Journal of Helminthology (2006) **80,** 281–290 DOI: 10.1079/JOH2005331

Molecular systematics of five *Onchocerca* species (Nematoda: Filarioidea) including the human parasite, *O. volvulus*, suggest sympatric speciation

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Abstract

The genus *Onchocerca* (Nematoda: Filarioidea) consists of parasites of ungulate mammals with the exception of *O. volvulus*, which is a human parasite. The relationship between *O. volvulus*, *O. ochengi* and *O. gibsoni* remains unresolved. Based on morphology of the microfilariae and infective larvae, vector transmission and geographical distribution, *O. ochengi* and *O. volvulus* have been placed as sister species. Nevertheless, the cuticle morphology and chromosomal data (*O. volvulus* and *O. gibsoni* have n = 4 while *O. ochengi* is n = 5) suggest that *O. gibsoni* could be more closely related to *O. volvulus* than *O. ochengi*. Sequences from the 12S rRNA, 16S rRNA and ND5 mitochondrial genes have been used to reconstruct the phylogeny of five *Onchocerca* species including *O. volvulus*. Analyses with maximum likelihood and maximum parsimony showed that *O. ochengi* is the sister species of *O. volvulus*, in accordance with the classification based on morphology and geographical location. The separate specific status of the species *O. gutturosa* and *O. lienalis* was supported, although their phylogenetic relationship was not well resolved. The analyses indicated that the basal species was *O. gibsoni*, a South-East Asian and Australasian species, but this result was not statistically significant. The possible involvement of sympatric speciation in the evolution of this group of parasites is discussed.

Introduction

The genus *Onchocerca* (Nematoda: Filarioidea: Onchocercidae) consists of 28 parasitic species (Chabaud & Bain, 1994) which, with one exception, infect ungulate mammals. The exception is *O. volvulus*, the causative agent of human onchocerciasis or 'river blindness', which has no known wild animal reservoir (Crosskey, 1990). The parasites of ungulates cause lesions which can affect animal health and diminish the value of carcasses (Muller, 1979). Human onchocerciasis is a severely debilitating disease widespread in West, Central and East Africa, and it is also present in smaller foci in Yemen and Latin America (Duke, 1990). The number of infected people is over 17 million, of whom more than 500,000 are visually impaired (Hoerauf *et al.*, 2003). The importance of this disease led in 1974 to the creation of a major World Health Organization (WHO)-sponsored programme (The Onchocerciasis Control Programme) to eliminate blindness in West Africa by vector control and its success subsequently resulted in the creation of two more

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programmes, one for Africa (The African Programme for Onchocerciasis Control) and another in America (The Onchocerciasis Elimination Programme in the Americas) both of which aim to eliminate the disease by community-wide chemotherapy.

Research into *O. volvulus* has been very extensive (for recent reviews see Burnham, 1998; Hoerauf *et al.*, 2003). The use of cattle models for treatment research and the development of markers that differentiate *O. volvulus* from the cattle parasites transmitted by the same vector species of blackflies (Diptera: Simuliidae), has been of major interest (Muller, 1979; Copeman, 1993). The presumed close taxonomic relationship between *O. volvulus*, *O. gibsoni* and *O. ochengi* (Bain, 1981) has stimulated the use of the two cattle parasites as model species for research into human onchocerciasis treatment and detection (Vankan *et al.*, 1988; Garate *et al.*, 1991; Trees *et al.*, 2000). However, the taxonomic relationships between *O. volvulus*, *O. ochengi* and *O. gibsoni* are controversial and remain to be clarified. Bain (1981, 2002) saw *O. ochengi* as the sister species of *O. volvulus* based on the morphology of the microfilariae and infective larvae, vector transmission (both species are transmitted by the same species of simuliids) and overlapping geographical distribution of the two species. On the other hand, Muller (1979, 1983) considered *O. gibsoni* to be closer to *O. volvulus* based on the annulation structure of the cuticle, and this appears to be supported by karyotype similarities. *Onchocerca ochengi* has a chromosome complement of $n = 5$ while both *O. volvulus* and *O. gibsoni* have a reduced karyotype of $n = 4$ with supernumerary (B) chromosomes (Post *et al.*, 1989). Also, there are some old records of the presence of *O. gibsoni* in Africa that could cast doubt on the zoogeographical evidence, although Bain & Beveridge (1979) consider that these identifications are likely to be erroneous.

Two further species parasitizing cattle in Europe and North America, *O. gutturosa* and *O. lienalis*, have been studied extensively (Muller, 1979) and in the past their validity as species has been controversial. Differences in morphology had previously been considered to be the result of adaptations to different sites within hosts, rather than reflect species differences (Eichler, 1973), but Bain *et al.* (1978) from a careful reconsideration of their morphology concluded that they were two differentiated species. Genetic evidence presented by Flockhart (1982) and Andrews *et al.* (1989) indicated fixed allelic differences between the two species in 35% of 23 enzyme loci.

