Prevalent sex ratio bias in *Caenorhabditis* nematodes

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Abstract

Although equal sex ratio represents an equilibrium in evolutionary theory, cases of sex ratio bias are accumulating, mostly reported in single species. Here, we surveyed progeny sex ratios in 23 species of the nematode genus *Caenorhabditis*. In experiments where males and females had unlimited access to each other (unlimited mating), we found 15 species out of the 23 species had female (hermaphrodite)-biased sex ratios. Phylogenetic mapping indicates female-bias to represent the ancestral state, with the occurrence of seven independent transitions from female-bias to non-bias along the phylogeny. As sperm competition could underlie the female bias, we also assayed progeny sex ratios in an experiment where mating was limited to a few hours. Of the 15 species that showed female-biased ratios under unlimited mating, six species showed no sex ratio bias when mating was limited. This result is consistent with sperm competition whereby X-bearing sperm take precedence over nullo-X during fertilization, yielding more female progeny when sperm are unlimited but equal sex ratio when sperm are limited. The other nine species showed sex ratio bias in both experiments, but the day-by-day profiles suggest sperm competition may also play a role. Our study shows that sex ratio evolution within *Caenorhabditis* nematodes is dynamic and that sex ratio bias is common not only in parasites as previously found but also in free-living nematodes. Our results also suggest that sperm competition could be a mechanism underlying sex ratio bias.

Key words:

sex ratio bias, sperm competition, mating, *Caenorhabditis*
Introduction

Sex ratio theory is at the core of evolutionary biology. First proposed by Charles Darwin (1, 2), and later formulated by Ronald Fisher (3), sex ratio theory posits that in a large, random mating population, when the sex ratio departs from equality, the rarer sex would have better mating prospects. Thus, genetic elements that favor the rarer sex would be favored by natural selection. Consequently, equal sex ratio should be restored and represents an evolutionary stable strategy. However, as many organisms have unique life histories, social structures and parental behaviors that do not meet Fisher’s assumptions, biased sex ratios exist and are intriguing examples to study the evolution of sex ratio (4).

Theories have been proposed to explain sex ratio bias based on the asymmetry of sex allocation and reproductive return between the two sexes in different scenarios (5, 6). Hamilton’s theory of local mate competition (LMC) predicts that in cases of highly structured populations and local mating, sex ratios would be biased toward females so that just enough males are produced to inseminate their female siblings (4). Similarly, theories predict that sex ratio bias may arise due to local resource competition, where the sex that only consumes local resources is reduced (7). In the case of cooperative breeding, sex ratio would be biased toward the helping sex (local resource enhancement, LRE) (8, 9). Moreover, Trivers and Willard proposed that females in good condition or of high social ranking would produce more male offspring as their sons would inherit their advantages and have above-average breeding success (10). These theories have successfully explained many empirical findings of sex ratio bias.

Examples of sex ratio bias across the unicellular and metazoan world are plentiful. For instance, female-biased sex ratios are commonly found in Apicomplexan parasites such as *Plasmodium malariae* (11-13) and *Toxoplasma gondii* (14), as well as in intestinal parasitic nematodes of *Heligmosomidae* (15, 16). In Hymenoptera insects, female-biased sex ratios are found and/or tested in fig wasps (17), parasitoid wasps of *Nasonia vitripennis* (18, 19), and the *Bethylidae* family (20). These female-biased sex ratios are good examples of LMC as these parasites are confined within their hosts. In birds, the Seychelles warbler shows facultative sex ratio bias in their offspring as they adjust production of helpers in relation to the quality of their territories (21, 22). Male-biased sex ratio was found in African wild dogs because male helpers
contribute to raising pups in the dens and increase pup survivorship (23). These are two typical examples of LRE. Female-biased sex ratios have also been observed in red deer and wild spider monkeys with subordinate females producing more daughters but high-ranking females producing more sons (24, 25), which fits the Trivers-Willard hypothesis.

Despite the mature theories for sex ratio bias and plenty of empirical examples, the evolutionary dynamics of sex ratio change above the species level is less understood. Because sex ratio is generally selected to maximize reproductive success, it can be regarded as an adaptive trait of sexually reproducing organisms. But how often and how fast do sex ratios evolve among diverging species, especially when they adapt to new environments and adopt new life histories? Does sex ratio bias evolve reversibly and repeatedly? To answer these questions, one needs to examine sex ratios across a lineage.

