability (1.57% to 1.96%), revealing two subpopulations as previously suggested (Labelle et al., 2013); one does not seem to follow a geographical pattern, while the other consists only of specimens from Portugal (Sreter-Lancz et al., 2007; Mutafchiev et al., 2013; Otranto et al., 2015a). However, two clusters of *Onchocerca* spp. are not clearly identified by the *cox*I analysis. Firstly, the one composed of *O. ochengi*, *O. volvulus* and *Onchocerca* sp. "Siisa": the characterization of *Onchocerca* sp. "Siisa" specimens was exclusively based on molecular analyses (i.e. *cox*I, 12S rDNA, 16S rDNA or *ND5*) and although these specimens constitute a clade (Krueger et al., 2007; Ferri et al., 2009; Eisenbarth et al., 2013), the data do not support the existence of a proper species. In addition, detection of mixed infections of *Onchocerca* sp. "Siisa" and *O. ochengi* in the same *Simulium* flies, as well as the presence of the two "species" in the same nodule (Eisenbarth et al., 2013), in conjunction with their genetic similarity, suggest that they are likely to be a single species. Therefore, a revision of the taxonomic position of *Onchocerca* sp. "Siisa" with morphological data combined with molecular data is essential in the future. Regarding *O. volvulus* and *O. ochengi* specimens, the morphology of microfilariae or infective larvae of both species is indistinguishable and adults stages share very similar

morphology (Bain, 1975; Denke and Bain, 1978; Bain and Chabaud, 1986). However, morphology of the female cuticle (Bain, 1975; Bain et al., 1976a), host specificity (Ferri et al., 2009) and L3 size distributions (McCall et al., 1992) allow the separation of *O. volvulus* and *O. ochengi*. Whether this morphological difference of the cuticle is due to adaptations to the host species needs to be addressed.

Second, there is the cluster with the different specimens of *O. lienalis*. The two specimens identified as *O. lienalis* do not form a monophyletic group (Figs. 1, 3), and none of the sequences is closely similar to another *Onchocerca* sp. (Fig. 1). Additional sequences for *O. lienalis* in public databases are only available for 12S rDNA and support a high genetic variability between the specimens identified as *O. lienalis* (mean 4.36%; range: 0.74% to 10.37%) (Supplementary Table S4). More specifically, the specimen 98YT appears more closely related to other specimens of *O. lienalis* than the specimen 413YU. It is interesting to note that the microfilarial specimens of *Onchocerca* sp. previously isolated from deer in the USA (McFrederick et al., 2013) are more closely related to several *O. lienalis* specimens (such as 98YT specimens) than *O. lienalis* specimens are between themselves (Supplementary Table S4). Surprisingly, this divergence was not previously discussed, and as molecular analyses were mainly based on microfilarial identification, a misidentification may have occurred. Taken together, if we consider *cox*I as an accurate identification marker, these two exceptions may be due to either a misidentification of samples, a mismatch in entries in the databases, or an incorrect delimitation of species including cryptic species (in cases where morphological analyses have been sufficiently thorough).

Our multi-locus phylogeny shows three strongly supported clades of *Onchocerca* spp., and this is the first known time that the phylogenetic resolution is sufficient to identify two of them (Fig. 3). Indeed, previous analyses were either based on a single gene and had low phylogenetic resolution (Sreter-Lancz et al., 2007; Fukuda et al., 2010; Ferri et al., 2011;

Lefoulon et al., 2012; McFrederick et al., 2013; Otranto et al., 2015a), or the *Onchocerca* species sampling was too narrow (mainly focused on bovine and human parasites) (Krueger et al., 2007). The first clade pulls together six species: *O. cervipedis*, *O. boehmi*, *O. dewittei japonica*, *O. armillata*, *O. suzukii* and *L. caprini*, confirming that *L. caprini* should be included within the *Onchocerca* genus as previously suggested (Bain et al., 2008; Lefoulon et al., 2015). *Loxodontofilaria caprini* is morphologically close to *O. suzukii* (Uni et al., 2006), although it presents some morphological traits characteristic of the genus *Loxodontofilaria* (e.g., a complex vagina, well-developed oesophagus and caudal lappets) (Bain et al., 1982). However, the taxonomic status of this species would need to be revised, especially as no males of *Loxodontofilaria* spp. (apart from *L. caprini*) have been described (Bain et al., 1982; Uni et al., 2006), depriving this genus of essential morphological criteria for systematics. The second clade groups together *O. eberhardi*, *O. flexuosa* and *O. skjrabini*. This close relationship was neither suggested by molecular nor morphological analysis previously (Uni et al., 2007).

The third clade collates five species: *O. lupi*, *O. gutturosa*, *O. lienalis*, *O. ochengi* and *O. volvulus*. Unlike the two other clades, some evolutionary relationships were previously identified such as *O. gutturosa* being sister to *O. volvulus*, *O. ochengi* and *O. lienalis* (Morales-Hojas et al., 2006; Krueger et al., 2007), or *O. volvulus* being closely related to parasites of African Bovidae, in particular *O. ochengi* (Bain, 1981). Our results now underline that *O. lupi* is also sister to the set *O. volvulus*, *O. ochengi*, *O. lienalis* and *O. gutturosa*.

Although we clearly identified three *Onchocerca* clades, our phylogenetic analyses do not allow us to determine which clade diverged early. A common misinterpretation of the phylogenetic trees is to associate an isolated taxon, which is positioned as a sister group of the other taxa, as so-called “independent basal lineages” (Krell and Cranston, 2004, Crisp and Cook, 2005). Indeed, there are at least two sister groups for every node of the phylogenetic

tree, and the group including the lowest number of species is often misinterpreted as being basal (Crisp and Cook, 2005). For example, *O. flexuosa* and *O. armillata* were previously identified as a sister-group of other *Onchocerca* spp. and they were described as "independent basal lineages" of the genus (Krueger et al., 2007). However, our current analysis with a larger sampling of *Onchocerca* spp. shows that these species belong to two different clades and none can be interpreted as 'early diverging'.

To decipher the evolutionary relationships between these clades, we compared the *Onchocerca* phylogeny with the evolutionary hypotheses made on morphological traits which were selected for their phyletic value in the *Onchocerca* genus (Bain et al., 1976b; Bain, 1981) (Fig. 7, Supplementary Table S5). Such a comparison is challenging due to the disparities in morphological descriptions (such as *O. cervipedis* (Wehr and Dikmans, 1935; Caballero, 1945; Yagi et al., 1994)), lack of information on certain taxa, and the bias in the interpretation of these hypotheses depending on the authors. Nevertheless, taking into account only the morphology, a cluster composed by *O. volvulus*, *O. ochengi*, *O. lienalis* and *O. gutturosa* presents mainly morphological traits considered derived (e.g., rectangular disposition of head papillae, undivided or weakly divided oesophagus, posterior position of vulva, complex female cuticle and reduced number of caudal papillae (Bain, 1981)) (Fig. 7). Conversely, most of the morphological traits described as an ancestral character state are associated with species belonging to the two other clades *e.g. O. armillata*, *O. suzukii* and *O. flexuosa* (Fig. 7) (Bain and Schulz-Key, 1974; Bain, 1975; Yagi et al., 1994). Interestingly, although our phylogenetic analysis shows that *O. skrjabini* is closely related to *O. flexuosa* and *O. eberhardi*, this species presents many morphological traits described as derived character states. However, morphological descriptions of *O. skrjabini* show variability (Bain and Schulz-Key, 1974; Yagi et al., 1994) (Fig.7). Thus, the combination of molecular and morphological data show that the speciation within the clade composed of *O. volvulus*, *O.*

ochengi, *O. lienalis*, *O. gutturosa* and *O. lupi* would be more recent than speciation which led to the two other clades.

