Bayesian paternity analysis and mating patterns in a parasitic nematode, *Trichostrongylus tenuis*

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Abstract

Mating behaviour is a fundamental aspect of the evolutionary ecology of sexually reproducing species, but one that has been under-researched in parasitic nematodes. We investigated mating behaviour in the parasitic nematode *Trichostrongylus tenuis* by performing a paternity analysis in a population from a single red grouse host. Paternity of the 150 larval offspring of 25 mothers (sampled from one of the two host caeca) was assigned among 294 candidate fathers (sampled from both caeca). Each candidate father's probability of paternity of each offspring was estimated from 10-locus microsatellite genotypes. Seventy-six (51%) offspring were assigned a father with probability greater than 0.8, and the estimated number of unsampled males was 136 (95% CI: 77, 219). The probability of a male from the one caecum fathering an offspring in the other caecum was estimated as 0.024 (95% CI: 0.003, 0.077), indicating that the junction of the caeca is a strong barrier to dispersal. Levels of promiscuity (defined as the probability of two of an adult's offspring sharing only one parent) were high for both sexes. Variance in male reproductive success was moderately high, possibly due to a combination of random mating and high variance in post-copulatory reproductive success. These results provide the first data on individual mating behaviour among parasitic nematodes.

Introduction

Animal parasitic nematodes are among the most widespread and diverse animal groups (Blaxter, 2001). Their hidden lifestyle, however, presents difficulties in observing their behaviour in the wild, and many areas of their ecology remain unexplored (Criscione *et al*, 2005). One area where progress has been made is in the application of molecular ecology methods to the inference of large-scale host-mediated ecological processes such as colonisation and dispersal (Criscione *et al*, 2005; Grillo *et al*, 2007; Nejsum *et al*, 2005; Nieberding *et al*, 2005; Troell *et al*, 2006; Webster *et al*, 2007; Wielgoss *et al*, 2008). However, no studies have yet used molecular tools to analyse patterns of parasite mating behaviour at the individual level. Mating behaviour has important consequences for understanding how this group of animals has evolved as well as for predicting how parasite populations will respond to changes in their environment. For example, deviation from random mating in the form of reproductive skew reduces the effective population size and increases the opportunity for sexual selection (Crow, 1958).

In the absence of specific data, parasitic nematodes have generally been assumed to mate randomly and promiscuously (e.g. Barnes *et al*, 1995; Churcher and Basáñez, 2008; Dobson *et al*, 1987; Saul, 1995; Smith *et al*, 1999 but for exceptions see Braselton *et al*, 2004; Churcher *et al*, 2008). In nonparasitic taxa, predictions about mating systems and the distribution of

reproductive success have been successfully tested via parentage analysis using molecular markers. Indeed, molecular markers have transformed the study of animal mating systems, often overturning conclusions based on observational evidence (e.g. Griffith *et al*, 2002; Jones and Avise, 2001), and would appear to be ideal tools for studying the concealed mating behaviour of animals such as parasitic nematodes. Paternity analysis (the most common type of parentage analysis) is conventionally performed by analysing the genotypes of a sample of mothers, their known offspring, and, ideally, all plausible candidate fathers. When applied to a parasitic nematode, paternity analysis presents particular technical and statistical challenges, including raising offspring *in vitro*, obtaining DNA of sufficient quantity and quality from microscopic larvae, and accurately assigning paternities among potentially hundreds or thousands of candidate fathers, some of whom might already have been shed from the host. These difficulties may explain the current lack of parentage analyses in parasitic nematodes.

We conducted a paternity analysis in a single host-population (or infrapopulation) of *Trichostrongylus tenuis*, a parasitic nematode of galliform and anseriform birds. Our aim was to provide the first data on patterns of parentage in a sexually reproducing animal parasitic nematode, and to shed light on the assumption that mating in these parasites is random.

T. tenuis has a direct life cycle and obligate sexual reproduction. Adult *T. tenuis* reside and mate in the host's two caeca; these are blind guts of about 75 cm in length that extend from the junction of the small and large intestines (Hudson, 1992). Candidate fathers for a *T. tenuis* offspring fall into three categories. The first and most obvious category is males present at the time of sampling in the caecum where the mothers were sampled. Second, males from the neighbouring caecum must be considered, although we know nothing about movement of adults between caeca. The third category is unsampled males, including candidate fathers shed prior to sampling and those present in the caeca that had escaped detection. Information about the number of unsampled males is essential for paternity assignment, which in *T. tenuis* will depend on both the longevity of males and the duration of sperm storage. *T. tenuis* infecting naive captive hosts can live for more than two years (Shaw and Moss, 1989), and most sexually reproducing nematodes are thought to store sperm (Bird and Bird, 1991), but we know nothing about these parameters in wild populations. Given perfect genotypic data, the number of unsampled males could be estimated relatively easily by partially inferring the genotypes of missing fathers from the genotypes of mothers and offspring. In practice, the presence of data errors makes this challenging (Emery *et al*, 2001).

In this study we jointly estimated paternity, the number of unsampled males and the probability of mating across caeca using a

Bayesian method tolerant of genotyping error (Hadfield *et al*, 2006). Our aim was to investigate four questions relevant to mating and reproduction in *T. tenuis*: (1) How are paternities distributed among sampled and unsampled males? (2) Among sampled males, what proportion of fathers was sampled in the same caecum as the mother? (3) How promiscuous are males and females? (4) Is reproductive success randomly distributed among males?