The genus *Caenorhabditis* provides a good opportunity to study the evolution of sex ratios as it is a species-rich genus (26), comprising ecologically diverse species with various life histories, population structures, and even reproductive modes (27). Most *Caenorhabditis* species have standard female-male reproduction (dioecy), however, three species, *C. elegans*, *C. briggsae* and *C. tropicalis*, have independently evolved reproduction through hermaphrodites and facultative males (androdioecy) (28, 29). Species of both reproductive modes in *Caenorhabditis* have the same chromosomal sex determination system with females (or hermaphrodites) being XX and males being XO (30). But unlike the male-female species that have obligate out-crossing, the androdioecious species have two ways of reproduction: a hermaphrodite can either self or out-cross with a male. Self-fertilization produces mostly hermaphrodites while males are produced by spontaneous nondisjunction of the X chromosome during meiosis at very low rates (31). On the other hand, outcrossing with a male should produce hermaphrodites and males in a 1:1 ratio, according to Mendel’s first rule. The common lab model, *C. elegans*, progenies derived from outcrossing display an equal sex ratio (selfing produces >99.5% hermaphrodites)(31, 32). In contrast, *C. briggsae*, yields a hermaphrodite-biased sex ratio upon outcrossing, putatively due to sperm competition where the male X-bearing sperm outcompeting the nullo-X counterpart (33). Knowledge of sex ratios and the underlying mechanisms in the other species of this genus is so far scarce.
In this study, we examined differences in progeny sex ratio across the genus *Caenorhabditis*. We first assayed the sex ratios where males and females (or hermaphrodites) have continuous access to each other throughout their lifetime ("unlimited mating experiment"). We mapped these observed sex ratios onto the phylogeny and inferred the ancestral state of sex ratio within this genus. As LaMunyon and Ward (1997) found sperm competition between male X-bearing and nullo-X sperm as a putative mechanism underlying the hermaphrodite-biased sex ratios in *C. briggsae* (33), we also investigated the role of sperm competition in sex ratio bias in the other *Caenorhabditis* species. In contrast to the unlimited mating experiment, we assayed the sex ratios by limiting mating to a short time interval so that with limited amounts of sperm, when X-bearing sperm are used up, the nullo-X sperm have a chance to catch up. Species exhibiting female-biased sex ratios in the unlimited mating experiment but no bias in the limited mating experiment suggest sperm competition as a mechanism explaining sex ratio bias. These surveys and analyses would enhance our understanding of the evolutionary dynamics of sex ratio within this diverse genus and also shed light on the mechanisms underlying sex ratio bias.
Results

Sex ratio bias is common in Caenorhabditis

To survey sex ratio bias within the Caenorhabditis genus, we conducted crosses in 23 species where females and males had continuous access to each other (unlimited mating experiment) and counted the numbers of female (hermaphrodite) versus male progeny produced. Fifteen of the 23 species tested exhibited sex ratio bias in this experiment (combined and adjusted \( P < 0.05 \), binomial test, Figure 1a, Table 1), all of which were female (hermaphrodite)-biased, with the median sex ratios ranging from 0.524 (C. latens) to 0.708 (C. doughertyi). In contrast, the eight species that did not show sex ratio bias had the median sex ratios ranging from 0.493 (C. elegans) to 0.550 (C. castelli).

Species with sex ratio bias do not cluster by phylogeny or reproductive mode

The species with sex ratio bias were scattered across the phylogeny, i.e., across the Elegans group, the Japonica group, and the Drosophilae supergroup. Ancestral trait reconstruction analysis assigned female-biased sex ratio as the ancestral states of the Elegans group, Japonica group, and the Drosophilae supergroup. Furthermore, the ancestral state of the Caenorhabditis genus was also assigned as female-biased (Figure 1b). Across the phylogeny, there was a total of seven transitions from female-biased to non-biased sex ratios, with four transitions in the Elegans group, one in the Japonica group, and two in the Drosophilae supergroup (Figure 1b). A single transition in the Elegans group resulted in one monophyletic clade of equal sex ratio, i.e., clade of C. wallacei and C. tropicalis. The other six transitions resulted in singleton species with equal sex ratio: C. sp. 41, C. brenneri, and C. elegans (Elegans group), C. panamensis (Japonica group), and C. virilis and C. castelli (Drosophilae supergroup).
Figure 1. Progeny sex ratios and the ancestral states of 23 *Caenorhabditis* species. (a) The proportion of females (hermaphrodites) in total progeny in the unlimited mating assay. For the species with multiple strains, only one tester strain is included in this figure. Each circle represents a replicate mating pair of the tester strain and the circle size denotes the total number of progeny. Bars represent median sex ratios. The asterisks indicate significant female (hermaphrodite) bias (combined and adjusted *P* < 0.05, binomial test). (b) The ancestral states of sex ratio bias constructed based on the phylogeny of the 23 species. The black branches represent sex ratio bias in the child nodes whereas the white branches represent equal sex ratio.
Furthermore, the sex ratio bias was not consistent across the hermaphroditic species. Of the three hermaphroditic species that independently evolved in the *Elegans* group (29), *C. briggsae* showed a hermaphrodite-biased sex ratio whereas *C. elegans* and *C. tropicalis* did not show sex ratio bias.

**Testing multiple strains within species reveals consistent sex ratios**

For the species with multiple strains, the sex ratios from different strains showed consistent bias or non-bias within the species. For example, for the four female-male species (*C. nigoni*, *C. latens*, *C. remanei*, and *C. sp. 33*), all strains from the respective species showed consistently biased sex ratios (*P* < 0.05 for the strains), except the *C. remanei* strain JU1084, which had a marginally non-biased sex ratio (*P* = 0.058). However, *C. nigoni*, *C. remanei*, and *C. sp. 33* had significantly different sex ratios between strains (*P* < 0.05, ANOVA test), whereas *C. latens* did not have significantly different sex ratios between strains (*P* = 0.824, ANOVA test).