To further elucidate the evolutionary relationships between *Onchocerca* groups, we performed cophylogenetic analyses between *Onchocerca* spp. and their vertebrate hosts. Co-speciation between *Onchocerca* spp. and their vertebrate hosts was not supported, although multiples events of host switching were identified as previously suggested (Krueger et al., 2007) (Fig. 4). Cophylogenetic analyses clearly supported an independent speciation in cervid/antilocaprid hosts on one hand, and in domestic bovine, canid and felid hosts on the other hand. Indeed, parasites of domestic bovines, canids, felids and humans seem to be derived from the same lineage (with the exception of *O. armillata*), suggesting an effect of domestication in the host switch. More specifically, a host switching event between domestic bovine and canid/felid hosts and another event between domestic bovines and humans appears to have occurred. This supports the hypothesis that the human parasite *O. volvulus* would have been derived from an ancestral bovine parasite, most likely in Africa (Bain, 1981; Krueger et al., 2007). As domestication of *Bos taurus* may have occurred in the Near-East 10,100–37,600 years ago (Troy et al., 2001), acquisition of *O. volvulus* would have to be very recent. In addition, it has been suggested that the domestication of cattle in Africa (especially sub-Saharan Africa) occurred later (4,000-1,500 years ago) (Marshall and Hildebrand, 2002). Intolerance of human patients to *O. volvulus* microfilariae is commonly reported and it could be associated with a suboptimal, rather recent adaptation to their human hosts (Bain, 1981). The co-speciation analyses also present a host switching event between carnivores and domestic bovines, but do not clearly determine whether this event followed the route from cattle to carnivores or vice-versa. However, the first scenario appears more biologically parsimonious, as all the other *Onchocerca* spp. infect ungulates. Regarding *O. lupi*, it infects not only domestic animals, as it was originally described from a wolf, *Canis lupus cubanensis*

(Rodonaja, 1967). Gravid females were identified in cases from wolves (Rodonaja, 1967), dogs (Mutafchiev et al., 2013) and cats (Labelle et al., 2011), suggesting that they all represent the definitive host of *O. lupi*. The infection in dogs and cats might be more recent; thus the adaptation of *O. lupi* with their different carnivore hosts could be related to domestication (respectively, estimated around 15,000-12,500 years ago for the dog (Frantz et al., 2016) and 11,000–4,000 years ago for the cat (Driscoll et al., 2007, 2009)). Moreover, it is interesting to note that *O. armillata*, a parasite of domestic bovines, *O. boehmi*, a parasite of domestic equids, and *O. dewittei japonica*, a parasite of wild boar, could be derived from an independent acquisition from wild fauna. In particular, the host association of *O. armillata* appears to have derived from a host switching event between the Cervidae and the Bovinae, while for *O. dewittei japonica*, the putative host switch was from the Caprinae to the Suidae.

The emergence of the *Onchocerca* genus in Africa has been dated back to the Pleistocene period based on morphological characters (Bain, 1981). This hypothesis was sugested because a significant number of *Onchocerca* spp. were described in the continent and *O. raillieti*, a parasite of the domestic donkey, harbours what are considered to be the most ancestral morphological traits (Bain et al., 1976b; Bain, 1981). More precisely, it has been hypothesised that two independent *Onchocerca* lineages may have evolved in Africa: one emerging from an ancestral speciation in forested regions, and one derived from savanna regions which switched to the human host, leading to *O. volvulus* speciation (Chabaud and Bain, 1994). Data presented herein do not support such a geographical pattern for the evolution of *Onchocerca* spp. (Supplementary Fig. S5). However, our sampling only includes a few *Onchocerca* spp. mainly restricted to Africa (other than *O. volvulus* and *O. ochengi*), and multiple lineages may have evolved in Africa as previously suggested (Chabaud and Bain, 1994). In addition, the ancestral speciation of *Onchocerca* was hypothesized to be related to the ancestral speciation of horses and donkeys (Bain, 1981), which may have

occurred in the Pliocene (4 - 4.5 million years ago) (Orlando et al., 2013). However, *O. boehmi* is not ancestrally derived. The cophylogenetic analyses do not allow determination of some strongly supported parsimonious host switching events which could have led to *O. boehmi* speciation. However, it seems that independent host switching events occurred in equids. Our results suggest a primary association with the Bovidae and the Cervidae, and would support a Eurasian origin where diversification for both groups has occurred (Petronio et al., 2007; Bibi, 2013).

In agreement with previous studies (Plenge-Bonig et al., 1995; Determann et al., 1997; Bandi et al., 1998; Henkle-Duhrsen et al., 1998; Neary et al., 2010; Ferri et al., 2011; Lefoulon et al., 2016), 15 out of the 16 *Onchocerca* spp. analysed in our study harboured *Wolbachia* from supergroup C, *O. flexuosa* being ancestrally infected but now aposymbiotic. The global-fit analyses clearly indicate that the associations between *Onchocerca* spp. and their *Wolbachia* symbionts have the strongest co-evolutionary pattern of all the filariae-*Wolbachia* associations, as previously suggested (Lefoulon et al., 2016); and within *Onchocerca* spp., the clade composed of *O. lupi*, *O. gutturosa*, *O. lienalis*, *O. volvulus* and *O. ochengi* shows the strongest co-evolutionary pattern with their *Wolbachia* symbionts. Furthermore, *Wolbachia*-like gene transcripts and peptides were detected in adult *O. flexuosa* worms, suggesting that perhaps the ancestral function of the symbiont is maintained in this species (McNulty et al., 2013). Recently, it has been underlined that genomes of the endosymbiotic *Wolbachia* from *Diroflaria immitis* and from *O. ochengi*, both within supergroup C, present similarly reduced genomes (with a low number of insertion sequence elements or genomic rearrangements), which are characteristic of an ancient relationship with their filarial hosts (Comandatore et al., 2015). This further supports the strong co-evolutionary pattern between these species and their *Wolbachia* symbionts.

To conclude, we have identified three clades of *Onchocerca* spp., and identify *L. caprini* as an *Onchocerca* sp. Thus, this species should be transferred to *Onchocerca* with the following new combination: *Onchocerca caprini* (Uni & Bain, 2006) n. comb. The genus *Loxodontofilaria* also needs to be revised. The clade with *O. cervipedis*, *O. boehmi*, *O. dewittei japonica*, *L. caprini*, *O. suzukii* and *O. armillata* is the most diverse regarding their host range (the Antilocapridae, the Cervidae, the Bovidae, the Equidae and the Suidae). Host switching events clearly occurred into new host groups. The clade composed of *O. eberhardi*, *O. flexuosa* and *O. skrjabini* includes the only known *Wolbachia*-free species. Finally, the clade with *O. volvulus*, *O. ochengi, O. lienalis*, *O. gutturosa* and *O. lupi* is mainly composed of parasites of domestic animals or humans. The process of domestication in bovines, dogs and cats is likely to have contributed to host switching events that led to speciation within this clade. Interestingly, the acquisition of *O. volvulus* in humans from domestic bovines could be very recent and related to this process of domestication. Multi-locus phylogeny, combined with morphological data and co-evolutionary analyses of either filariae and their vertebrate hosts, or filariae and their *Wolbachia* symbionts, indicate that this clade was probably derived from a more recent speciation than the other two clades.

Acknowledgements

This work was supported the Interdisciplinary Programs of the Muséum National d'Histoire Naturelle (MNHN), France, (ATM Taxonomie moléculaire: DNA Barcode et gestion durable des collections). This work was partly supported by the Ministry of Higher Education, Malaysia (FRGS EP020-2012) to Shigehiko Uni. The authors have declared that no competing interests exist.

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Legends to Figures

Fig. 1. Comparison of nucleotide sequence divergences in the cytochrome oxidase subunit I (*cox*I) gene among 19 *Onchocerca* spp. Pairwise comparisons between 59 *cox*I sequences are separated into two categories: differences between individuals of the same species and differences between individuals of different species. The divergence between sequences is estimated by the number of base differences per site between two sequences (p-distance) using MEGA version 6.

Fig. 2. Bayesian phylogram based on cytochrome oxidase subunit I (*cox*I) gene sequences from 80 onchocercid specimens including 19 *Onchocerca* spp. The total length of datasets is 632 bp. *Loxodontofilaria caprini*, *Dirofilaria immitis* and *Dirofilaria repens* were included in addition to *Onchocerca* spp. The topology was inferred using Bayesian inference. Nodes are associated with Bayesian posterior probabilities based on one run of five million generations. Bayesian posterior probabilities inferior to 0.70 are not shown. Countries of collection are indicated by a flag for *Onchocerca lupi* specimens. The scale bar indicates the number of nucleotide substitutions. Newly sequenced specimens are in bold.