Materials and methods

Sampling of study population

A single male red grouse was harvested at a grouse-shooting estate in Lauderdale, Scotland at 1200 hours on 29th October 2004 (because the timing of events such as the death of the host and the isolation of the females influences the interpretation of the results, we give the local time [British Summer Time] at each stage). The two caeca were removed (1230 hours) and stored in M9 buffer (Hope, 1999) at approximately 30 °C during transport to the laboratory. On arrival (1500 hours), the diluted contents and the mucosal and submucosal surface of one caecum (designated the *local* caecum, as distinct from the *neighbouring* caecum) were examined for *T. tenuis* adults under a dissecting microscope. The neighbouring caecum was stored at –20 °C. All 108 female and 122 male adult *T. tenuis* found in the local caecum were removed. Male *T. tenuis* are easily distinguished from females by their shorter length and the presence of bursate claspers. Fifty-

138 one live females were isolated in 250 µl M9 buffer in separate wells of a
139 covered transparent polystyrene 96-well flat-bottom microtitre plate
140 (Greiner, UK) at 25 °C (1730 hours). After 2.5 hours all 51 females were
141 transferred to a fresh microtitre plate containing M9 (2000 hours), allowing
142 eggs and larvae to be categorised as laid early (1730–2000 hours) or late
143 (after 2000 hours). Both plates were incubated at 25 °C for 48 hours (by
144 which time egg laying had ceased) and stored overnight at 4 °C. The
145 number of eggs laid was 987, of which 154 (16%) hatched and moulted to
146 infective stage 3 larvae (iL3) stage (Table 1). Approximately equal numbers
147 of eggs were laid in each time period, but early eggs were 4 times more
148 likely to develop to iL3 than late eggs. A number of factors could explain
149 the low hatch rate, including shedding of unfertilized eggs and mortality of
150 fertilized eggs *in vitro*. Likewise the decline in hatch rate over time could be
151 related either to worsening condition (of the mother or of stored sperm) or to
152 a dwindling supply of stored sperm. There was substantial variation in
153 fecundity among females, whether measured in terms of numbers of eggs
154 (mean 19.3, SD 13.1, range 0–45) or larvae (mean 3.0, SD 4.1, range 0–16).
155 The 29 mothers that had produced at least one larva and all of their 154
156 larval offspring were then removed from the microtitre plate for immediate
157 DNA extraction.

158 The neighbouring caecum was searched for adult *T. tenuis*, as
159 described for the local caecum, leading to the recovery of 183 male and 200

female *T. tenuis*. In total, 305 males were preserved for genotyping in 95% ethanol at 4 °C, 122 and 183 from the local and neighbouring caeca, respectively.

Microsatellite genotyping

All females, males and offspring were genotyped at 10 microsatellite loci, including replicate genotyping of DNA-poor samples likely to incur genotyping errors, as follows. Template DNA for PCR was prepared from the mothers, offspring and candidate fathers using the worm lysis method (Grillo *et al*, 2006; Williams *et al*, 1992). Males and larvae were lysed whole, while only the heads (the anterior 10–20%) of females were used, in order to minimize the risk of contamination from sperm or fertilized eggs (Anderson *et al*, 2003). Live larvae were ex-sheathed under a dissecting microscope in 0.2% sodium hypochlorite solution. Females, males and larvae were rinsed in ddH_2O then placed into either 10 µl (larvae and females) or 20 µl (males) of lysis buffer (50 mM KCl, 10 mM Tris pH 8.0, 2.5 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween-20, 0.01% gelatine and 120 µg/ml proteinase K) in 96-well PCR plates. The plates were frozen at –80 °C for 10 minutes to lyse the tissues, incubated overnight at 65 °C then heated to 95 °C for 15 minutes. The resulting 488 lysates were diluted with ddH_2O by a factor of five (females and larvae) or 10 (males) and stored at –20 °C.

The *T. tenuis* females, larvae and males were genotyped using 10 autosomal microsatellite markers (Table 2) as described by Johnson *et al.* (2006) with the exceptions that primers were redesigned for two loci. For Tte303 the reverse primer was 5'-ACGTTCCCTGGCCTAAATAC and for Tte365 the primers were 5'-GGTGTCTTTTGCGTGTTAGTG (forward) and 5'-GATCGTCAGCAGCCTCG (reverse). Microsatellite genotyping was attempted for all 488 lysates. Rates of PCR failure and genotyping error were high in the genotypes from females and larvae but low in males, possibly due to the differences in relative tissue quantities available (males are around 5 mm long, compared with 1 mm for females' heads and 0.5 mm for larvae). An alternative method of nematode lysis (Floyd *et al*, 2002) was optimized but did not reduce error rates. Therefore, to reduce the number of missing genotypes and allow accurate estimation of error rates, all females and larvae were genotyped three times, and 68 (23%) males were genotyped twice. Allele lengths were measured using an ABI3730 DNA Analyzer (Applied Biosystems) by The Sequencing Service (University of Dundee, UK) and analyzed using GeneMapper 4.0 (Applied Biosystems).

Paternity analysis

The approach to paternity assignment followed here is fractional allocation of paternity (Jones and Ardren, 2003). For each offspring a probability of paternity is estimated for every candidate male, which, together with the

probability that the father was not sampled, must sum to one. This approach is contrasted with categorical allocation, most widely implemented in the software CERVUS (Marshall *et al*, 1998), where the paternity is assigned to the single most likely candidate that passes a likelihood threshold. Categorical allocation is useful when a single "accepted" pedigree is required, but because it ignores the uncertainty in estimating the pedigree, it results in optimistic assessment of precision and biased parameter estimates (Hadfield *et al*, 2006; Jones and Ardren, 2003). By contrast, fractional methods aim to avoid this systematic bias and optimism by effectively averaging over all possible pedigrees in proportion to their probability. The method used here further reduces bias by simultaneously estimating individual paternity probabilities with the overall effect of caecum of origin on probability of paternity (Hadfield *et al*, 2006).

For each offspring we estimated the probability of paternity by each of 294 candidate fathers (the 305 sampled males minus 11 from the neighbouring caecum that provided no genotypic data) as well as the probability that the true father was not sampled. For the purpose of illustration, the informativeness of these 295 probabilities can be intuitively summarised by the maximum posterior probability. A high maximum probability of paternity (e.g. > 0.9) implies that a single male (or the unsampled males) has been "assigned" paternity of the offspring with high

225 confidence. A low maximum probability of paternity (e.g. < 0.5) implies
226 that no single male is a strong candidate.

227 Paternity probabilities, the number of unsampled males (Koch *et al*,
228 2008), and the probability of paternity from the neighbouring caecum were
229 estimated from the microsatellite genotypes (including all replicate
230 genotypes) by a Bayesian Markov Chain Monte Carlo (MCMC) approach
231 implemented in MASTERBAYES (available at http://www.R-project.org)
232 according to Hadfield *et al.* (2006).