To test the possibility that sex ratio bias was due to sex-specific consequences of inbreeding depression (34), for the four female-male species, for which we had multiple strains, (*C. nigoni*, *C. latens*, *C. remanei*, and *C. sp. 33*), we performed inter-stain crosses. The inter-strain crosses yielded consistent female-biased sex ratios, congruent with the intra-strain experiments above (combined *P* < 0.05, Table S1).

For the three androdioecious species, the multiple tester strains also showed consistent bias (for *C. briggsae*) or non-bias (for *C. elegans* and *C. tropicalis*). All the strains from *C. elegans* and *C. tropicalis*, respectively, did not have hermaphrodite-biased sex ratios (*P* > 0.05 for the strains), and the sex ratios were not significantly different between strains for both of these two species (*P* = 0.658 for *C. elegans* and *P* = 0.441 for *C. tropicalis*, ANOVA test). For 31 *C. briggsae* strains we surveyed in addition to the common lab strain AF16, 19 strains showed significant hermaphrodite-biased sex ratios (combined and adjusted *P* <0.05, binomial test,
Table S2), consistent with AF16. For the other 12 strains that did not have significant sex ratio bias, most of them still had more hermaphrodites than males for all replicates, except 1 out of 4 replicates in BRC20095, 1 out of 5 in BRC20299, 1 out of 5 in BRC20324, 1 out of 5 in BRC20334, 2 out of 5 in BRC20234 and 4 out of 8 in BRC20339. Thus, the majority of the C. briggsae strains had significantly hermaphrodite-biased sex ratios, whereas for the other strains, although there was no significant bias, there was a general tendency toward bias, except for BRC20234 and BRC20339.

Sex ratios in the limited mating experiment

To test whether sperm competition between X and nullo-X plays a role in progeny sex ratio bias, we conducted limited mating experiments to assay sex ratios. When exposed to males for only up to 5 hours, the females (or hermaphrodites) sired on average 51% (range 20-80%) fewer progeny (outcrossed progeny for androdioecious species) compared to unlimited mating, except for C. sinica and C. latens, which had comparable numbers of progeny between the two mating experiments (Table 1 and 2). Ten of the 23 species examined showed female(hermaphrodite)-biased sex ratios in the limited mating experiment (combined and adjusted $P < 0.05$, binomial test; Figure 2a, Table 2). Out of the 15 species that showed female-biased sex ratio in the unlimited mating experiment, nine were also found with female(hermaphrodite)-biased sex ratios in the limited mating experiment. The other six species (C. nigoni, C. zanzibari, C. sinica, C. remanei, C. becei, and C. portoensis) did not show significant sex ratio bias in the limited mating experiment. In contrast, C. sp. 41, which did not show sex-ratio bias in the unlimited mating experiment, showed significant female-biased sex ratio in the limited mating experiment (adjusted $P = 0.0002$). The remaining seven species consistently showed no bias in both the unlimited and limited mating experiments.
sex ratio bias in two experiments

- female bias in both
- non-bias in unlimited mating but female bias in limited mating
- non-bias in both

brood size

- 50
- 100
- 500

unlimited mating

| Species         | D0 | D1 | D2 | D3 | D4 | D5 |
|-----------------|----|----|----|----|----|----|
| nigoni          | 0  | 0  | 0  | 0  | 0  | 0  |
| briggsae        | 0  | 0  | 0  | 0  | 0  | 0  |
| zanzibari       | 0  | 0  | 0  | 0  | 0  | 0  |
| sp41            | 0  | 0  | 0  | 0  | 0  | 0  |
| latens          | 0  | 0  | 0  | 0  | 0  | 0  |
| remanei         | 0  | 0  | 0  | 0  | 0  | 0  |
| sp33            | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboe        | 0  | 0  | 0  | 0  | 0  | 0  |
| brentesi        | 0  | 0  | 0  | 0  | 0  | 0  |
| dougheerti      | 0  | 0  | 0  | 0  | 0  | 0  |
| sp44            | 0  | 0  | 0  | 0  | 0  | 0  |
| elegans         | 0  | 0  | 0  | 0  | 0  | 0  |
| brock           | 0  | 0  | 0  | 0  | 0  | 0  |
| imperialis      | 0  | 0  | 0  | 0  | 0  | 0  |
| virility        | 0  | 0  | 0  | 0  | 0  | 0  |
| wittapini       | 0  | 0  | 0  | 0  | 0  | 0  |
| castelli        | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboebuli    | 0  | 0  | 0  | 0  | 0  | 0  |