Fig. 3. Phylogeny of *Onchocerca* genus based on partitioned concatenated datasets of seven markers. Analysis is based on 12S rDNA, cytochrome oxidase subunit I (*cox*I), RNA polymerase II large subunit (*rbp1*), heat shock protein (*hsp70*), myosin heavy chain (*myoHC*), 18S rDNA and 28S rDNA sequences. The total length of the datasets is approximately 4,600 bp. Twenty-four onchocercid specimens (representing 20 species) were analysed. *Oswaldofilaria chabaudi, Icosiella neglecta* and *Setaria labiatopapillosa* were used as outgroups. The topology was inferred using Bayesian inference. Nodes are associated with

Bayesian posterior probabilities based on one run of five million generations (in black). An independent run is processed using Maximum Likelihood inference. Nodes are associated with Bootstrap values based on 1,000 replicates (in grey). The scale bar indicates the number of nucleotide substitutions. The host vertebrate family (or subfamily) for each filarial species is indicated using the specified symbols. Newly sequenced specimens are in bold.

Fig. 4. Parsimonious co-evolutionary reconstructions between *Onchocerca* spp. and their vertebrate hosts using an event-based method. (A) Co-evolutionary reconstructions by an event-based method with the vertebrate hosts from which the filarial specimens were recovered. Three different isomorphic solutions (representing 12 scenarios) associated with the lowest cost (= 17) were established. (B) Co-evolutionary reconstructions by an event-based method with the whole known vertebrate host spectrum. Eight different isomorphic solutions (representing 83 scenarios) associated with the lowest cost (= 34) were established. The presented co-evolutionary scenario represents the majority of all the less costly scenarios. *Loxodontofilaria caprini*, *Dirofilaria immitis* and *Dirofilaria repens* were included in addition to *Onchocerca* spp. The event-based method was performed with the default settings for cost regimes ("co-speciation" event = 0 cost; a "duplication" event = 1; "loss" event = 1; "duplication then host switching" event = 2) using Jane 4.0 (Conow et al., 2010). All analyses were performed with a number of generations of 5,000 and a population of 500.

Fig. 5. Procrustean superimposition plot of *Wolbachia* and their filarial host phylogenies. Representative plot of a Procrustes superimposition analysis which minimizes differences between the two partners' principal correspondence coordinates of patristic distances. For each vector, the starting point represents the configuration of *Wolbachia* and the arrowhead

the configuration of filarial hosts. The vector length represents the global fit (residual sum of squares) which is inversely proportional to the topological congruence. (A) Analysis of co-evolution between 67 filariae specimens and their *Wolbachia* symbionts (only 44 filariae harbouring *Wolbachia*). (B) Analysis of co-evolution between *Onchocerca*, *Dirofilaria* and *Loxondotofilaria caprini* spp. and their *Wolbachia* symbionts.

Fig. 6. Contribution of each *Wolbachia*-filaria association to a general pattern of co-evolution. Each bar represents a Jack-knifed squared residual and error bars represent upper 95% confidence intervals from applying PACo to patristic distances. *wb*, *Wolbachia*. (A) Analysis of co-evolution between 67 filariae of which 44 specimens were infected, and their *Wolbachia* symbionts. (B) Analysis of co-evolution between *Onchocerca*, *Dirofilaria* and *Loxondotofilaria caprini* spp. and their *Wolbachia* symbionts.

Fig. 7. Graphical representation of morphological traits and comparison with molecular cladogram. The cladogram of evolutionary history of *Onchocerca* spp. (with *Loxodontofilaria caprini* sp.) is shown. The species *Onchocerca raillieti* is included because it is thought to present a mostly ancestral state of morphological characters (Bain et al., 1976b), but its phylogenetic position remains hypothetical (represented by a dashed grey line). Hypothetical cladograms based on morphological traits are presented. Six different morphological traits are compared with the molecular phylogeny: i) the head papillae: a squared pattern of labial or cephalic papillae represents an ancestral state, whereas a laterally or dorsoventrally elongated rectangle is interpreted as a derived state (Chabaud, 1955); ii) the oesophageal morphology: clearly divided with well-distinct muscular and glandular portions represents an ancestral state, whereas undivided (without distinct portions) is defined as a derived state (Anderson,

1957); and a poorly divided oesophagus characterized by ill-defined muscular and glandular portions was classified as an intermediate state; iii) the position of the vulva: an anterior position (ratio of the distance from anterior end to the vulva/length of oesophagus < 0.5) represents an ancestral state, while a position near to the oesophagus-intestine junction (ratio close to 1) is defined as a derived state (Anderson, 1957); and a vulva situated at the mid-length of the oesophagus was classified as an intermediate state; iv) the female somatic-musculature at mid-body: a well-developed musculature was considered an ancestral state while weakly-developed musculature is defined as a derived state (Bain, 1981); v) the presence of external ridges of the female cuticle: a striation without ridges represents an ancestral state, whereas the presence of prominent ridges is defined as a derived state (Bain et al., 1976b; Bain, 1981), and undulations or fine ridges on the female cuticle were classified as intermediate; vi) the caudal papillae of males: none or weak reduction of caudal papillae number (10 to nine) was associated with the ancestral state, while a strong reduction of caudal papillae number (seven pairs) is defined as a derived state (Chabaud and Petter, 1961). Species with eight caudal papillae were classified as intermediate.

Supplementary data Legends

Supplementary Fig. S1. Phylogeny of the *Onchocerca* genus based on partitioned concatenated datasets of 12S rDNA, cytochrome oxidase subunit I (*coxI*), RNA polymerase II large subunit (*rbp1*), heat shock protein (*hsp70*), myosin heavy chain (*myoHC*), 18S rDNA and 28S rDNA sequences masking with Gblock. The alignments of genes 12S rDNA, 18S rDNA, 28S rDNA and *hsp70* sequences were masked using Gblock version 0.91b (Castresana, 2000) to remove the effect of ambiguously aligned positions. The total length of the datasets is approximately 4,140 bp. Twenty-four onchocercid specimens (representing 20 species) were analysed. *Oswaldofilaria chabaudi, Icosiella neglecta* and *Setaria labiatopapillosa* were used as outgroups. The topology was inferred using Bayesian inference. Nodes are associated with Bayesian posterior probabilities based on one run of five million generations (in black). An independent run was processed using Maximum Likelihood inference. Nodes are associated with Bootstrap values based on 1,000 replicates (in grey). The scale bar indicates the number of nucleotide substitutions. Newly sequenced specimens are in bold.

Reference

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Supplementary Fig. S2. Phylogeny of the Onchocercidae based on partitioned concatenated datasets of 12S rDNA, cytochrome oxidase subunit I (*coxI*), RNA polymerase II large subunit (*rbp1*), heat shock protein (*hsp70*), myosin heavy chain (*myoHC*), 18S rDNA and 28S rDNA

sequences. The total length of the datasets is approximately 4,870 bp. Sixty-seven onchocercid specimens (representing 54 species) were analysed. *Filaria latala* and *Protospirura muricola* were used as outgroups. The topology was inferred using Bayesian inference. Nodes are associated with Bayesian posterior probabilities based on one run of five million generations. The scale bar indicates the number of nucleotide substitutions. The onchocercid clades are indicated as ONC1 to ONC5 according to Lefoulon et al., (2015). Newly sequenced specimens are in bold.

Reference

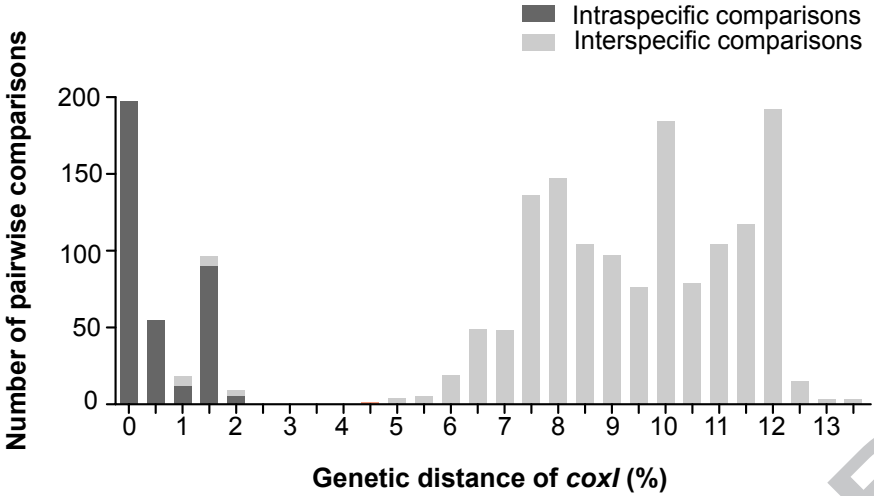
Lefoulon, E., Bain, O., Bourret, J., Junker, K., Guerrero, R., Canizales, I., Kuzmin, Y., Satoto, T.B., Cardenas-Callirgos, J.M., de Souza Lima, S., Raccurt, C., Mutafchiev, Y., Gavotte, L., Martin, C., 2015. Shaking the Tree: Multi-locus Sequence Typing Usurps Current Onchocercid (Filarial Nematode) Phylogeny. PLoS Negl Trop Dis 9, e0004233.