233 MASTERBAYES estimates the total number of unsampled males, not
234 unsampled fathers. The data only contains information about unsampled
235 fathers, but we can extrapolate to the number of unsampled males by
236 assuming that the proportion of unsampled males with no offspring is the
237 same as the proportion observed among the sampled males. The probability
238 of paternity from the neighbouring caecum is the probability that a male
239 from the neighbouring caecum will gain paternity ahead of an otherwise
240 identical male from the local caecum, and effectively quantifies the degree
241 to which free interbreeding is restricted by the subdivision of the population
242 into two caeca. A probability of 0.5 would suggest that there is no barrier to
243 mating between the caeca, while a probability close to zero would indicate
244 that mating between *T. tenuis* from opposite caeca is very unlikely. For a
245 precise definition of these two parameters and how they are estimated, see
246 Hadfield *et al.* (2006).

A particularly difficult problem for paternity assignment can occur when the true father has not been sampled and has close relatives among the sampled males. MASTERBAYES could potentially be led astray by this problem, although it is robust to the presence of related males among the sampled males. To assess the frequency of close relationships (e.g. parent-offspring pairs, full-sibs and half-sibs) among the sampled males, we estimated pairwise relatedness (r) among according to Lynch & Ritland (1999) using the software GENALEX (Peakall and Smouse, 2006).

Hadfield *et al.* (2006) account for stochastic genotyping errors by modelling two separate classes of error: allelic dropouts (where one allele fails to amplify, causing a heterozygote to be recorded as a homozygote) and false alleles (any other stochastic genotyping error) (Wang, 2004). Allelic dropout and false allele genotyping error rates were estimated according to Hadfield *et al.* (2006) with the exception that, rather than assuming that genotyping errors are independently distributed across all loci and individuals, we estimated two separate pairs of error rates, one for males and the other for females and larvae. Two categories of error rate were required because of the wide disparity in error rates observed between these two groups. The prior distributions of the four error rates were uniform in the range 0–1.

For the paternity analysis the Markov chain was run for 250 million iterations with a thinning rate of 225,000 and burn-in of 2.5 million. The

prior distribution of the probability of paternity across sampled and unsampled males was left unspecified. Estimation of the probability of paternity from the neighbouring caecum was based on a model with a normally distributed prior on the logit scale with a mean of zero and variance of 3.06. This is the closest logit parameterisation to a uniform prior on the probability scale, although it differs from the uniform distribution in having very low density for extreme probabilities. In consequence, probabilities outside the range 0.01–0.99 are heavily penalised and probabilities of zero or one are impossible.

The distributions of further parameters of interest (see below) were estimated directly from 1000 MCMC samples from the posterior distribution of the pedigree, and summarised by the median and the range between quantiles 2.5% and 97.5% (referred to as a 95% credible interval or 95% CI).

Reproductive promiscuity

Promiscuous mating systems are those in which pair bonds are not formed. Promiscuous mating can be easily diagnosed from a pedigree. However, it would be more useful to be able to quantify the degree of promiscuity. (Because we are unable to observe mating directly, but only the pedigree, we are referring here to "reproductive" promiscuity, or the tendency to produce offspring by different mates.) An intuitive measure of reproductive

promiscuity would be the number of mates that contributed to a mother's brood (for simplicity we describe only female promiscuity, although the same arguments apply to males). This measure is unsatisfactory because it is related to brood size: a mother might appear more promiscuous simply because we sampled a larger number of her offspring. We propose a measure of reproductive promiscuity that can be estimated from pedigree data and that is not biased by brood size: the probability, p, that two siblings chosen at random from a mother's offspring have different parents, or are half-sibs. We can treat the observed set of genotyped offspring as a random sample from a larger population consisting of all offspring that could have been produced by the mother during the sampling period. A sample of size n from this population of offspring consists of k full sibships (by k fathers), each sibship having frequency (proportion) x_i, $i = 1, 2, ..., k$. This is analogous to a classic scenario in population genetics, where a sample of n chromosomes consisting of k different alleles at frequencies x_i is drawn from a larger gene pool. The probability, p, that any two sibs have different fathers is analogous to the expected heterozygosity of a gene locus, which is the probability that two randomly sampled alleles are different. p is estimated by

$$\hat{p} = \frac{n}{n-1}\left(1 - \sum x_i^2\right)$$

(Nei and Roychoudhury, 1974). $\hat{p}$ is unbiased at any n, which is important as broods could be as small as $n = 2$. Unless broods are large, individual

estimates will be very variable, but averaging over a large pedigree will give a reliable pedigree-wide estimate of reproductive promiscuity.

Distribution of male reproductive success

The observed distribution of paternities among males was compared to the distribution expected under a null hypothesis of random allocation. Treating paternities as random events, the number of paternities allocated to a male under the null hypothesis is a Poisson random variable with parameter λ, where λ is the average number of paternities per male. Deviation from random mating was estimated by the standardized variance in reproductive success, I, which is the variance in reproductive success divided by the mean (Boness *et al*, 1993), and is equivalent to the index of dispersion (Krebs, 1989). A wide variety of statistics are available to quantify variance in reproductive success (Kokko *et al*, 1999). For the purpose of quantifying deviation from randomly allocated reproductive success, I is a natural choice because of its relationship to the Poisson distribution. Estimates of I in males can be related to a continuum from strict monogamy ($I = 0$) to random allocation ($I = 1$, i.e. the mean equals the variance, as expected under the null hypothesis of Poisson-distributed reproductive success) to high variance in reproductive success ($I >> 1$). Because I is sensitive to mean reproductive success, to allow comparison with other studies we also estimated the standardized variance in reproductive success as the variance