limited mating

| Species         | D0 | D1 | D2 | D3 | D4 | D5 |
|-----------------|----|----|----|----|----|----|
| nigoni          | 0  | 0  | 0  | 0  | 0  | 0  |
| briggsae        | 0  | 0  | 0  | 0  | 0  | 0  |
| zanzibari       | 0  | 0  | 0  | 0  | 0  | 0  |
| sp41            | 0  | 0  | 0  | 0  | 0  | 0  |
| latens          | 0  | 0  | 0  | 0  | 0  | 0  |
| remanei         | 0  | 0  | 0  | 0  | 0  | 0  |
| sp33            | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboe        | 0  | 0  | 0  | 0  | 0  | 0  |
| brentesi        | 0  | 0  | 0  | 0  | 0  | 0  |
| dougheerti      | 0  | 0  | 0  | 0  | 0  | 0  |
| sp44            | 0  | 0  | 0  | 0  | 0  | 0  |
| elegans         | 0  | 0  | 0  | 0  | 0  | 0  |
| brock           | 0  | 0  | 0  | 0  | 0  | 0  |
| imperialis      | 0  | 0  | 0  | 0  | 0  | 0  |
| virility        | 0  | 0  | 0  | 0  | 0  | 0  |
| wittapini       | 0  | 0  | 0  | 0  | 0  | 0  |
| castelli        | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboebuli    | 0  | 0  | 0  | 0  | 0  | 0  |

unlimited mating

| Species         | D0 | D1 | D2 | D3 | D4 | D5 |
|-----------------|----|----|----|----|----|----|
| nigoni          | 0  | 0  | 0  | 0  | 0  | 0  |
| briggsae        | 0  | 0  | 0  | 0  | 0  | 0  |
| zanzibari       | 0  | 0  | 0  | 0  | 0  | 0  |
| sp41            | 0  | 0  | 0  | 0  | 0  | 0  |
| latens          | 0  | 0  | 0  | 0  | 0  | 0  |
| remanei         | 0  | 0  | 0  | 0  | 0  | 0  |
| sp33            | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboe        | 0  | 0  | 0  | 0  | 0  | 0  |
| brentesi        | 0  | 0  | 0  | 0  | 0  | 0  |
| dougheerti      | 0  | 0  | 0  | 0  | 0  | 0  |
| sp44            | 0  | 0  | 0  | 0  | 0  | 0  |
| elegans         | 0  | 0  | 0  | 0  | 0  | 0  |
| brock           | 0  | 0  | 0  | 0  | 0  | 0  |
| imperialis      | 0  | 0  | 0  | 0  | 0  | 0  |
| virility        | 0  | 0  | 0  | 0  | 0  | 0  |
| wittapini       | 0  | 0  | 0  | 0  | 0  | 0  |
| castelli        | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboebuli    | 0  | 0  | 0  | 0  | 0  | 0  |

limited mating

| Species         | D0 | D1 | D2 | D3 | D4 | D5 |
|-----------------|----|----|----|----|----|----|
| nigoni          | 0  | 0  | 0  | 0  | 0  | 0  |
| briggsae        | 0  | 0  | 0  | 0  | 0  | 0  |
| zanzibari       | 0  | 0  | 0  | 0  | 0  | 0  |
| sp41            | 0  | 0  | 0  | 0  | 0  | 0  |
| latens          | 0  | 0  | 0  | 0  | 0  | 0  |
| remanei         | 0  | 0  | 0  | 0  | 0  | 0  |
| sp33            | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboe        | 0  | 0  | 0  | 0  | 0  | 0  |
| brentesi        | 0  | 0  | 0  | 0  | 0  | 0  |
| dougheerti      | 0  | 0  | 0  | 0  | 0  | 0  |
| sp44            | 0  | 0  | 0  | 0  | 0  | 0  |
| elegans         | 0  | 0  | 0  | 0  | 0  | 0  |
| brock           | 0  | 0  | 0  | 0  | 0  | 0  |
| imperialis      | 0  | 0  | 0  | 0  | 0  | 0  |
| virility        | 0  | 0  | 0  | 0  | 0  | 0  |
| wittapini       | 0  | 0  | 0  | 0  | 0  | 0  |
| castelli        | 0  | 0  | 0  | 0  | 0  | 0  |
| wallaboebuli    | 0  | 0  | 0  | 0  | 0  | 0  |
Figure 2. Progeny sex ratios in the limited mating assay. (a) The proportion of females (hermaphrodites) in total progeny after limited mating. Each circle represents a replicate mating pair of the tester strain of the species and the circle size denotes the total number of progeny. Black bars represent median sex ratios. The asterisks indicate significant female (hermaphrodite) bias (combined and adjusted $P < 0.05$, binomial test). The 23 species are categorized into 4 groups: significant female bias in both unlimited mating and limited mating experiments (red), significant female bias in unlimited mating but not in limited mating (purple), significant female bias in limited mating but not in unlimited mating (brown), and no bias in both experiments (blue). (b) Day-by-day sex ratios of species of the 4 categories. Each line represents a species. The points represent mean sex ratios per day across replicates and the error bars represent standard error of the mean. The day-by-day sex ratios calculated from less than 10 progeny are excluded. Note that for the red, purple and blue panels, only 4 exemplary species are displayed. Other species are presented in Figure S1.