Apart from taxonomic studies (Bain, 1981), few studies on the evolutionary history and systematics of the genus have been published (e.g. Chabaud & Bain, 1994; Bain, 2002). Xie *et al.* (1994) included three *Onchocerca* species (*O. volvulus*, *O. ochengi* and *O. gutturosa*) in their phylogenetic analysis of filarial parasites. Their results suggested that *O. gutturosa* was basal to *O. volvulus* and *O. ochengi*. Zimmerman *et al.* (1994), in a study of the evolutionary history of *O. volvulus* using the O-150 repetitive DNA sequence, concluded that the American strain of *O. volvulus* had been introduced from the savannah region of West Africa. Bandi *et al.* (1998) and Casiraghi *et al.* (2001) estimated the phylogeny of some filarial parasites in relation to that of their *Wolbachia* endosymbionts using mitochondrial DNA sequences (part of the COI gene) including *O. volvulus*, *O. ochengi*, *O. gibsoni* and *O. gutturosa*. The relationships within the *Onchocerca* branch were not very well resolved and, depending on the phylogenetic method used, the position of *O. gutturosa* and *O. gibsoni* changed (they were either shown as sister species or basal to the other species). Using mitochondrial 12S rRNA gene sequences, however, Casiraghi *et al.* (2004) obtained a phylogeny in which *O. gutturosa* and *O. gibsoni* formed a well supported sister clade to that formed by *O. volvulus* and *O. ochengi* independently of the method used. A recent molecular characterization study of a parasite ascribed to the genus *Onchocerca* (and found infecting dogs) along with its *Wolbachia* endosymbionts included a phylogenetic analysis of partial COI sequences of *O. volvulus*, *O. ochengi*, *O. gibsoni*, *O. gutturosa* and the '*Onchocerca* sp.' recovered from dogs (Egyed *et al.*, 2002). The monophyly of these species was not well supported (52% bootstrap support), with the dog '*Onchocerca* sp.' as the most distinct species, and *O. gibsoni* being basal, but with no bootstrap support, to the monophyletic clade *O. volvulus*-*O. ochengi*. It is not clear whether this '*Onchocerca* sp.' is a new species or an aberrant infection of *O. lienalis* (Eberhard *et al.*, 2000).

Impoverishment of the morphology of the Onchocercidae and the absence of a fossil record have been a hindrance to the testing of hypotheses on the origin and evolution of the group (Chabaud & Bain, 1994). Geographical distribution (Holarctic, African and Asian-Australasian) has been suggested to be more important in the evolution of this genus than host-tracking (e.g. the taxonomic and evolutionary relationship of the hosts) (Chabaud & Bain, 1994). This is because *Onchocerca* species seem to group according to the geographic region where they are found rather than in relation to the group of ungulates they infect. Thus, three main branches might be expected in the phylogeny of the genus, each corresponding to a geographical location. Nevertheless, this is not clearly observed in the phyletic tree of Bain (1981). Also, the origin of the genus has been placed in Africa because it is in this continent where the majority of the species occur and the most morphologically primitive species (*O. raillieti*, a parasite of the African wild ass) is present (Bain, 1981). It has also been suggested that the origin of the genus took place in recent geological time (during the Pleistocene) with the establishment of the Equidae in that continent (Bain, 1981), although Bain (2002) recently referred to the Miocene radiation of the cervids and bovids, which form the majority of hosts. In either case, the rate of evolution in this group appears to be high. Furthermore, *O. ochengi* presumably speciated by host switch into domestic cattle in Africa, and cattle did not appear in areas of Africa where *O. ochengi* (and *O. volvulus*) is found until 5000–2500 BP (Marshall & Hildebrand, 2002). Subsequently, *O. ochengi* underwent a second host switch into humans to become *O. volvulus*. These speciation events are very recent and seem to have occurred very fast, suggesting an increase in the rate of evolution at least in these species, and recent bottlenecks at speciation could explain the observed low level of molecular variation (Unnasch & Williams, 2000).

The genus *Onchocerca* is also a good group to test the hypothesis of sympatric evolution. Host–parasite

https://doi.org/10.1079/JOH2006331 Published online by Cambridge University Press

co-speciation events are likely to be infrequent because in some cases the age of the parasite species is younger than that of the host it is infecting and the oldest hosts do not necessarily harbour the oldest parasites (Bain, 1981; Chabaud & Bain, 1994). These observations point towards host switch and site shift as important factors in the evolution of the genus. Given that some species of *Onchocerca* not only share the same geographical distribution but also have common life cycles in common host and vector species, host and site shifts in some of the *Onchocerca* species could be indicative of sympatric speciation events.

In the present paper a molecular phylogenetic analysis of three mitochondrial DNA gene fragments [12S and 16S rRNA genes, and NADH dehydrogenase subunit 5 (ND5)] from five species of *Onchocerca* is presented, using maximum likelihood (Felsenstein, 1981) and maximum parsimony (Farris, 1970). The hypothesis that *O. gibsoni* could be the sister species of *O. volvulus*, as opposed to *O. ochengi*, is tested and the possibility of sympatric speciation is discussed.