335 divided by the square of the mean, I_s, which is also known as an index of the
336 opportunity for sexual selection (Crow, 1958; Wade and Arnold, 1980).
337 Since variation in male fecundity (the number of offspring resulting
338 from a single copulation) will produce a non-random distribution of
339 paternities even when copulation is random, we also tested the allocation of
340 copulations for deviation from randomness as described above for
341 paternities. In order to infer the number of copulations it was necessary to
342 make the assumption that all multiple paternities by a given male with a
343 given female arose from a single copulation. This assumption seems
344 reasonable for two reasons. First, all nematodes are thought to have the
345 capacity to store sperm (Bird and Bird, 1991), even if the longevity of stored
346 sperm is unknown in *T. tenuis*, which increases the potential for multiple
347 fertilisations from a single copulation. Second, at 613 adults the population
348 within the sampled host was large enough that females were likely to
349 encounter many potential mates.
350 We did not assess variation in female reproductive success as this is
351 likely to reflect variation in egg and larva viability *in vitro*.
352 Except where otherwise stated, data analyses were performed in R
353 (R Development Core Team, 2008).
354

Results

Genotyping

Of the 488 adults and larvae genotyped, 469 (96%) yielded genotypic data from at least one locus (Table 3). No genotypes were recovered from four larvae and 11 of the males sampled from the neighbouring caecum. Each of the four larvae was the sole offspring of its mother, so in effect four mothers were also lost from the paternity analysis, although their genotypes nevertheless contributed to the estimation of the population allele frequencies and genotyping error rates. Thus, the main task of the analysis was to allocate paternity of 150 offspring of 25 mothers among 294 candidate fathers and an unknown number of unsampled males.

Data quality varied widely between males on the one hand and mothers and larvae on the other (Table 3). Among males, 97% of loci on average yielded genotypic data, rising to 98% when including repeat genotypes. Both allelic dropout and false allele error rates were low (<2%) in males. Averaged across loci, 84% of mothers and larvae yielded genotypic data from at least one of the three repeat genotypes. Mothers and larvae incurred high allelic dropout (18%) but relatively low false allele (1.9%) error rates.

Pairwise relatedness

Median pairwise relatedness (r) among the 294 candidate fathers was 0.00. The 2.5% and 97.5% quantiles were –0.19 and 0.19 and the maximum and minimum values were –0.45 and 0.43. The distribution of relatedness was smooth, symmetrical and typical of normally distributed noise, with a single mode at zero and no modes characteristic of first- ($r = 0.5$) or second-degree relatives ($r = 0.25$). We conclude that problems caused by the presence of closely related candidate fathers are unlikely to have affected this analysis.

Patterns of paternity

The posterior probability of paternity from the neighbouring caecum was 0.024 (95% CI: 0.003, 0.077). Even this low probability may be an overestimate given the strong prior odds against probabilities very close to zero or one. The 95% credible interval for the number of unsampled males ranged from 77 to 219, with a median of 136 (Figure 1). Combining this estimate with the 294 sampled males reveals that 21–43% (median 32%) of adult males went missing between mating and sampling. Even if we assume that 10% of males were lost during sampling, the bounds of the credible interval fall only slightly to 13–37% (median 25%).

Two patterns emerge from the inferred distribution of paternity confidence (Figure 2). First, it shows a strong bias toward high probabilities, indicating that paternity has been determined with high probability for a

large proportion of offspring. One hundred and twelve (75%) of the 150 larvae were assigned a "probable" father, i.e. maximum probability of paternity was greater than 0.5, while 76 (51%) were assigned a father with probability greater than 0.8. Second, no male from the neighbouring caecum was a probable father of any of the larvae, reflecting the lack of support in the data for cross-caeca paternity.

Although fractional assignment of paternity yields no single "accepted" pedigree, for illustration a pedigree constructed using the paternity assignments with the highest probabilities is presented in Figure 3. This pedigree displays two main features: (1) a high degree of promiscuity among both sexes; and (2) moderately high variance in reproductive success, indicated by the observation that while many males have contributed offspring to the next generation, only a few (e.g. M13) have fathered considerably more than the average.

Promiscuity

Mean reproductive promiscuity was 0.75 (95% CI: 0.69, 0.80) for females and 0.64 (95% CI: 0.52, 0.75) for males, and was 17% (95% CI: 1%, 42%) higher in females compared with males.

Distribution of male reproductive success

We investigated the distribution of reproductive success among the 122 sampled males from the local caecum, measured in terms of both paternities and copulations. Only males from the local caecum were considered because the opportunity to father offspring appears to be largely restricted to males sharing a caecum with the mothers (see above), and therefore to include males from the neighbouring caecum would conflate the effects of inter- and intra-caecal barriers to random mating. The distribution of the paternities among the 122 sampled males is shown in Figure 4A. The distribution is overdispersed and clearly deviates from the null distribution under random allocation, there being more males than expected with no paternities, fewer with 1–2 paternities and more males with at least four paternities than expected. This pattern of high variance in reproductive success is confirmed by an I estimate of greater than 1 (2.9; 95% CI: 2.4, 3.6).

If the deviation from random allocation of paternities was caused by fecundity differences, the estimate of I will be artificially inflated by variation in female fecundity and by differential mortality due to *in vitro* conditions. To investigate the impact of these potentially confounding factors, we recalculated I after setting variance in female reproductive success to zero. This was achieved by including in the pedigree only females with the mean number of offspring. Females with fewer offspring

were excluded and a random sample of offspring was used from females with more offspring (mean *n* offspring per female = 4.4; mean *n* females included = 11). Error due to sampling offspring was removed by averaging over 100 random samples in each of the 1000 pedigrees in the MCMC output. Because the mean number of offspring was generally not an integer, the *I* estimate was a weighted average of estimates obtained by rounding up and down (e.g. if the average number of offspring was 4.4, the *I* estimate would be $0.6I^{(4)} + 0.4I^{(5)}$ where the superscript denotes the number of offspring per female used to estimate *I*). The resulting estimate of 1.6 (95% CI: 1.4, 1.8) is considerably lower than the unadjusted estimate, and indicates that variation in female reproductive success inflated the *I* estimate by about 80%. Nevertheless, this caveat applies only to the size of the estimate and not to the conclusion that allocation is non-random, because if paternities are allocated randomly there will be no female-level correlation between paternity assignments, so that the expected value of *I* will be one regardless of variation in female reproductive success.