Examine sex ratios by day shows heterogenous profiles across species. For the nine species with female(hermaphrodite)-bias in both the unlimited mating and limited mating experiments, the sex ratios per day show strongest female(hermaphrodite)-bias at the beginning and then a diminishing bias (with some fluctuations) to about or below 50% at the end of reproduction in both experiments (Figure 2b red panel). For the six species with female bias in the unlimited mating experiment but not in the limited mating experiment, the sex ratios were female-biased throughout the reproductive period under unlimited mating but fluctuating around 50% under limited mating (Figure 2b purple panel). Amongst the species with no sex ratio bias in unlimited mating, C. sp. 41 was the only species that had female bias in limited mating. The unlimited mating of C. sp. 41 showed a fluctuating sex ratio around 50% throughout the reproductive period, while the two-day profile of 5-hour mating showed a female bias on the first day and a decrease to 50% on the second day (Figure 2b brown panel). The seven species with no sex ratio bias in either experiment had sex ratios fluctuating around 50% throughout the reproductive period in both experiments (Figure 2b blue panel).
Discussion

We found prevalent female sex ratio bias (15 out of 23) among the *Caenorhabditis* species and none in the other direction. The ancestral state was inferred to be female biased, with seven transitions from female bias to no bias in the phylogeny. While most species (16 out of 23) showed consistent sex ratio bias or no bias between the unlimited mating and limited mating experiments, six species had a female(hermaphrodite)-biased sex ratio in the unlimited experiment but no bias in the limited mating experiment, consistent with sperm competition as a possible explanation. Our genus-wide survey of progeny sex ratios sheds light on the evolution of sex ratio bias in this genus.

Female-biased sex ratios are frequently found in parasitic nematodes (15, 35-37). Theory predicts female-biased sex ratios in parasites due to confined dispersion and high inbreeding (4, 38, 39). We found that the species of the free-living nematode *Caenorhabditis* also show prevalent female-biased sex ratios. Though being free-living and dwelling in diverse habitats, many *Caenorhabditis* species probably share the features of life history such as boom-and-bust population growth in ephemeral habitats, active dispersal seeking, and strong founder effect followed by population re-expansion, such as found in extensive sampling of *C. elegans* (40-42). These life history features may result in high inbreeding rates and intense local competition for mating between kin. Hence, a female(hermaphrodite)-biased sex ratio may be favored, according to the theory of LMC. Compared to their parasitic relatives, despite the very distinct life styles, sex ratio bias may have evolved in parallel in the free-living *Caenorhabditis* species. Alternatively, sex ratio bias is widely conserved across diverse nematode taxa. This hypothesis could be tested with a broader survey of sex ratios in free-living nematode species outside of *Caenorhabditis* genus.

Based on the prevalent sex ratio bias detected in these 23 species, the ancestral state was inferred to be female-biased, with seven transitions from female bias to equal sex ratio along
the phylogeny. This suggests that sex ratio bias could be a phenotype that can be frequently gained or lost, possibly reflecting the adaptation to the respective habitats and life histories in these species. Despite common features of their life styles, Caenorhabditis species dwell in ecologically diverse habitats, ranging from cattle auditory canals to rotting fruits and man-made compost (27). Except for a few species that have been sampled extensively, such as C. elegans, C. briggsae, and C. remanei, (40, 43-45), the natural habitats of most Caenorhabditis species are largely unknown, mainly because they have been sampled very rarely. More knowledge about the ecology and natural history of Caenorhabditis species might thus be able to explain the driving forces of sex ratio evolution in these species.

Of the three androdioecious Caenorhabditis species, C. elegans, C. briggsae, and C. tropicalis, only C. briggsae showed a hermaphrodite-biased sex ratio in out-crossed progeny. As the hermaphrodites can self-fertilize, the role of males in these species is obscure (46) but is likely important for rapid adaptation, such as to pathogens (47). Production of male progeny would take up brood "quota" but does not directly contribute to population growth (48). Field studies of C. elegans have rarely found males in the wild, and wild C. elegans largely suffer from outcrossing depression (49-51). Thus, a hermaphrodite-biased sex ratio may be favored in androdioecious species. However, C. elegans and C. tropicalis showed no hermaphrodite-biased sex ratio, suggesting unknown ecological factors or historical contingencies that may be contributing to offspring sex ratios in these hermaphroditic species.

Our strategy of a broad survey across species with limited diversity of strains within species assumes that the sex ratio status of the tester strain is representative for each species. An alternative scenario is that sex ratio is a trans-species polymorphism in the genus. As we used a single strain for the sex ratio assays for most species (16 of 23), we cannot exclude the possibility that, for some of these species, the sex ratio bias we detected was specific to those
strains. However, for the four female-male species for which we did have multiple strains, we tested sex ratios for multiple strains as well as for inter-strain crosses. We found that both the intra-strain and inter-strain crosses yielded qualitatively consistent sex ratio bias within species. For the three androdioecious species, the sex ratios of multiple strains also showed consistent non-bias in *C. elegans* and *C. tropicalis*. With an extensive survey of *C. briggsae* strains, we found that most of the strains had significantly hermaphrodite-biased sex ratios, consistent with the common lab strain AF16. Although the other *C. briggsae* strains did not have significant sex ratio bias, they mostly had more hermaphrodites than males in the replicates, except a few outlier strains. These results together suggest that the prevalent sex ratio bias in *Caenorhabditis* nematodes is rather a stable trait within species rather than a trans-species polymorphic trait.