Materials and methods

Samples and DNA extraction

Due to the difficulty in obtaining material from *Onchocerca*, the present study was restricted to five species: one sample of adult *O. volvulus* dissected out of nodules removed surgically from patients in the Kati District, Mali, in December 1986; adult *O. gibsoni* dissected out of two nodules removed from cattle from Australia in 2002 (provided by Professor Bruce Copeman); one sample of adult *O. ochengi* from cattle post-mortem at an abattoir in Bamako, Mali, in November 1986; an *O. lienalis* sample of pooled microfilariae from Shrewsbury, UK, obtained by Dr P.J. McCall (Liverpool School of Tropical Medicine) in February 1988; and a sample of adult *O. gutturosa* obtained at an abattoir in Bamako, Mali in November 1987. All these samples were preserved in ethanol. As an outgroup, a sample of adult worms of *Litomosoides sigmodontis* maintained in jirds, *Meriones* sp., and obtained from Dr M.A. Beg (University of Salford, UK) in 1991 was used.

The DNA extraction method used was a cetyltrimethyl ammonium bromide (CTAB) treatment followed by a phenol:chloroform:isoamyl alcohol extraction and an ethanol precipitation (Murray & Thompson, 1980).

PCR and sequencing

Adult worms of *Onchocerca* sp. occur in various connective tissues within their hosts in which several females and males may be in close contact. Because of this, separation of individual worms clear of host DNA or that of other worms is difficult (Muller, 1979). Therefore, the strategy of cloning polymerase chain reaction (PCR) products prior to sequencing was adopted to ensure that nucleotide sequences were each derived from a single individual.

PCR amplification of different mitochondrial gene sequences was done separately, using primers shown in table 1, in an Applied Biosystems GeneAmp PCR System 9700 thermocycler. Primer design was based on the *O. volvulus* mitochondrial genome (AF015193; Keddie *et al.*, 1998). Reactions were performed in a total volume of 25 μl each containing 1 × buffer (Promega), 3 mM $MgCl_2$ (Promega), 200 μM of each dNTP (Promega), 0.2 μM of each primer, 1 unit of *Taq* DNA polymerase (Promega) and 1.5 μl of the DNA extraction. Amplifications consisted of a first denaturation step at 94°C for 3 min followed by 35 cycles of 45 s at 94°C, 1 min at 50°C (annealing) and 30 s at 72°C, with a final extension step of 5 min at 72°C. PCR amplification products were run on 1% (w/v) agarose gels stained with ethidium bromide, and visualized with uv light. Negative controls for the PCR were always run to control for contamination. Amplification products were extracted directly from the gel using the Geneclean II kit from Anachem.

PCR products were cloned using the TOPO TA Cloning Kit for Sequencing from Invitrogen, and clones were extracted from transformed bacteria using the S.N.A.P. MiniPrep kit also from Invitrogen. Clones were cut with EcoRI and run on agarose gels 1% (w/v) to check that transformants contained inserts of the appropriate size. Clones were sent to the Advanced Biotechnology Centre, Imperial College School of Medicine, for sequencing in both directions using the universal primers T3 and T7 (as provided in the TOPO TA Cloning Kit for Sequencing, Invitrogen). Big Dye v3.1 chemistry (Perkin Elmer/Applied Biosystems) was used and products were run in ABI 3100 and ABI 377 automated sequencers.

Table 1. Primers designed for the amplification of the different mitochondrial gene fragments used in the study. These primers are based on the mitochondrial genome of *Onchocerca volvulus* (Keddie *et al.*, 1998).

Name	Sequence[a]	Direction	Gene	Position[b]
12SOvC	TCGGCTATGCGTTTTAATTTT	Forward	12S	7496-7517
12SOvB	CAACTTACGCCCCTTTAGGC	Reverse	12S	7996-8015
16SOvC	AGCCTTAGCGTGATGGCATA	Forward	16S	10976-10995
16SOvB	ACCCACATTGCATTCCTTTC	Reverse	16S	11442-11461
ND5OvA	TTGGTTGCCTAAGGCTATGG	Forward	ND5	12697-12716
ND5OvC	CCCCTAGTAAACAACAAACCACA	Reverse	ND5	13145-13167

[a] Sequence given in the 5′ → 3′ direction.
[b] Position of the primer in the mitochondrial genome of *O. volvulus*, reference number AF015193.

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Phylogenetic analyses

Sequences were visually inspected for reading errors using TraceViewer 3.0.2 (CodonCode). Sequences were aligned using ClustalX v1.83 (Thompson *et al.*, 1997) and corrected by eye using the sequence editor Se-Al v2.0a11 (Rambaut, 1996).

To check the phylogenetic signal of sequences, likelihood mapping (LM) tests (Strimmer & von Haeseler, 1997) were performed using Tree-Puzzle 5.0 (Strimmer & von Haeseler, 1996). Likelihood mapping is a graphical method of visualizing the phylogenetic signal content of a set of sequences. It is based on the spatial location of the maximum likelihoods of each of the three possible trees computed for four sequences. The frequency of the unresolved quartets is a measure of the phylogenetic 'noise' in the data (Strimmer & von Haeseler, 1996). In general, when more than 10–15% of the quartets are unresolved the tree will not be completely resolved. An example of the use of LM for testing the phylogenetic signal of a sequence data set can be seen in Farias *et al.* (2001). For all four analyses (each gene plus the combined data set) the substitution model used for the LM was the Hasegawa-Kishino-Yano (HKY) (Hasegawa *et al.*, 1985), with the transition/transversion ratio, the nucleotide frequencies, and the parameter alpha of the gamma distribution estimated from the data set. All possible quartets were analysed.