By contrast, when reproductive success was defined as the minimum possible number of copulations that could have given rise to the observed distribution of paternities (i.e. assuming that all full-sib larvae arose from a single copulation), it did not deviate significantly from random allocation (Figure 4B). The *I* estimate for the number of copulations of 1.1 (95% CI: 0.9, 1.3) is close to one, showing no substantial deviation from random

mating. Thus, the distribution of paternities is consistent with random mating combined with post-copulatory differences in reproductive success.

Discussion

We have conducted the first paternity analysis in a parasitic nematode population, the four main outcomes of which are: (1) a large fraction of candidate fathers were not sampled, probably having been ejected by the host; (2) mating was predominantly (and possibly exclusively) within, not between, caeca; (3) both sexes mated promiscuously; and (4) variance in male reproductive success was higher than expected under random allocation, possibly as a result of some males receiving more mating opportunities, but more likely because of differences in male fecundity following random mating. An additional outcome of this study is the demonstration that paternity analysis in parasitic nematodes is feasible, in spite of the technical and statistic challenges imposed by their endoparasitic lifestyle, microscopic larvae, large population of candidate fathers and unknown number of unsampled males.

Perhaps the most surprising finding was the large proportion (21–43%) of males that were not sampled. Could we have overestimated the proportion of unsampled males? A potential source of overestimation is violation of the assumption that all samples are equally prone to genotyping error. Although error parameters were specific to mothers and larvae on the

483 one hand and fathers on the other, sample quality and therefore error rates
484 will have varied within these groups. However, analysis of simulated data
485 where the samples were mixtures of two unobserved and highly disparate
486 error rates suggested that even extreme error rate variation results only in a
487 moderate (14%) upward bias in the estimated number of unsampled males
488 (JDH, unpublished data). Another possible source of overestimation is the
489 assumption that sampled and unsampled males contribute equally to
490 paternity. If unsampled males have a higher probability of gaining paternity,
491 then those offspring assigned to unsampled males will be distributed across
492 fewer males then expected. This will result in overestimation of the
493 unsampled population size even though the number of offspring assigned to
494 unsampled versus sampled males should be accurate. A tendency for
495 reproductive success to increase with age (for example, due to a period of
496 sexual immaturity in newly moulted adult males) would therefore bias the
497 estimate upwards, assuming that older males are more likely to have been
498 lost from the caeca. Although there are no data on how male fecundity
499 changes over time in *T. tenuis*, senescence and declining mating frequency
500 with age is suspected in males of *Onchocerca volvulus* (Karam *et al*, 1987).
501 Older populations of *T. tenuis* produce fewer eggs (Shaw and Moss, 1989),
502 but this is likely to be caused by declining female fecundity. Bias aside, the
503 unsampled male population will also tend to be high if male longevity is low
504 and the duration of sperm storage is high. Experimental infections of *T.*

505 *tenuis* in captive red grouse can survive for more than two years without
506 substantial mortality (Shaw and Moss, 1989), although their longevity in
507 wild populations is unknown. The duration of sperm storage is also
508 unknown in any parasitic nematode. Thus, at present there is insufficient
509 information to explain the surprisingly large number of unsampled males in
510 this study.

511 Among the sampled males, it was less surprising to discover that a
512 male sharing a caecum with a female is much likelier to gain paternity over
513 her offspring than a male from the other caecum. This result suggests that
514 the passage of adults or sperm between caeca occurs very rarely if at all.
515 The capacity for oriented movement is thought to be widespread among
516 nematodes (Burr and Robinson, 2004), so it is plausible that, given
517 sufficient motivation, *T. tenuis* adults would be capable of moving between
518 caeca. Such motivation may therefore have been absent in the population of
519 613 adult *T. tenuis* from which our sample was taken, although populations
520 at extremes of population density—burdens of *T. tenuis* can range from
521 fewer than ten to tens of thousands of adults—may experience greater
522 pressures to emigrate in search of mates or food.

523 In addition to the population-level processes discussed above we
524 made inferences from the estimated pedigree about the mating behaviour of
525 individual nematodes. Obligately sexual parasitic nematodes have long been
526 thought to mate randomly and promiscuously, and this assumption of

527 randomness is generally extended to cover allocation of reproductive
528 success (Barnes *et al*, 1995; Churcher and Basáñez, 2008; Dobson *et al*,
529 1987; Saul, 1995; Smith *et al*, 1999). Deviation from random mating will
530 usually reduce the effective population size, which will in turn reduce the
531 amount of genetic variation that a population can maintain, accelerate
532 genetic drift and reduce the effectiveness of selection on traits such as drug
533 resistance. Our results from *T. tenuis* are consistent with random mating and
534 promiscuity, but not with random allocation of reproductive success.
535 However, the deviation from random allocation observed here should be
536 interpreted with caution because it is effectively instantaneous. Ideally,
537 measures of reproductive success are taken over an organism's lifetime,
538 whereas this study was restricted to observing a few hours of reproductive
539 output from the time of host sampling until the females ceased laying viable
540 eggs *in vitro*. It is possible that the degree of variance in male reproductive
541 success observed here might average out over the lifespan of a *T. tenuis* to
542 produce a distribution of male reproductive success more indicative of
543 random allocation of paternities, although such data would be practically
544 impossible to acquire in an endoparasite. In species where lifelong
545 observations are feasible, short term estimates of standardized variance in
546 male reproductive success (I_s) have been found to be unreliable indicators of
547 long term I_s, either by underestimating (Partridge, 1988) or overestimating it
548 (Fincke, 1988).