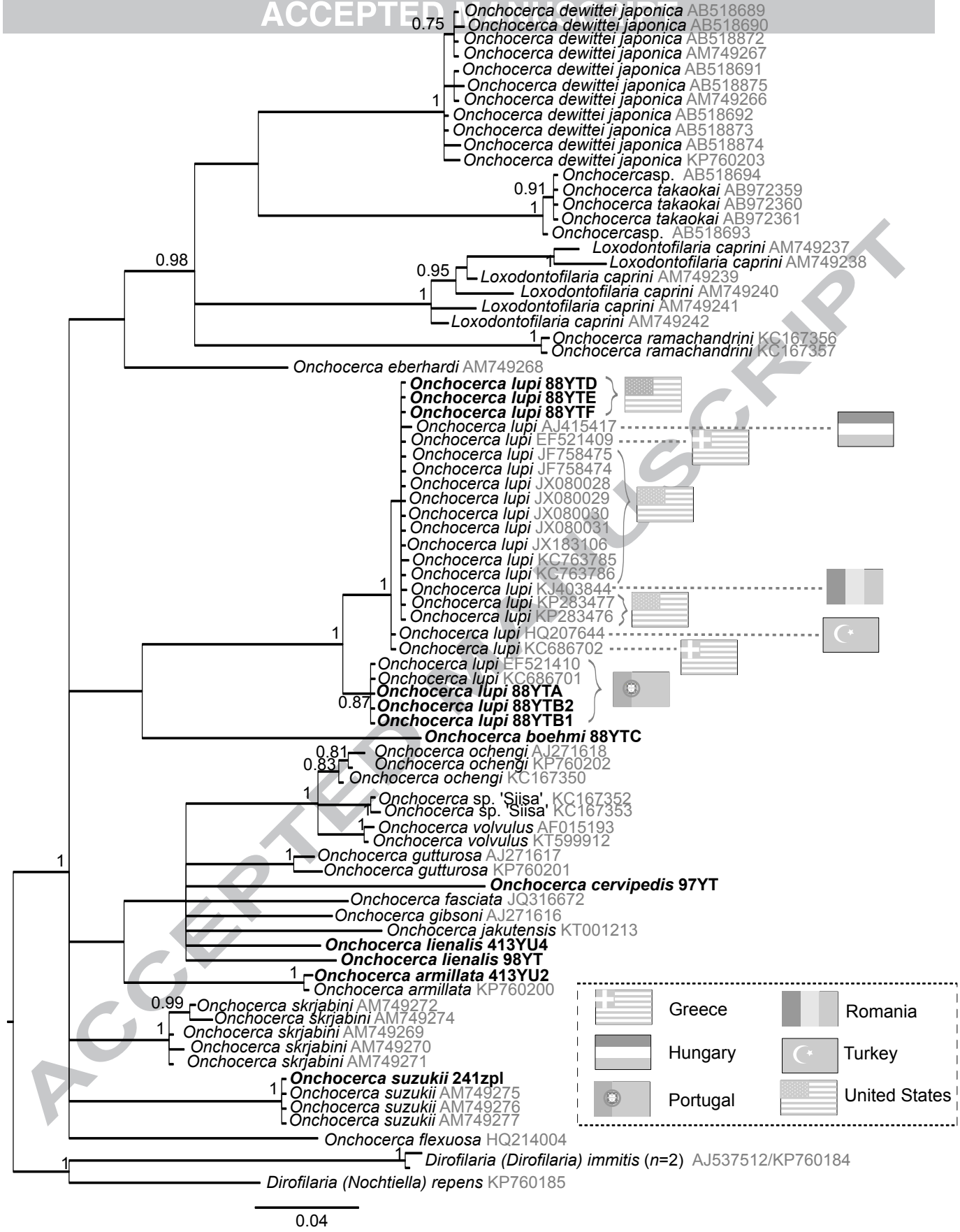
Supplementary Fig. S3. *Wolbachia* immunostaining of a *Onchocerca lupi* female. Sections of *O. lupi* female specimens were stained with a rabbit polyclonal antiserum against *Wolbachia* Surface Protein (WSP) of *Brugia pahangi Wolbachia* (Wol-Bp-WSP, dilution 1:2,000). A) Section of the entire female. B) Focus on uterus and hypodermal lateral chords. Presence of *Wolbachia* (small red dots) is indicated by an arrow. U, uterus; c, cuticle; h, hypodermal lateral chords; m, muscles. Hypodermal lateral chord delimited by stars; *, lateral plane. Scale bars: A 100 µm and B 40 µm.

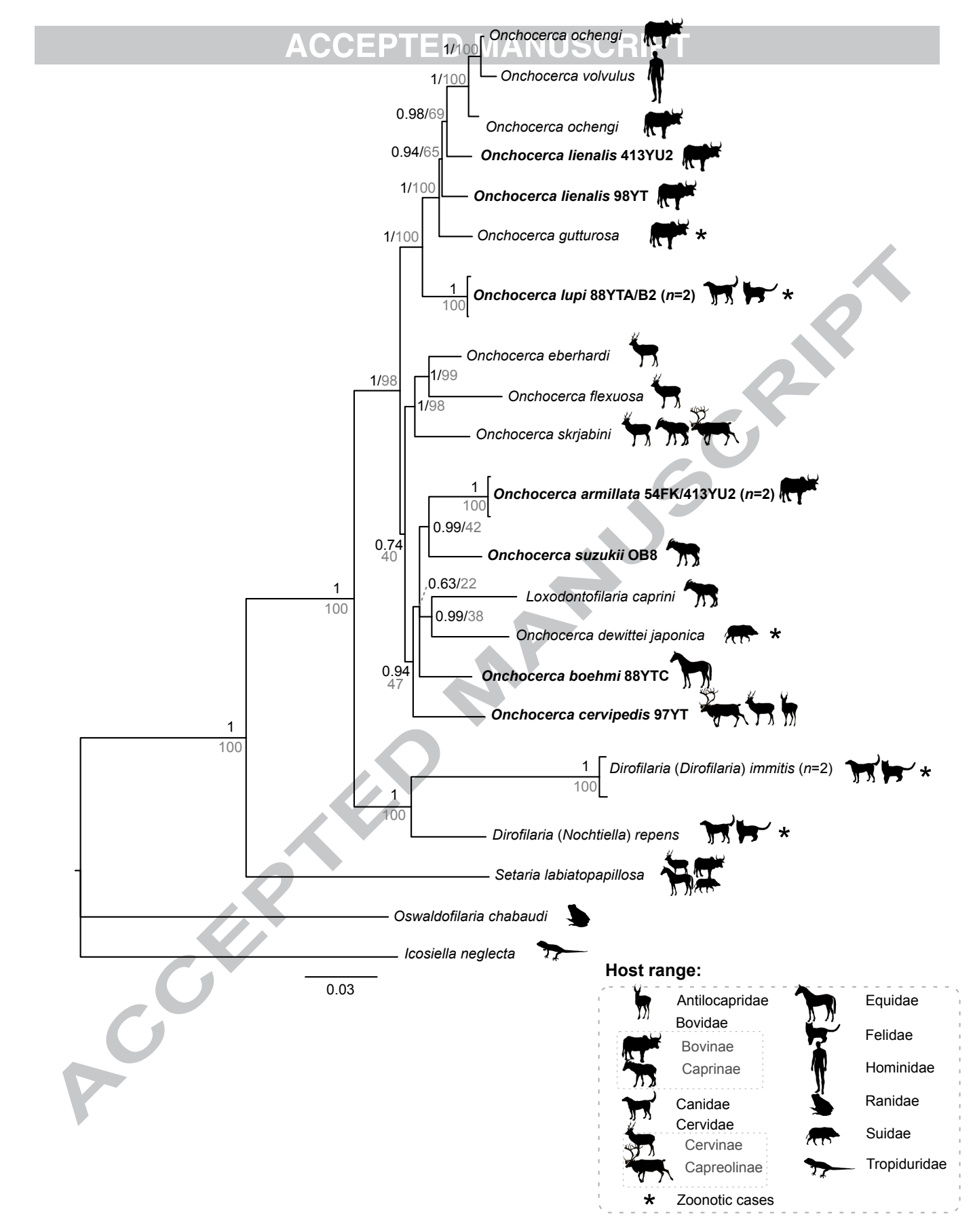
Supplementary Fig. S4. Phylogenetic trees of *Wolbachia* based on seven markers by Maximum Likelihood (ML). (A) Phylogenetic tree of *Wolbachia* restricted to supergroup C.