Before sequence data were combined into a single matrix, a homogeneity partition test, the incongruence length difference test (ILD) (Mickevich & Farris, 1981; Farris *et al.*, 1994, 1995), was performed as implemented in PAUP* v4.0b10 (Swofford, 2002) to check for possible incongruencies between phylogenetic signals of three sequence partitions, corresponding to the mitochondrial gene sequences. One thousand replicates were performed with parsimony as the optimality criterion.

Maximum likelihood (ML) (Felsenstein, 1981) and maximum parsimony (MP) (Farris, 1970) phylogenetic analyses were run in PAUP* v4.0b10. Before ML analyses were run, the Akaike information criterion (AIC) (Akaike, 1974) was used to find the substitution model that fitted the data best as implemented in the program Modeltest v3.06 (Posada & Crandall, 1998). To obtain the ML tree, heuristic searches were run with the starting tree obtained via stepwise addition, with random addition of sequences and ten replicates. Tree-bisection-reconnection was used as the branch-swapping algorithm. ML trees were obtained without a molecular clock and enforcing it to test the molecular evolutionary clock hypothesis (see below). To obtain MP trees, an heuristic search, with tree-bisection-reconnection as the branch-swapping algorithm was used. The starting tree was obtained via stepwise addition with random addition of sequences and 100 replicates. All characters were treated as unordered and given equal weights, and gaps were treated as missing data.

To test the robustness of the MP trees 1000 bootstrap replicates were run (Felsenstein, 1985) and in the case of the ML tree, zero-branch-length tests were performed. The Bremer support or decay index (DI) (Bremer, 1994) was estimated for the MP tree to measure levels of support at the nodes using TreeRot v2.0 (Sorenson, 1999). This measure is calculated by subtracting the length of the tree constrained not to contain the node of interest to the length of the most parsimonious unconstrained tree. The partition Bremer support (PBS) (Baker & DeSalle, 1997; Baker *et al.*, 1998) measures the support of each partition (in this case the genes) to the nodes. The sum of all PBS will equal the total DI. The PBS also gives an approximation to the disagreement between the signals of the partitions. If one gene supports one node the value will be positive and if the other partition supports an alternative node, the value will be negative.

In order to investigate the hypothesis of an increase of the evolutionary rate in *O. ochengi* and *O. volvulus*, the molecular evolutionary clock hypothesis, which assumes that the different species show a homogeneous rate of evolution, was tested using a likelihood ratio test (LRT) (Goldman, 1993) and Tajima's 1D and 2D tests (Tajima, 1993). For the LRT, the log-likelihoods of the ML phylogenetic tree obtained enforcing a molecular clock or without enforcing it were used to estimate the likelihood ratio statistic. Since the clock hypothesis is the simpler model, the likelihood statistic is estimated as $2x(\ln L_{clock} - \ln L_{noclock})$. This statistic follows a chi-square distribution with n-2 degrees of freedom, where n is the number of taxa (sequences in this case). When the test is significant at or below 5%, the null hypothesis of equal rates of evolution among the branches of the phylogeny is rejected because the more complex model improves the log-likelihood significantly. Tajima's 1D and 2D tests are based on the expectation $E(n_{ijk}) = E(n_{jik})$, where n is the observed number of sites where three sequences carry nucleotides i, j and k, respectively, the third sequence being the outgroup. Using a chi-square statistic can test this equality and if it does not hold, then it can be concluded that the sequences do not conform to rate constancy. For the Tajima's 1D and 2D tests we used one sequence per species. All combinations of two *Onchocerca* species were tested with *L. sigmodontis* as outgroup (ten combinations) using the MEA program (written and provided by Etsuko Moriyama, University of Nebraska).

The basal position of *O. gibsoni* was tested against the alternative topology of *O. gibsoni* being sister species to the *O. volvulus* and *O. ochengi* cluster. To do this, the (*O. gibsoni*, (*O. volvulus*, *O. ochengi*)) constraint was introduced into the analyses and the MP and ML constraint trees recovered were compared to the MP and ML trees recovered without the constraint to evaluate if they were significantly different. Comparisons were performed with the Kishino-Hasegawa and Templeton tests implemented in PAUP* v4.0b10. Due to the criticism these tests have received (e.g. Shimodaira & Hasegawa, 1999), we also used the likelihood based Shimodaira-Hasegawa test also implemented in PAUP* v4.0b10. The question of which species, *O. gibsoni* or *O. ochengi*, is the sister taxon of *O. volvulus* was also evaluated using the same methods. Maximum parsimony and ML analyses were run with the constraint (*O. gibsoni*, *O. volvulus*). The MP and ML constraint trees obtained were then compared to those obtained without constraints and also

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to that containing the (*O. gibsoni*, (*O. volvulus*, *O. ochengi*)) constraint using the above tests.

Results

Sequences

The sequences of each mitochondrial gene obtained for each species have been deposited in the GenBank and have the accession numbers AY462875–AY462892 for the ND5, AY462893–AY462910 for the 16S rRNA gene and AY462911–AY462928 for the 12S rRNA gene. The combination of the different gene sequences into single sequence matrices resulted in two sequences for *O. gibsoni*, five for *O. ochengi*, three for *O. volvulus*, two for *O. gutturosa*, five for *O. lienalis* and one for the outgroup *L. sigmodontis* (more details available on request). The alignment file is available by anonymous FTP from ftp.ebi.ac.uk in directory/pub/databases/embl/align or via the EMBLALIGN database via SRS at ftp://ftp.ebi.ac.uk/pub/databases/embl/align/ under accession number ALIGN_000844.