549 It should also be remembered that only a single host was sampled,
550 and that the parameters estimated here might vary between hosts, possibly
551 in association with host-level factors, in particular parasite population size.
552 It should be further borne in mind that the posterior distribution of parentage
553 is likely to be biased towards random (Poisson) mating because the
554 Bayesian analysis assumes that parentage follows a Poisson process,
555 conditioning on the fixed effects (in this case the caecum). One option for
556 dealing with this bias would be to take a random effects approach to model
557 overdispersion in male fecundity, or even to model mate fidelity through
558 overdispersion at the level of parental combination, although this would add
559 further complexity to an already computationally complex analysis.
560 Fully random and promiscuous mating, where mate choice and pair
561 bonding are absent, is expected to be rare because both sexes should benefit
562 from the ability to choose mates that bring direct (e.g. high fecundity) and
563 indirect benefits ("good genes"). However, promiscuity can be favoured
564 under certain conditions: when there is no variation in fitness, or in
565 unpredictable environments where selective forces favour different genes in
566 different generations (Jennions and Petrie, 2000), or when monopolisation
567 of mates or resources is not feasible. Any of these factors might explain the
568 degree of promiscuity that we have observed in *T. tenuis*. A lack of variation
569 in fitness seems the least likely factor, for a variety of reasons. Firstly, a *T.*
570 *tenuis* population must have the ability to evade the potentially varied

571 immune defences of their hosts, and variation in immune evasion by
572 parasitic nematodes is likely to have a significant genetic component, as
573 suggested by variation in immunogenicity and other life history traits
574 between strains (Grant, 1994; Paterson and Barber, 2007). In addition,
575 variation in fitness is likely to be influenced by non-genetic factors such as
576 age, which is associated with declining fecundity in female *T. tenuis* (Shaw
577 and Moss, 1989). Other explanations are therefore more plausible. *T. tenuis*,
578 in common with most parasites of vertebrates, live in an unpredictable
579 environment in the sense that each new generation is likely to encounter a
580 new host with a different set of immune defences. Such unpredictability
581 should also make promiscuous mating a beneficial strategy by maximising
582 the genetic variation among offspring and improving the chances of creating
583 optimal genotypes for an unknown future environment.

584 Absence of mate choice could also result from mechanistic
585 limitations. It is not known to what extent parasitic nematodes are able to
586 distinguish between mates, nor if either sex has the ability to control access
587 to matings. Pheromone-mediated sex attraction of females by males and of
588 males by females is widespread in animal parasitic nematodes (Bone and
589 Shorey, 1978), so pheromones provide a potential mechanism through
590 which mate choice could operate. The existence of sperm storage organs
591 also provides the opportunity for cryptic post-copulatory mate choice in
592 females and sperm competition between males. All nematodes have large

amoeboid sperm that are thought to compete for access to the spermatheca. Indeed, the evolution of amoeboid sperm in nematodes is thought to have been driven by sperm competition (LaMunyon and Ward, 1999; Snook, 2005).

In summary, we have used paternity analysis of a *T. tenuis* population to reveal a large number of missing males and patterns of mating consistent with random and promiscuous mating within caeca followed by post-copulatory variance in male reproductive success. The extent to which these patterns reflect general processes in parasitic nematodes—and in particular whether the assumption of random mating is justified—is difficult to discover by studying populations in wild hosts, but could perhaps more easily be tackled by genotyping the shed eggs of experimentally infected captive hosts.

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824 **Titles and legends to figures**

825

826 Figure 1. Posterior probability density of the number of unsampled males.

827

828 Figure 2. The distribution of the maximum posterior probability of paternity
829 among 150 *T. tenuis* larvae.

830

831 Figure 3. A pedigree showing the parentage of 71 larvae by 19 females (left)
832 and 32 sampled males (right). Paternity assignments with a maximum
833 probability of paternity exceeding 0.64 are shown, resulting in a mean
834 paternity assignment confidence of 90%. Parentage is indicated by solid
835 lines linking males and females, with numbers of offspring given above the
836 lines.

837

838 Figure 4. The observed distribution of reproductive success among sampled
839 males in the local caecum, and the expected distribution under the null
840 hypothesis of a random (Poisson) allocation. The mean (± SD) number of
841 males achieving a given number of units of reproductive success (measured
842 in paternities, A, and copulations, B) was estimated from 1000 MCMC
843 samples. I, I_s: standardized variance in reproductive success (95% CI) as
844 defined by Boness *et al.* (1993) and Wade & Arnold (1980) respectively.

845

Tables

Table 1. Numbers of eggs laid and larvae hatched by 51 female *T. tenuis* within 2.5 hours of isolation (early) and in the subsequent 48 hours (late).

Laying period	*n* (%) females laying eggs	*n* eggs laid	*n* (%) females producing larvae	*n* larvae hatched	Mean (SD) hatch rate[1]
Early	38 (74.5%)	517	22 (43.1%)	122	0.20 (0.24)
Late	43 (84.3%)	470	12 (23.5%)	32	0.06 (0.13)
Combined	43 (84.3%)	987	29 (56.9%)	154	0.15 (0.19)

[1]Mean (SD) hatch rate was calculated using only females who laid eggs in both laying periods (n = 38), and differed significantly between periods (P = 0.001, paired t-test).

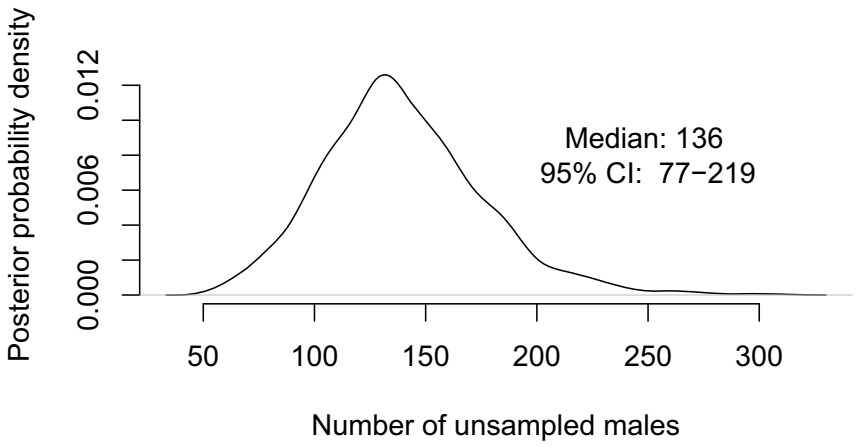
Table 2. Genetic diversity at the ten microsatellite marker loci assayed: number of individuals typed (*n*), number of alleles observed (N_a), expected heterozygosity (H_e; Nei, 1978), polymorphic information content (*PIC*; Botstein *et al*, 1980).