Despite the qualitative consistency in female bias, *C. nigoni*, *C. remanei*, and *C. sp. 33* showed quantitative differences in sex ratios between strains, as indicated by ANOVA tests, suggesting a contribution of genetic differences in the female-biased sex ratios. On the other hand, *C. latens*, *C. elegans*, and *C. tropicalis* had quantitatively constant sex ratios among strains, suggesting genetic constraints that govern the bias or non-bias.

To investigate sperm competition as a potential mechanism underlying the sex ratio bias, as previously found in *C. briggsae* (33), we conducted mating experiments where mating was limited for a few hours as opposed to the unlimited mating experiments where mating was allowed for the entirety of adulthood. A contrast of female biased sex ratios in unlimited mating experiment and an equal sex ratio in limited mating experiment suggests sperm competition. In the unlimited mating experiments, the couples probably mated repeatedly and the X-bearing sperm would be refilled and therefore X-bearing sperm would always take precedence over the nullo-X sperm, resulting in an overall female-biased sex ratio. In contrast,
in the limited mating experiments, the amount of sperm transferred was limited and all sperm were presumably used, resulting in an overall equal sex ratio. Consistent with this sperm competition model, six of the 15 species that showed significant female-biased sex ratio in unlimited mating did not have a sex ratio bias in limited mating. The day-by-day sex ratios of these six species also show female bias in unlimited mating throughout the reproductive period and equal sex ratio in limited mating, consistent with the sperm competition scenario. On the other hand, nine species showed female bias in both mating experiments. The day-by-day profile of these nine species in both experiments showed female bias at the beginning and then declined to equal sex ratio or male bias in the following days. These observations suggest that sperm competition plays, at least, a partial role in contributing to female-biased sex ratios in both experiments. In *C. briggsae*, LaMunyon and Ward (1997) conducted 3-hour mating as well as 8-hour mating experiments and found the overall sex ratio was equal after 3-hour mating whereas it was hermaphrodite-biased after 8-hour mating (33). Here, we found a hermaphrodite-biased sex ratio after 5 hours of mating. These together suggest that the amount of sperm ejaculated into the hermaphrodite is a limiting factor for sperm competition and hence sex ratio bias. In addition, we found one species, *C*. sp. 41, which had no sex ratio bias under unlimited mating conditions while limited mating yielded a female bias. The day-by-day sex ratios show a slightly female-biased sex ratio on the first day in both experiments, suggesting sperm competition may be present but very weak in this species. Finally, the remaining nine species did not have sex ratio bias in either mating experiments, and the day-by-day sex ratios fluctuated around 50:50. Thus, there is no indication of sperm competition in these species.

The presence of selfish genetic elements could provide an alternative mechanism for sex ratio bias where the proportion of X-bearing sperm is selfishly enhanced in some species. Examples include *segregation distorter* on the X chromosome of *Drosophila simulans* (52) and
asymmetric division in spermatocytes in a *Rhabditis* nematode (53). So far, several selfish elements have been discovered in *Caenorhabditis* nematodes, but they all reside on autosomes (54-57).

While equal sex ratios are presumably predominant in nature, biased sex ratios may be largely underappreciated. In this study, we carried out a broad survey of sex ratio bias in outcrossed progeny across *Caenorhabditis* nematodes. Our findings of prevalent sex ratio bias add to the limited knowledge about sex ratio bias in the animal kingdom, and provide evidence that sex ratio bias can evolve rapidly within a single genus.

**Materials and Methods**

**Species and culture**

We examined 23 *Caenorhabditis* species for progeny sex ratio in this study (Table 1), including four new species: C. sp. 33, C. sp. 41, C. sp. 44, and C. sp. 45. These four new species were placed onto the phylogenetic tree based on their ITS2 sequences (the intergenic region between the 5.8S and LSU rRNA genes) (58). These 23 species comprise 17 species from the *Elegans* supergroup and 6 from the *Drosophila* supergroup. Of the 17 species from the *Elegans* supergroup, three were from the *Japonica* group and the rest were from the *Elegans* group, including the three androdioecious species (*C. elegans*, *C. briggsae*, and *C. tropicalis*). All species were grown at room temperature (23-24°C) on nematode growth media agar plates seeded with OP50 *Escherichia coli* bacteria.

**Sex ratio assay**

**Mating experimental design**

For the female-male species, sex ratios were assayed by intra-strain crosses, i.e., females and males from the same wild-type isofemale strains (tester strains). For most of the
female-male species, we had only one tester strain, except for four of the species (C. nigoni, C. latens, C. remanei, and C. sp. 33). For these four species we had more than one strain available at the start of this experiment, so the sex ratios of intra-strain crosses were tested for multiple strains. We also conducted inter-strain crosses for these four species to test the possibility that sex ratio bias was due to sex-specific consequences of inbreeding depression (34). For C. nigoni, for which we had two strains, we intercrossed the two strains and then crossed the heterozygous F1 males with the maternal strain. For the three species, C. latens, C. remanei, and C. sp. 33, for which we had three strains, we first crossed two strains and then crossed the heterozygous F1 males with the third strain (Table S1). We scored the two sexes in the F2 progeny.