Lengths of aligned fragments of the mitochondrial genes were 533 base pairs (bp) for the 12S, 493 bp for the 16S and 471 bp for the ND5. Alignment of the sequences required 57 gaps to be optimal. Of these, 28 fell in the 12S gene, ten were in the 16S gene and 19 in the ND5 gene. Most gaps were introduced to optimize alignment between *Onchcocerca* species and the outgroup. However, some deletions were observed within and between the ingroup species, which were mostly single deletions falling in poly-T runs. No gaps were included in the ND5 gene, with the exception observed in one *O. gutturosa* sequence (Ogut2c1), in which a gap of 19 nucleotides had to be included. This long deletion could indicate that the sequence was a nuclear pseudogene, but in this case we should also expect the rest of the gene to be dissimilar to the other *O. gutturosa* sequence. It could also be a sequencing error, but sequencing the clones in both directions independently should have controlled for this. The third possibility is that it is a PCR artefact. In any case, this deletion did not affect the outcome of the phylogenetic analyses and *O. gutturosa* sequences were placed together with high support (see below).

Phylogenetic analyses

Likelihood mapping (LM) analyses of the individual genes showed that the 16S and ND5 genes contained a low phylogenetic signal, while the 12S had a higher signal. The total evidence LM analysis (fig. 1) resulted in a slightly improved signal in comparison with the individual genes, and since the partition-homogeneity test was not significant ($P = 0.169$) the three genes were combined for the analyses. The validity of the ILD test has been questioned based on high type I errors due to disparity in homoplasy levels between data sets, differences in lineage evolutionary rates and among site rate variation (Cunningham, 1997a,b; Dolphin *et al.*, 2000; Barker & Lutzoni, 2002; Darlu & Lecointre, 2002). However, we proceeded since the *P* value found was higher than the conservative significance threshold of 0.05, thus suggesting that phylogenetic signal at least would not reduce phylogenetic accuracy (Cunningham, 1997a).

The ML phylogenetic analysis was run with settings corresponding to the HKY + I + G substitution model, which best fitted the data. The parameters of the model were set to a transition/transversion ratio (ts/tv) of 3.0034, nucleotide frequencies of A = 0.23500, C = 0.07680, G = 0.20870 and T = 0.47950, an assumed proportion of invariable sites of 0.3707, a gamma distribution of variable sites with a shape parameter α of 0.3972, and four rate categories. The analysis was run without enforcing the molecular clock option. The resultant tree had a -ln likelihood = 4106.83 and it was well resolved in the branches between species, but much less resolved within species (fig. 2). The monophyly of *Onchocerca* with respect to *L. sigmodontis* was highly supported ($P < 0.001$) in the zero branch length test, as was the monophyly of each species. A surprising result was the basal placement of *O. gibsoni*, although the monophyly of the other *Onchocerca* species with respect to *O. gibsoni* was supported with a $P = 0.030$. Monophyly of

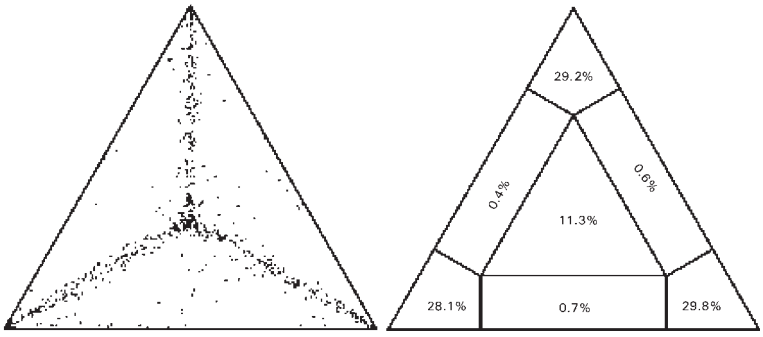

Fig. 1. Likelihood mapping of the phylogenetic signal of combined data. For the group of sequences, the likelihoods of each of the possible quartet trees are placed in the triangle. This triangle is subdivided in different regions according to the signal, one central region for star-like phylogeny, three regions in each corner for well-resolved phylogenies, and three other regions in the laterals between each corner which represent phylogenies that are not resolved for two of the three possible trees. Percentages shown indicate the proportion of all possible quartets that fall in each region of the triangle.