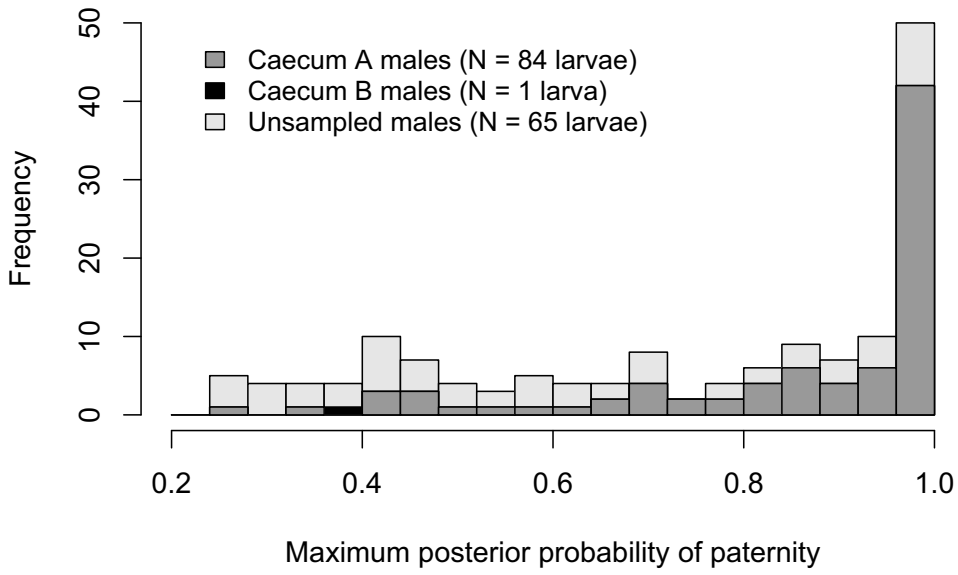
Locus	*n*	N_a	H_e	*PIC*
Tte002	453	14	0.72	0.68
Tte003	440	21	0.77	0.74
Tte057	428	18	0.78	0.76
Tte102	416	9	0.78	0.75
Tte134	437	15	0.85	0.83
Tte211	451	12	0.69	0.65
Tte254	420	19	0.80	0.77
Tte303	439	11	0.63	0.60
Tte335	458	9	0.75	0.72
Tte365	439	10	0.60	0.53
Mean	438	14	0.74	0.70

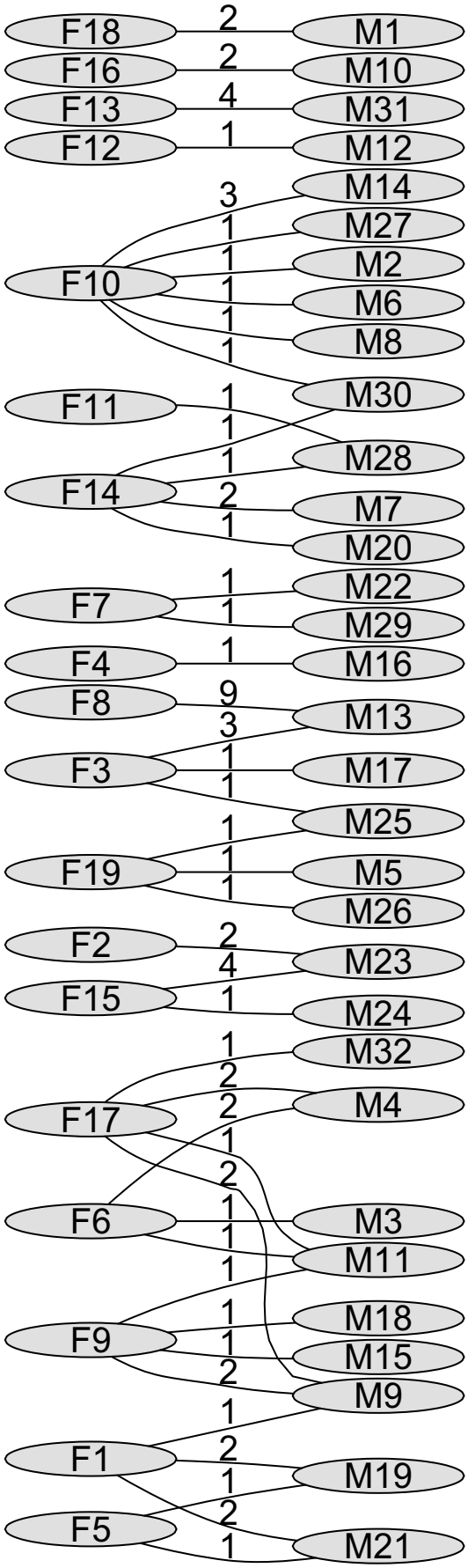
Table 3. Quantity and quality of microsatellite genotype data from *T. tenuis*: number of samples (*n*), number of genotypes attempted, proportion of loci and individuals successfully genotyped and genotyping error rates (with 95% credible intervals). Eleven males from the neighbouring caecum and four larvae that failed at all loci have been excluded from the table.

Generation	Caecum	*n*	*n* genotypes assayed (mean *n* per individual)	Proportion of loci typed, *n* = 469 individuals	Proportion of loci typed, *n* = 887 genotypes	*n* (%) individuals typed at ≥ 5 loci	Allelic dropout rate per allele (95% CI)	False allele rate per allele (95% CI)
Mother	Local	25[1]	75 (3.0)	84%	53%	22 (88%)	0.177 (0.163, 0.191)	0.019 (0.015, 0.023)
Larval offspring	Local	150	450 (3.0)	84%	55%	133 (89%)		
Candidate father	Local	122	148 (1.2)	96%	96%	122 (100%)	0.014 (0.009, 0.020)	0.006 (0.004, 0.010)
Candidate father	Neighbr.	172	214 (1.2)	98%	97%	170 (99%)		
All	Both	469	887 (1.9)	92%	72%	449 (96%)	0.082 (0.075, 0.090)	0.013 (0.010, 0.016)

[1]An additional four mothers whose offspring yielded no genotype data have been omitted from the table. They were nevertheless retained in the analysis because they provided additional information for estimating allele frequencies and error rates.