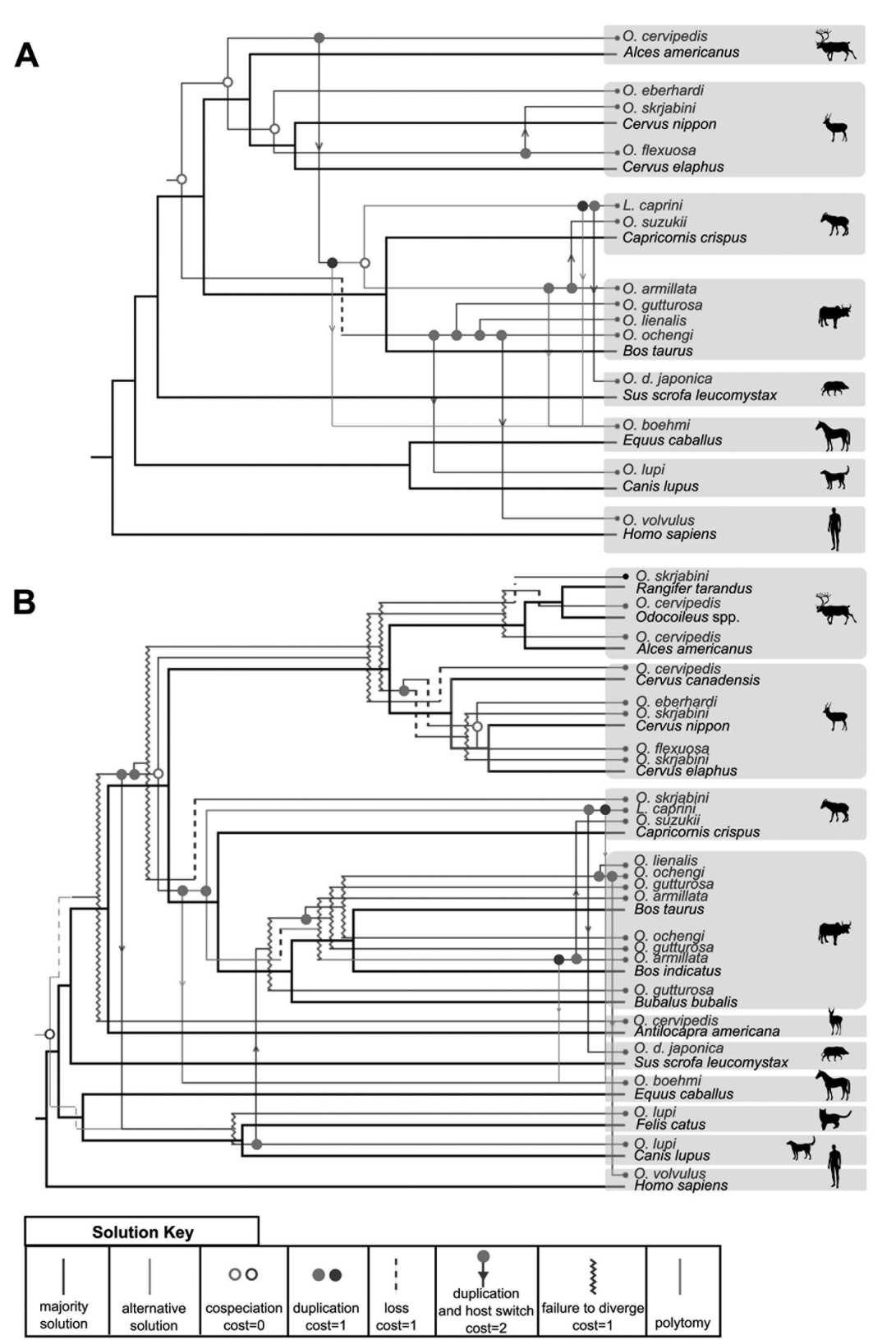
Twenty *Wolbachia* strains were analysed, including strains from 12 different *Onchocerca* spp. *Wolbachia* from *Dirofilaria* spp. were used as outgroups. (B) Phylogenetic tree of *Wolbachia* from filariae including 44 *Wolbachia* strains with strains from 12 different *Onchocerca* spp. Analyses based on concatenation of 16S rDNA, *dnaA*, *groEL*, *ftsZ*, *coxA*, *fbpA* and *gatB*. The total length of the datasets is approximately 4,170 bp. The topology was inferred using ML inference using RaxML. Nodes are associated with Bootstrap values based on 1,000 replicates. Bootstrap values below 70 were not shown. The scale bar indicates the number of nucleotide substitutions. Newly sequenced specimens are in bold. *wb*, *Wolbachia*; .

Supplementary Fig. S5. Phylogeny of the *Onchocerca* genus based on partitioned concatenated datasets of 12S rDNA, cytochrome oxidase subunit I (*coxI*), RNA polymerase II large subunit (*rbp1*), heat shock protein (*hsp70*), myosin heavy chain (*myoHC*), 18S rDNA and 28S rDNA sequences with an indication of geographical distribution. The total length of datasets is approximately 4,600 bp. Twenty-four onchocercid specimens (representing 20 species) were analysed. *Oswaldofilaria chabaudi, Icosiella neglecta* and *Setaria labiatopapillosa* were used as outgroups. The topology was inferred using Bayesian inference. Nodes are associated with Bayesian posterior probabilities based on one run of five million generations (in black). An independent run was processed using Maximum Likelihood (ML) inference. Nodes are associated with Bootstrap values based on 1,000 replicates (in grey). The scale bar indicates the number of nucleotide substitutions. Newly sequenced specimens are in bold. The known geographical distribution for each filarial species is indicated using the specified coloured symbols: green for Neartic; red for Palearctic; dark purple for Neotropic; orange for Afrotropic; yellow for Australasia and brown for Indomalaya.