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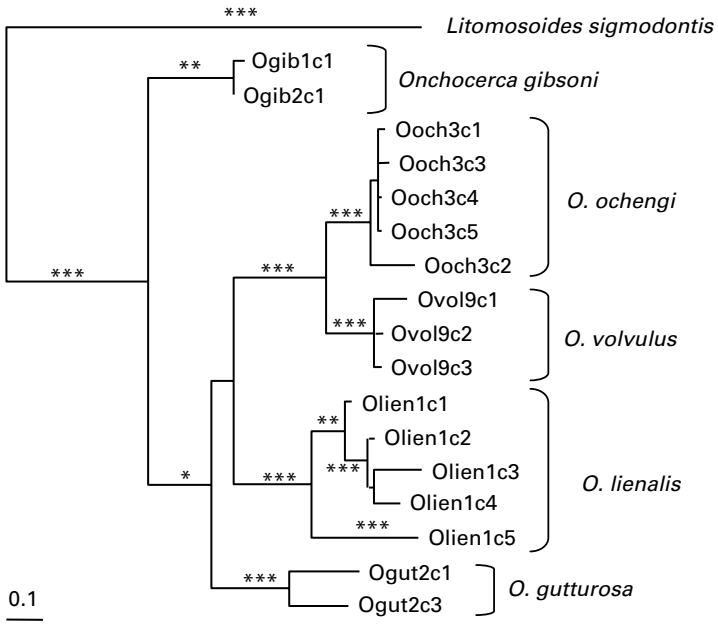

Fig. 2. Phylogenetic reconstruction using the maximum likelihood method. Asterisks shown on the branches are the results of the zero branch length, a measure of the robustness of the branches (*** represents $P < 0.001$; ** $P < 0.01$; and * $P < 0.05$).

a group formed by *O. ochengi*, *O. volvulus* and *O. lienalis*, with *O. gutturosa* as basal to it was not supported ($P = 0.086$) suggesting that the branch should be collapsed. *Onchocerca volvulus* and *O. ochengi* formed a monophyletic group with a strong support ($P < 0.001$).

Maximum parsimony analysis resulted in three most parsimonious trees that only differed in the placement of the *O. volvulus* sequences within the species group. The number of variable characters was 311 of which 135 were parsimony-informative. The trees had a length of 428, a consistency index (CI) of 0.8154 (CI excluding uninformative characters 0.6762, rescaled CI 0.6831), and a retention index (RI) of 0.8378. The strict consensus tree is presented in fig. 3. The relationships between species were resolved but those within species were not entirely resolved. Each species's monophyly had 100% bootstrap support and also high Bremer decay values (fig. 3). *Onchocerca gibsoni* was again also placed basal to the remaining *Onchocerca* species with strong bootstrap support (82%), although the Bremer decay value for the monophyly of these with respect to *O. gibsoni* was not very high (5) (fig. 3). In contrast with the ML results, *O. gutturosa* and *O. lienalis* were placed as a monophyletic sister group to the also monophyletic *O. volvulus*/*O. ochengi* group. The bootstrap support for these monophyletic clusters was strong, 100% and 72%, respectively. Nevertheless, while the monophyly of *O. volvulus* and *O. ochengi* is strongly supported by the Bremer decay index (11), that of *O. gutturosa* and *O. lienalis* is not (Bremer decay value 3), suggesting that the branch should be collapsed (fig. 3).

The estimated -ln L of the ML tree when the molecular clock option was enforced was 4130.06. In order to test the molecular clock hypothesis to check if different species were evolving at different rates, this value was contrasted to that obtained without the molecular clock option giving a likelihood ratio test statistic value of 46.46 ($2 \times [4130.06 - 4106.83]$). The critical significance level with 16 degrees of freedom ($n - 2$, where n is the number of OTUs) is 0.0000, thus rejecting the molecular clock hypothesis. This meant that the different OTUs were evolving at different rates. Furthermore, Tajima's 1D and 2D tests were non-significant for all the combinations of *Onchocerca* spp. using *L. sigmodontis* as outgroup, indicating that the evolutionary rates were similar between the *Onchocerca* species. Thus, these results together point to a difference in the rate of evolution between the *Onchocerca* clade and *L. sigmodontis*, species of *Onchocerca* evolving at similar rates within the genus.

Running the analyses with the constraint (*O. gibsoni*, (*O. volvulus*, *O. ochengi*)) resulted in three MP parsimony trees of 435 steps and a ML tree with a -ln likelihood = 4116.80. Comparison of the lengths of the constraint trees with that of the ML and MP trees without constraint resulted in non-significant differences (Kishino-Hasegawa test: MP $P = 0.1445$ and ML $P = 0.323$; Templeton test: MP $P = 0.1444$; Shimodaira-Hasegawa test: ML $P = 0.383$), indicating that *O. gibsoni* is not necessarily basal and could also be the sister species to *O. ochengi* and *O. volvulus*. The hypothesis of *O. gibsoni* being the sister species of *O. volvulus* as opposed to *O. ochengi* was also investigated in a similar way. Analyses with the constraint (*O. gibsoni*, *O. volvulus*) enforced resulted in six MP trees with a length of 448 and a ML tree with a -ln L = 4168.91. These trees were significantly worse (Kishino-Hasegawa, Templeton and Shimodaira-Hasegawa tests $P < 0.001$) than the MP and ML trees without