Posterior probability density
0.000
0.006
0.012
Median: 136
95% CI: 77−219
50
100
150
200
250
300
Number of unsampled males


Caecum A males (N = 84 larvae)
Caecum B males (N = 1 larva)
Unsampled males (N = 65 larvae)
Frequency
0
10
20
30
40
50
0.2
0.4
0.6
0.8
1.0
Maximum posterior probability of paternity


F18
M1
2
F16
M10
2
F13
M31
4
F12
M12
1
M14
M27
M2
M6
M8
M30
F10
3
1
1
1
1
1
F11
M28
F14
M7
M20
1
1
1
2
1
M22
M29
F7
1
1
F4
M16
1
F8
M13
9
M17
M25
F3
3
1
1
M5
M26
F19
1
1
1
F2
M23
2
F15
M24
4
1
M32
M4
F17
1
2
2
1
F6
M3
M11
2
1
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M9
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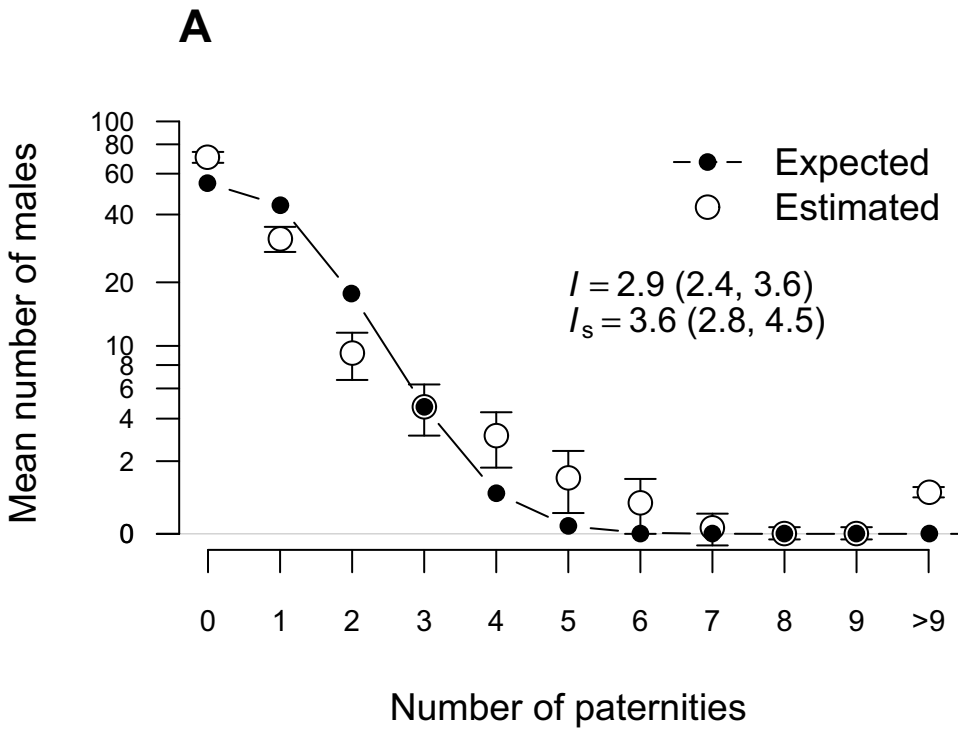
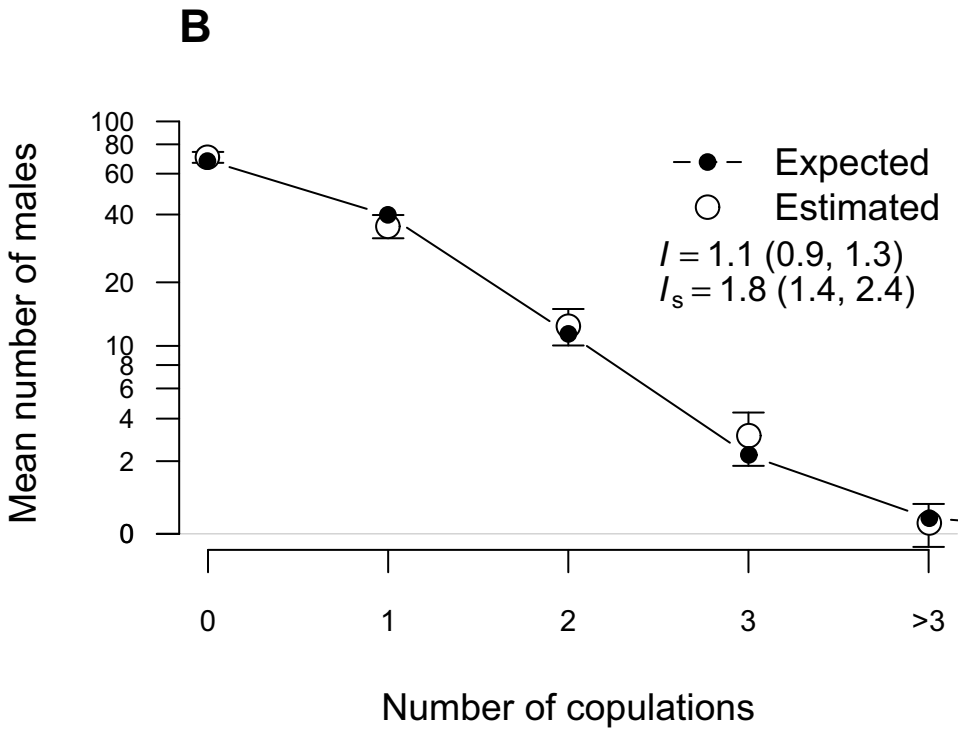
F1
M19
2
1
F5
M21
2
1


A
Mean number of males
100
80
60
40
20
10
8
6
4
2
0
Expected
Estimated
I = 2.9 (2.4, 3.6)
Is = 3.6 (2.8, 4.5)
0
1
2
3
4
5
6
7
8
9
>9
Number of paternities


B
Mean number of males
100
80
60
40
20
10
8
6
4
2
0
Expected
Estimated
I = 1.1 (0.9, 1.3)
Is = 1.8 (1.4, 2.4)
0
1
2
3
>3
Number of copulations