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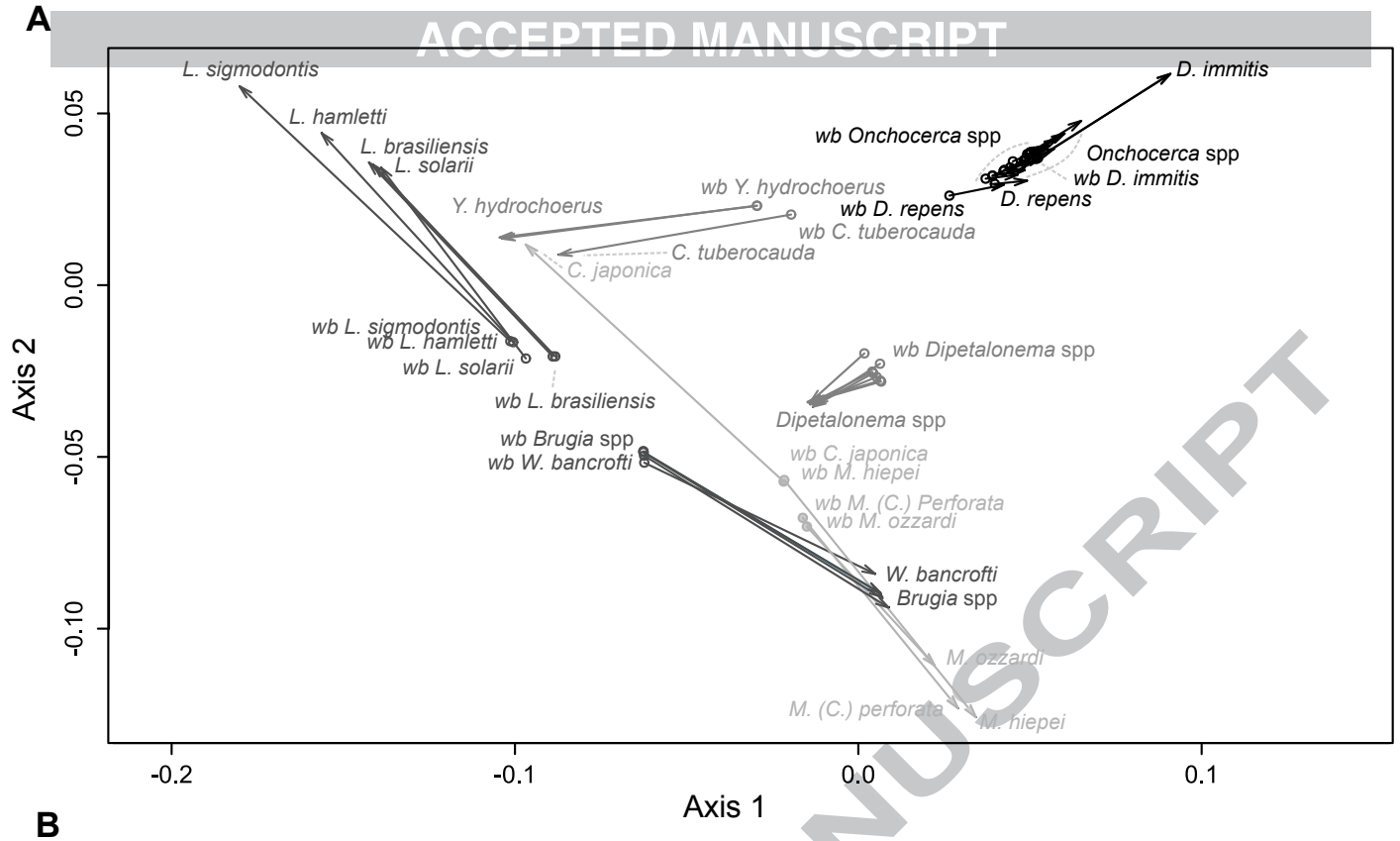
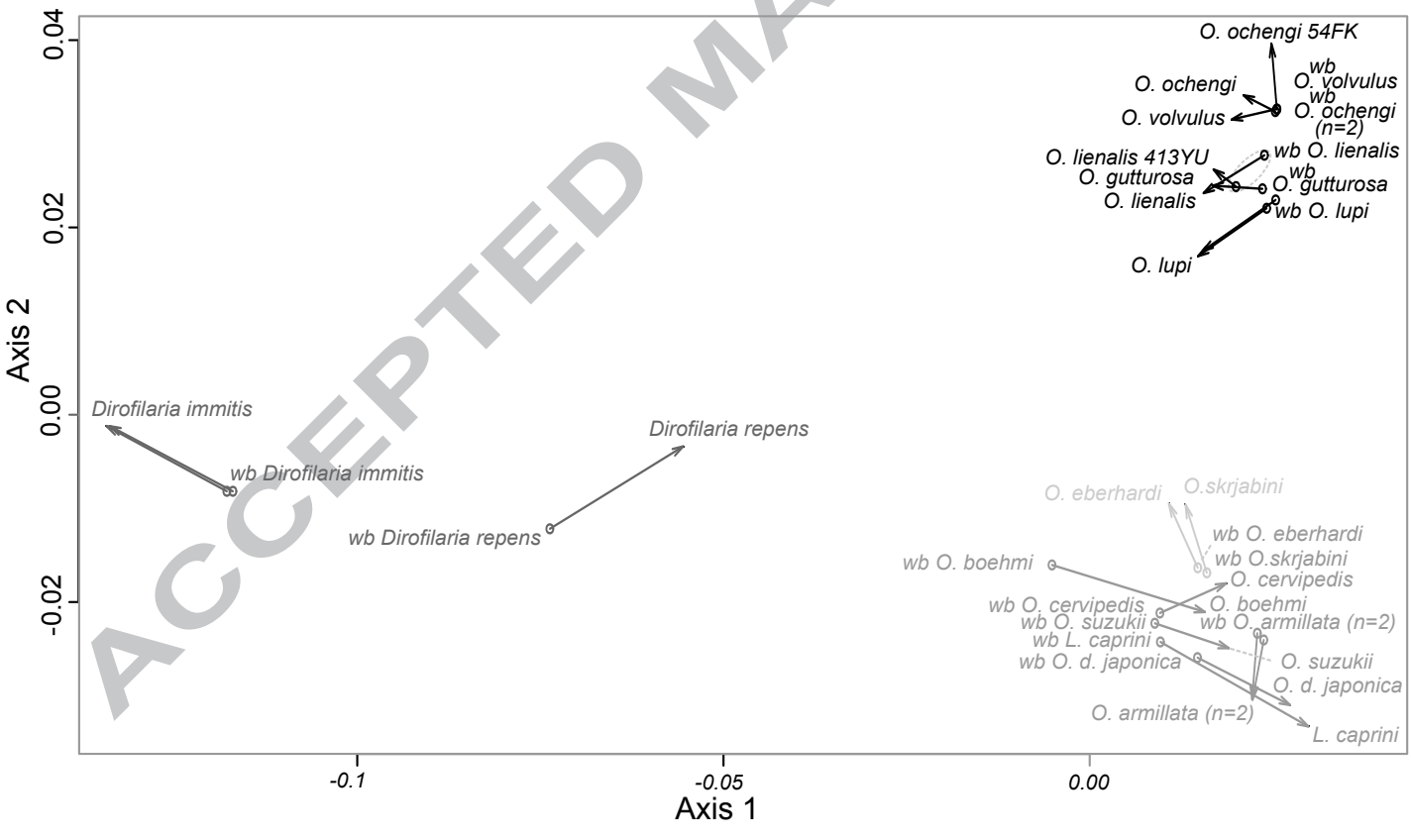
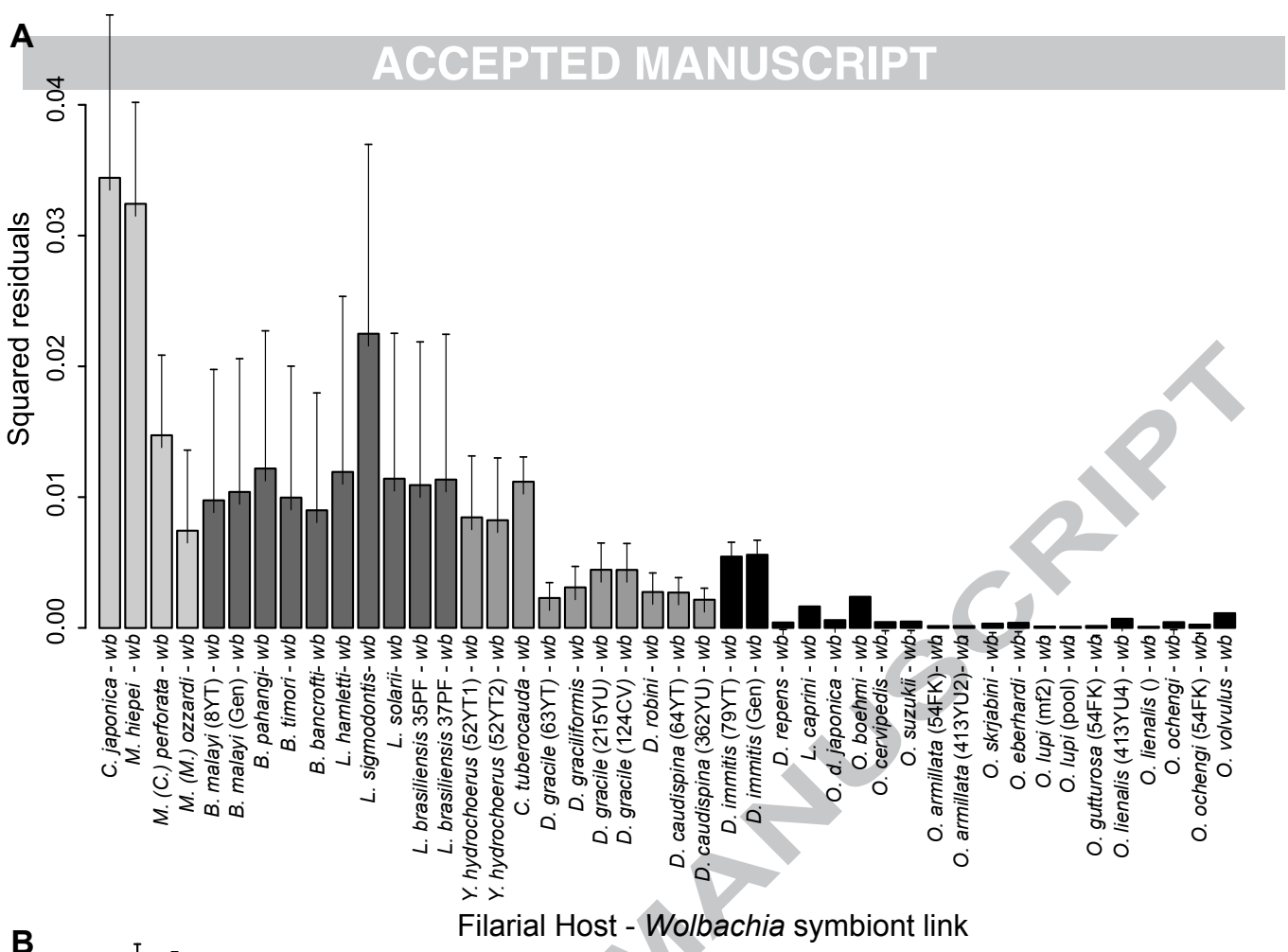
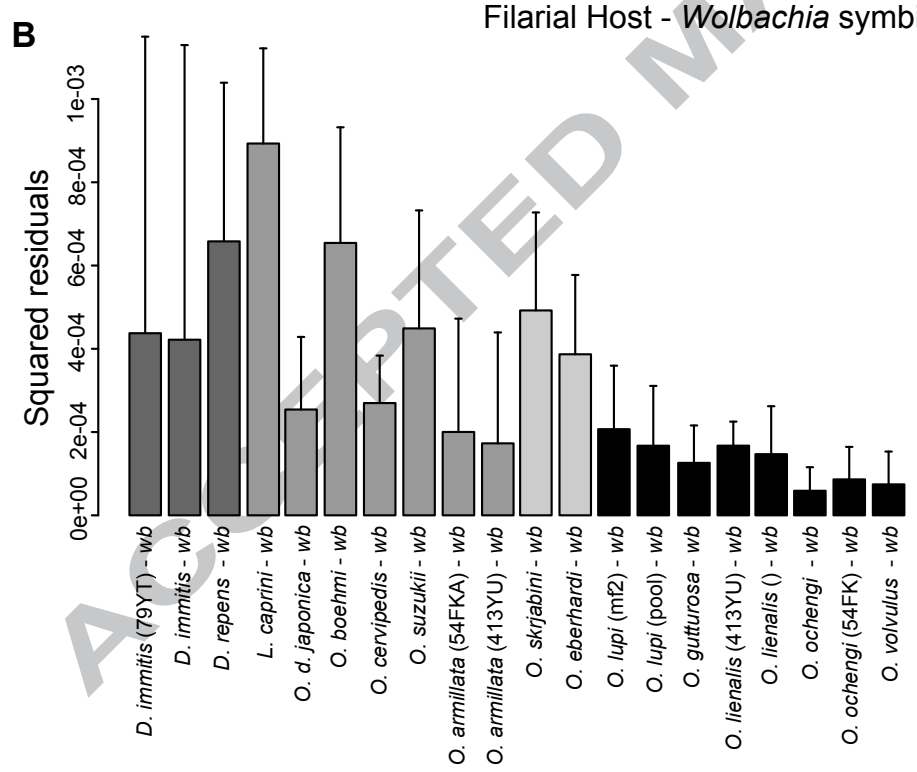
O. cervipedis
Alces americanus
O. eberhardi
O. skrjabini
Cervus nippon
O. flexuosa
Cervus elaphus
L. caprini
O. suzukii
Capricornis crispus
O. armillata
O. gutturosa
O. lienalis
O. ochengi
Bos taurus
O. d. japonica
Sus scrofa leucomystax
O. boehmi
Equus caballus
O. lupi
Canis lupus
O. volvulus
Homo sapiens
B
O. skrjabini
Rangifer tarandus
O. cervipedis
Odocoileus spp.
O. cervipedis
Alces americanus
O. cervipedis
Cervus canadensis
O. eberhardi
O. skrjabini
Cervus nippon
O. flexuosa
O. skrjabini
Cervus elaphus
O. skrjabini
L. caprini
O. suzukii
Capricornis crispus
O. lienalis
O. ochengi
O. gutturosa
O. armillata
Bos taurus
O. ochengi
O. gutturosa
O. armillata
Bos indicatus
O. gutturosa
Bubalus bubalis
O. cervipedis
Antilocapra americana
O. d. japonica
Sus scrofa leucomystax
O. boehmi
Equus caballus
O. lupi
Felis catus
O. lupi
Canis lupus
O. volvulus
Homo sapiens
Solution Key
majority solution
alternative solution
cospeciation cost=0
duplication cost=1
loss cost=1
duplication and host switch cost=2
failure to diverge cost=1
polytomy