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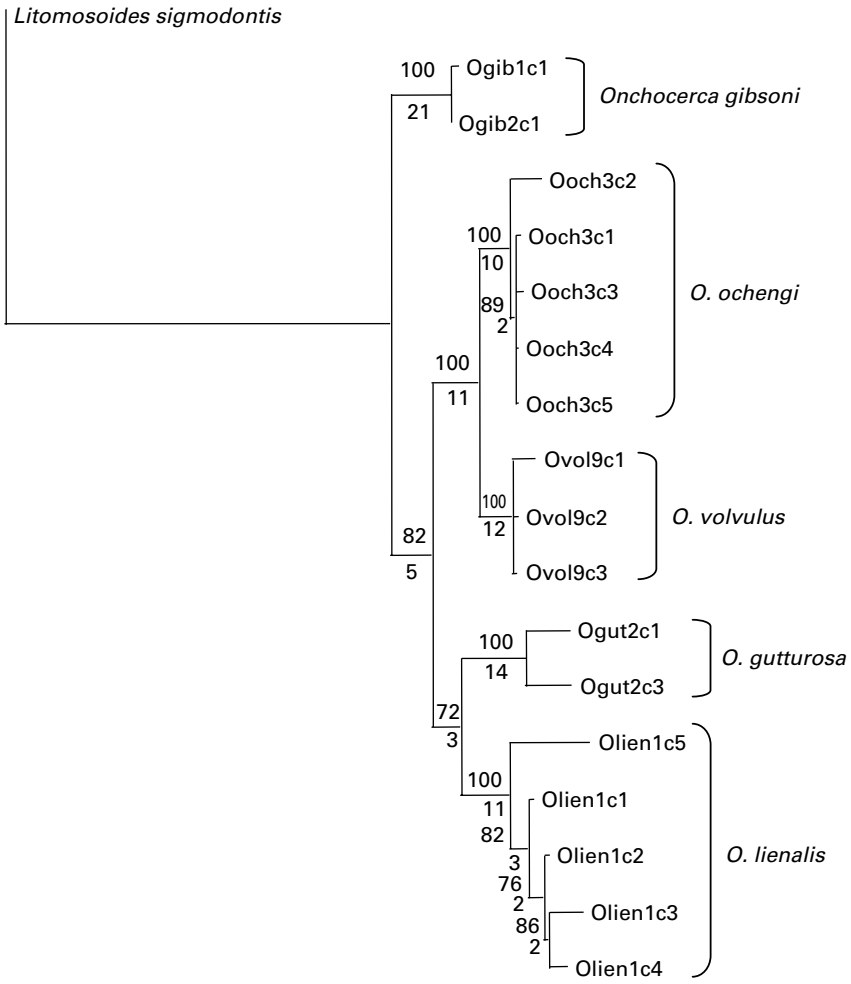

Fig. 3. Maximum parsimony tree. Values above branches show bootstrap support and those below are the decay indices (partition Bremer support (PBS) values are not shown).

constraints and the trees constrained to have (*O. gibsoni*, (*O. volvulus*, *O. ochengi*)).

Discussion

The main hypothesis to be tested was whether the sister group of *O. volvulus* was *O. ochengi* or *O. gibsoni*. All three phylogenetic reconstruction methods placed *O. volvulus* and *O. ochengi* as a monophyletic group with very high support. Furthermore, direct phylogenetic testing of these hypotheses supports the view that *O. ochengi* is the sister species of *O. volvulus* and rejects the alternative. This strongly indicates that *O. ochengi* is the sister species of *O. volvulus* as suggested by Bain (1981, 2002) and rejects the hypothesis of Muller (1979, 1983). It also indicates that the reduction of the karyotype in *O. volvulus* and *O. gibsoni* (Post *et al.*, 1989) is likely to be the result of two independent events. *Onchocerca dukei* is also closely related to *O. volvulus* (Bain, 1981), but no material was available for molecular analysis. However, there is no taxonomic opinion that *O. dukei* is the sister species to *O. volvulus*, and it does not have the reduced (n = 4) karyotype seen in *O. volvulus* (Post *et al.*, 1991).

The exact relationship between *O. lienalis* and *O. gutturosa* appears to be difficult to estimate. In the past, there has been debate regarding their status as separate or single species. Steward (1937) regarded the two names as synonymous and the differences in morphology as a result of adaptations to their different locations within the host (*O. gutturosa* in the nuchal ligament, *O. lienalis* in the gastro-splenic ligament). Eichler & Nelson (1971) and Eichler (1973) supported the view of a single species with morphological variation, but Bain *et al.* (1978) re-established the validity of the two species based on the re-examination of the morphology of adult specimens, the different width of the microfilariae of both species and their distinct location within the host, and placed them on a common branch in the phyletic tree (Bain, 1981). Their status as two independent species was finally confirmed by enzyme analyses, which showed that there were fixed differences between the two species in isoenzyme variation (Flockhart, 1982; Andrews *et al.*,

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1989). The present results further corroborate their status as separate species although their phylogenetic position was difficult to estimate.

A line of Asiatic and African species of *Onchocerca*, including *O. gibsoni*, *O. volvulus* and *O. ochengi* among others, is recognized by various authors based on morphological characters (Bain & Beveridge, 1979; Muller, 1979, 1983; Bain, 1981). Thus, the present results giving *O. gibsoni* a basal position to the rest of the *Onchocerca* species included is in contradiction with the accepted taxonomy. The basal position of *O. gibsoni* obtained in our analyses was moderately supported ($P = 0.03$ in ML and 82% bootstrap and a DI of 5 in MP) but the MP and ML trees obtained were not significantly better than those which placed *O. gibsoni* as sister species to *O. volvulus* and *O. ochengi*. The position of *O. gibsoni* is unlikely to be an artefact due to different rates of evolution because of the results of the LRT and Tajima's 1D and 2D tests. Nucleotide ratio differences can also be ruled out. However, placement of *O. gibsoni* as basal in the phylogeny could be an artefact due to the small sample size (see below). Other phylogenetic studies of filarial nematodes that included a few species of *Onchocerca* gave contradictory results concerning the placement of *O. gibsoni* (Casiraghi *et al.*, 2001, 2004; Egyed *et al.*, 2002).