For the androdioecious species, we crossed males from wild-type strains (tester strain) to hermaphrodite strains carrying a recessive mutation, so that the outcrossed progeny were visually identifiable. The recessive morphological mutant strains had Uncoordinated (Unc) or Dumpy (Dpy) phenotypes: C. elegans (BRC0189, unc-119(ed9)); C. briggsae (BRC0258, unc(ant10)); and C. tropicalis (BRC0419, dpy(ant23)). For each of these androdioecious species, we had multiple tester strains, especially for C. briggsae, for which we had many isolates collected in Taiwan. Because androdioecious species are normally inbred, we tested sex ratios using males from different strains but did not generate heterozygous F1 males to test an inter-strain effect.

Time limitations for mating

We set up mating experiments to assay sex ratio in the same manner across all the species tested. In the "unlimited mating" experiment, we placed one L4 female (or hermaphrodite) and one L4 male on a fresh 55 mm diameter Petri plate and then transferred them together every one or two days to fresh plates until they died or produced no more eggs. When the progeny reached the L4 or adult stage, the numbers of outcrossed females (or hermaphrodites) and males per plate were manually scored under the microscope. For the male-female species, all progeny were counted, while for the androdioecious species,
only wild-type cross progeny were counted whereas selfed Unc or Dpy progeny were ignored. Mating pairs with total number of out-crossed progeny smaller than 40 were excluded from further analyses to ensure adequate statistical power. For each test of the strains, we had at least three replicate mating pairs. The counts of progeny of the two sexes for each replicate were used for further statistical analyses (see below).

In addition to the unlimited mating experiment, to interrogate the potential role of competition between male X-bearing sperm and the nullo-X counterpart in progeny sex ratio bias, we conducted sex ratio assays by "limited mating" for one tester strain per species. To do so, L4 females (hermaphrodites) and L4 males were isolated one day prior to the cross to ensure their virginity and matured singly overnight. The next day, one male was added to one isolated female (hermaphrodite). The male was removed when mating plugs were observed on the females (hermaphrodites) or after five hours. Mated females (hermaphrodites) were transferred daily to new plates. For *C. tropicalis*, all pairs failed to mate within five hours, so we crossed one hermaphrodite with three males to increase the chance of mating. The progeny sex ratios were scored as above.

**Statistical analysis**

For each of the sex ratio assays, unlimited mating or limited mating, we tested whether the progeny sex ratio was biased. For each replicate within the tester strain, the counts of total females (hermaphrodites) and total males were used for the binomial test. As the majority of crosses yielded more female (hermaphrodite) than male progeny, we tested whether the proportions of females (hermaphrodites) significantly exceeded equality (R, `binom.test`, alternative = "greater"). The *P*-values of the replicates within a strain were corrected for multiple testing for the numbers of replicates using the Benjamini-Hochberg method (59) and then combined to yield the overall *P*-value for the strain (Fisher’s method, R package `metaseqR` (60)). For species with only one strain, the strain *P*-value was used to represent the species. For species with multiple strains, the *P*-values of the strains were again corrected for multiple testing using the Benjamini-Hochberg method and then combined using Fisher’s method to yield an overall *P*-value for the species.
corrected for multiple strains and then combined to yield the $P$-value for the species. The species $P$-values were corrected a final time for multiple testing for the 23 species. Species with the corrected $P$-values smaller than 0.05 were defined as having sex ratio bias. Based on the states of sex ratio bias or non-bias of the 23 species (unlimited mating) and the phylogenetic tree (61), we inferred the ancestral state of sex ratio bias and the evolutionary transitions between bias and non-bias among these species, using the maximum parsimony method in MESQUITE v. 3.10 (62). In addition, for species with multiple strains, we applied ANOVA to test if sex ratios are significantly different between strains. All statistical analyses were performed in R v.3.2.3 except otherwise indicated (63).

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Author contributions

Y.-H.L., and J.W. conceived and designed the study. Y.-H.L, T.S.L, J.-C.H., F.-J.Y., T.C., and J.W. conducted experiments. C.B. contributed strains and help revised the manuscript. Y.H. and Y.-H.L. analyzed the data. Y.-H.L., Y.H., and J.W wrote the manuscript with input from others.

Competing interest

The authors declare that they have no conflict of interest.