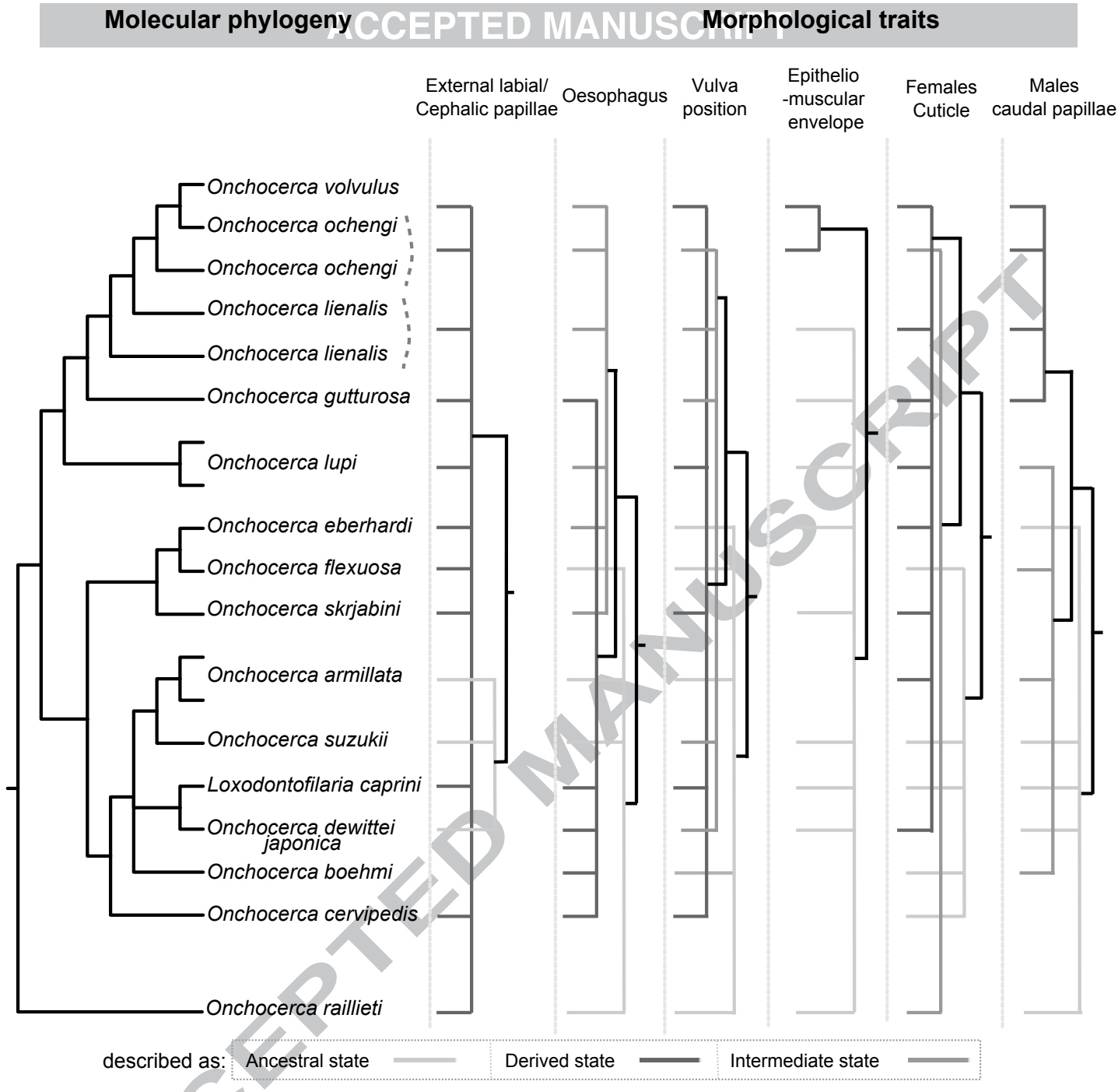


Table 1. Filarial nematode specimens for which new molecular and/or histological analyses were performed.