The positioning of *O. gibsoni* as basal to the other species examined in this study contradicts the taxonomy of the genus and is not very well supported. However, it is important to a whole set of interesting evolutionary questions. For example, it has been suggested that the evolution of this genus has been influenced more by the geographical distribution of the species than by the evolutionary relationship of the hosts (Chabaud & Bain, 1994). Although the phyletic tree in Bain (1981) does not clearly indicate it, the phylogeny obtained in this study could be reflecting this geographic division into African (*O. volvulus* and *O. ochengi*), European (*O. gutturosa* and *O. lienalis*) and Asian (*O. gibsoni*) branches. Another interesting question to address would be the origin of the genus, which has been traditionally placed in Africa (Bain, 1981). Nevertheless, amongst the species that we studied the African species were the most derived, with the European and Austral-Asian species more basal. If Africa is the centre of origin of the genus, our results also suggest the possibility of reverse migration back into Africa. There is a growing amount of evidence for an Australasian dispersal of some species of vertebrates into Africa, contradicting the previous hypotheses of an African origin for many species (e.g. Juste *et al.*, 1999; Bossuyt & Milinkovitch, 2001). Further analyses with additional sequences, preferably nuclear, and a more complete set of species (comprising different continents) would have to be conducted to test the above hypotheses. Among other species that would be interesting to include are *O. raillieti*, a parasite of the African wild ass, and considered to be the most primitive species of the genus (Bain, 1981); *O. dukei*, an African species taxonomically close to *O. volvulus* and *O. ochengi*, and which has been suggested to be the vicariant species of *O. gibsoni* (Bain, 1981); and *O. cebei*, a parasite of water buffalo in Asia, taxonomically close to *O. gibsoni* and vicariant of *O. ochengi* (Bain, 1981). European species like *O. cervicalis*, *O. flexuosa* and *O. tarsicola* would also be interesting to include, as well as the only species of North American origin, *O. cervipedis*.

The occurrence of sympatric speciation in nature is a central topic in evolutionary biology (e.g. Bush, 1994). In parasites, however, defining sympatry is not always straightforward (McCoy, 2003), and sympatric speciation could occur through host switch or site switch, although the former could also be seen as a mechanism of peripatric speciation since different hosts can be equivalent to different geographical regions in some circumstances (Brooks & McLennan, 1993). In the genus *Onchocerca* it is clear that co-speciation between hosts and parasites is not the dominant mode of speciation. The most ancient hosts, like camelids or suids, do not harbour the most primitive species of *Onchocerca* and the parasite genus is undoubtedly younger than the species they infect (Chabaud & Bain, 1994). The results showed some evidence of sympatric speciation both through host switch and site shift. The case of *O. volvulus* can be considered as an example of sympatric speciation through host switch because, apart from being sympatric (in strict geographical terms, see above), it also shares the same vector (members of the *Simulium damnosum* species complex) with its sister species, *O. ochengi*. The origin of *O. lienalis* and *O. gutturosa* is consistent with a model of sympatric speciation, since they occupy different sites in cattle and are both present in Europe (both species are considered European, Bain, 1981). Nevertheless, this hypothesis should be further tested using more species of the genus and additional sequences, preferably from the nuclear genome, so that the evolutionary relationship between them can be better resolved.

The present results also showed a difference in the rate of evolution between the outgroup, *L. sigmodontis*, and the *Onchocerca* species. It has been speculated (Bain, 1981) that the origin of this genus might be quite recent during the Pleistocene (1.8 million years to 11,000 years before present), although Bain (2002) recently referred to the Miocene radiation of the cervids and bovids, which form the majority of hosts, as the possible time of origin of *Onchocerca*. In any case, the *Onchocerca* species studied are likely to be of recent origin because their hosts are mostly domestic cattle, and cattle were domesticated no more than 10,000 years ago. Thus, for example, *O. ochengi* presumably speciated by host switch into domestic cattle in Africa, and cattle did not appear in areas of Africa where *O. ochengi* (and *O. volvulus*) is found until 5000–2500 BP (Marshall & Hildebrand, 2002). Subsequently, the most recent common ancestor of these species underwent a second host switch into humans to become *O. volvulus*. These recent speciation events are likely to have been associated with an accelerated rate of evolution in the *Onchocerca* genus in comparison with *Litomosoides*.

Acknowledgements

The authors are grateful to Professor Bruce Copeman (James Cook University, Townsville, Australia), Dr P.J.

McCall (Liverpool School of Tropical Medicine, UK) and Dr M.A. Beg (University of Salford, UK) for providing parasite material. R. M.-H. is grateful for post-doctoral support from the University of Greenwich (HEFCE funds). The authors also thank Dr Carlos Juan (Institut Mediterrani d'Estudis Avançats, Balearic Islands, Spain) for his useful comments on the manuscript.

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(Accepted 17 October 2005)