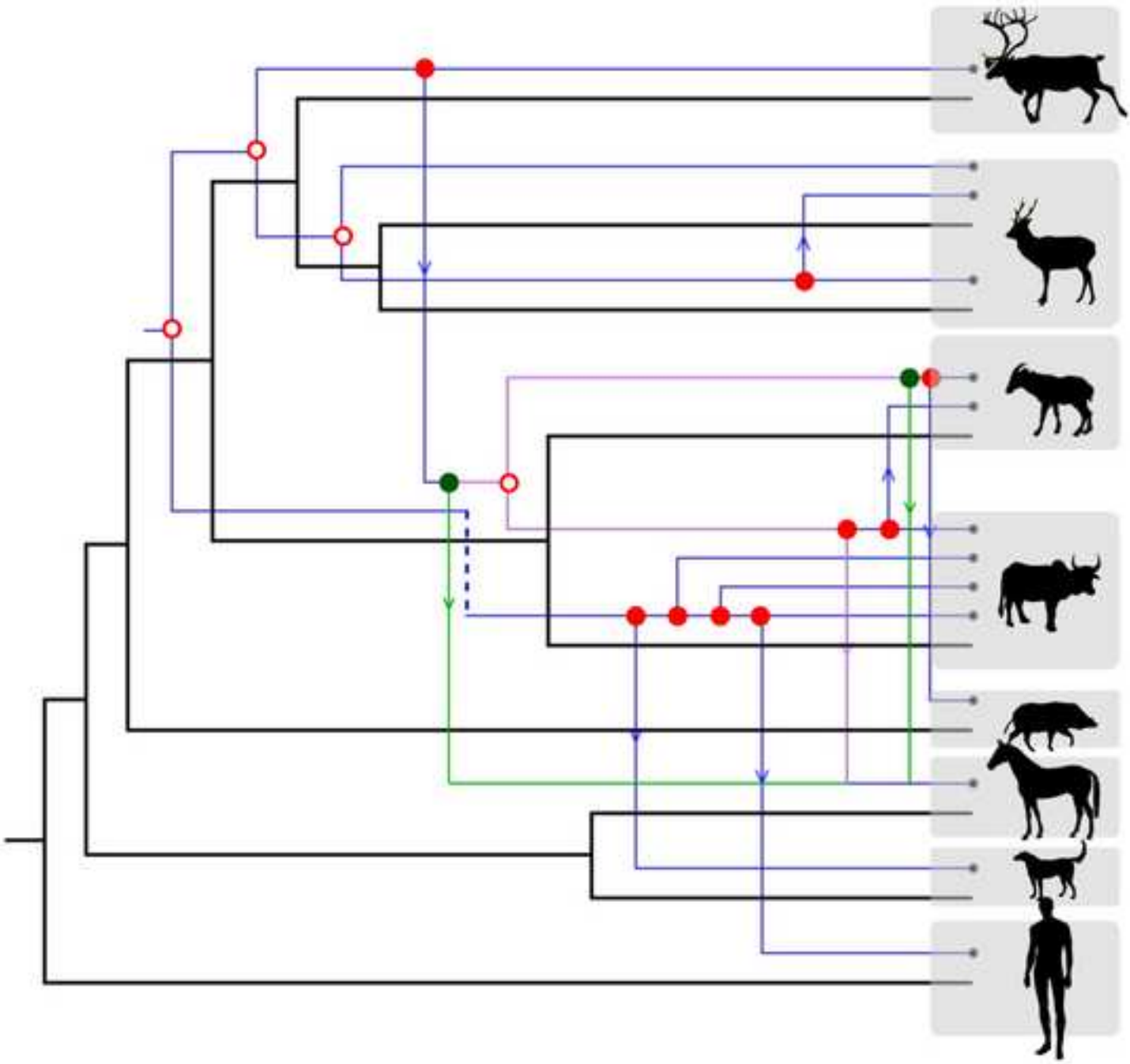
Species name, author and date	Host	MNHN N°	Collection place	Source
Loxodontofilaria caprini Uni & Bain, 2006	*Capricornis crispus*	YG2-58	Japan	DNA
Onchocerca armillata Railliet & Henry, 1909	*Bos taurus*	413YU2	Cameroon	DNA
Onchocerca boehmi (Supperer, 1953)	*Equus caballus*	88YT	Italy	adult
Onchocerca cervipedis Wehr & Dickmans, 1935	*Alces americanus*	97YT	Canada	adult
Onchocerca eberhardi Uni & Bain, 2007	*Cervus nippon*	S63-5	Japan	DNA
Onchocerca lienalis (Stiles, 1892)	*Bos taurus*	413YU4	Wales	DNA
		98YT	Wales	microfilariae
Onchocerca lupi Rodonaja, 1967	*Canis lupus familiaris*	88YTA (n=1)	Portugal	adult
		88YTB (n=2)	Portugal	microfilariae
		88YTD/E/F (n=3)	United States	adults
Onchocerca suzukii Yagi, Bain & Shoho, 1994	*Capricornis crispus*	S63-8	Japan	DNA

MNHN, Muséum National d'Histoire Naturelle

Table 2. List of cytochrome oxidase subunit I (*cox*I) inconsistent pairwise comparisons between *Onchocerca* specimens. The distance of the pairwise comparison estimated with the number of base differences per site between two sequences (p-distance); S.E. associated with the estimated distance using MEGA version 6. These comparisons are inconsistent with the estimated overlap between the distributions of both intraspecific and interspecific distances between *Onchocerca* spp. (between 2% and 4.5%). The listed interspecific pairwise comparisons (*Onchocerca* sp. 'Siisa', *Onchocerca volvulus* and *Onchocerca ochengi*) are associated with a lower distance than the estimated overlap. The listed intraspecific comparisons (*O. lienalis*) are associated with a higher distance than the estimated overlap.

Comparisons between sequences	***Onchocerca volvulus* AF015193**	***O. volvulus* KT599912**	***Onchocerca* sp. 'Siisa' KC167352**	***Onchocerca* sp. 'Siisa' KC167353**	***Onchocerca lienalis* 98YT**
Onchocerca ochengi KC167350	0.78 ± 0.55%	0.78 ± 0.55%			
O. ochengi AJ271618	1.18 ± 0.68 %	1.18 ± 0.68 %			
O. ochengi KP760202	1.18 ± 0.68 %	1.18 ± 0.68 %			
O. ochengi KC167350			1.57 ± 0.78%	1.57 ± 0.78%	
Onchocerca sp. 'Siisa' KC167352	1.57 ± 0.78%	1.57 ± 0.78%			
Onchocerca sp. 'Siisa' KC167353	1.57 ± 0.78%	1.57 ± 0.78%			
O. lienalis 413YU					7.06 ± 1.60%

*Graphical Abstract (for review)



Highlights

- *coxI* is a suitable marker for the identification of *Onchocerca* spp.
- Multi-gene phylogeny reveals three strongly supported clades of *Onchocerca*.
- Recent host switch events between Bovidae, Canidae and humans are observed.
- Potential role for the domestication of cattle in *Onchocerca* speciation.
- Cophylogenetic analyses of *Onchocerca*/*Wolbachia* show the strongest coevolution.