Tables
| species      | strain     | no. of replicates | median brood size | median sex ratio | binomial test p value (strain) | sex ratio (species) | combined p value (species) | female bias |
|--------------|------------|-------------------|-------------------|------------------|--------------------------------|---------------------|---------------------------|-------------|
| nigoni       | BRC20235   | 4                 | 379.5             | 0.590            | 0.000                          | 0.562               | 0.000                     | yes         |
|              | BRC20079   | 5                 | 695               | 0.534            | 0.004                          | 0.656               | 0.000                     | yes         |
| briggsae     | BRC20266   | 5                 | 107               | 0.656            | 0.000                          | 0.656               | 0.000                     | no          |
| zanzibari    | BRC20243   | 4                 | 149               | 0.525            | 0.037                          | 0.525               | 0.061                     | no          |
| sinica       | BRC20276   | 9                 | 647               | 0.523            | 0.372                          | 0.523               | 0.475                     | no          |
|              | NIC1200    | 6                 | 687.5             | 0.525            | 0.000                          | 0.524               | 0.000                     | yes         |
| latens       | NIC1201    | 5                 | 400               | 0.538            | 0.000                          | 0.524               | 0.000                     | yes         |
|              | NIC1207    | 6                 | 392.5             | 0.509            | 0.000                          | 0.509               | 0.000                     | yes         |
|              | BRC20108   | 4                 | 232               | 0.600            | 0.000                          | 0.600               | 0.000                     | no          |
| remanei      | JU1084     | 5                 | 854               | 0.511            | 0.058                          | 0.556               | 0.000                     | yes         |
|              | MY31       | 5                 | 563               | 0.559            | 0.000                          | 0.559               | 0.000                     | yes         |
|              | BRC20005   | 5                 | 539               | 0.540            | 0.002                          | 0.540               | 0.002                     | yes         |
| sp33         | BRC20258   | 5                 | 497               | 0.635            | 0.000                          | 0.635               | 0.000                     | yes         |
|              | BRC20273   | 5                 | 344               | 0.558            | 0.000                          | 0.558               | 0.000                     | yes         |
| wallacei     | JU1904     | 6                 | 109               | 0.516            | 0.978                          | 0.516               | 1.000                     | no          |
|              | BRC20400   | 8                 | 211               | 0.545            | 0.097                          | 0.545               | 0.097                     | no          |
| tropicalis   | JU1373     | 11                | 162               | 0.503            | 0.877                          | 0.533               | 0.652                     | no          |
|              | NIC58      | 3                 | 60                | 0.550            | 0.235                          | 0.550               | 0.235                     | no          |
| brenneri     | JU1397     | 5                 | 326               | 0.519            | 0.820                          | 0.519               | 0.993                     | no          |
| doughertyi   | JU1333     | 1                 | 265.5             | 0.745            | 0.000                          | 0.745               | 0.000                     | yes         |
| sp44         | BRC20300   | 5                 | 342               | 0.576            | 0.000                          | 0.576               | 0.000                     | yes         |
|              | AB1        | 4                 | 243               | 0.487            | 1.000                          | 0.487               | 1.000                     | yes         |
| elegans      | BRC20067   | 4                 | 123               | 0.490            | 0.997                          | 0.493               | 1.000                     | no          |
|              | N2         | 3                 | 549               | 0.503            | 0.828                          | 0.503               | 0.828                     | yes         |
| becei        | BRC20007   | 6                 | 706               | 0.600            | 0.000                          | 0.600               | 0.000                     | yes         |
| panamensis   | BRC20006   | 4                 | 681.5             | 0.510            | 0.222                          | 0.510               | 0.407                     | no          |
| imperialis   | JU1905     | 5                 | 402               | 0.570            | 0.000                          | 0.570               | 0.000                     | yes         |
| virilis      | JU1968     | 4                 | 274               | 0.521            | 0.337                          | 0.521               | 0.521                     | No          |
| vivipara     | NIC1070    | 4                 | 392               | 0.633            | 0.000                          | 0.633               | 0.000                     | yes         |
| castelli     | JU1427     | 4                 | 193.5             | 0.550            | 0.071                          | 0.550               | 0.167                     | no          |
| waitukubuli  | NIC564     | 5                 | 341               | 0.558            | 0.000                          | 0.558               | 0.000                     | yes         |
| portoensis   | EG4788     | 8                 | 419               | 0.591            | 0.000                          | 0.591               | 0.000                     | yes         |
| sp45         | NIC759     | 5                 | 369               | 0.618            | 0.000                          | 0.618               | 0.000                     | yes         |
Table 2. Sex ratios in the limited mating experiment

| species      | strain   | no. of replicates | median brood size | median sex ratio | binomial test combined p value | binomial test adjusted p value | female bias |
|--------------|----------|-------------------|-------------------|------------------|-------------------------------|-------------------------------|-------------|
| nigoni       | BRC20235 | 9                 | 162               | 0.54             | 1.34E-01                      | 2.57E-01                      | no          |
| briggsae     | AF16     | 8                 | 81                | 0.58             | 1.35E-02                      | 3.44E-02                      | yes         |
| zanzibari    | BRC20266 | 10                | 219.5             | 0.524            | 7.24E-01                      | 9.80E-01                      | no          |
| sinica       | BRC20243 | 9                 | 217               | 0.521            | 5.14E-01                      | 7.88E-01                      | no          |
| sp41         | BRC20276 | 8                 | 191.5             | 0.546            | 1.42E-04                      | 1.09E-03                      | yes         |
| latens       | NIC1207  | 10                | 378               | 0.521            | 6.20E-03                      | 2.04E-02                      | yes         |
| remanei      | BRC20108 | 11                | 90                | 0.508            | 6.50E-01                      | 9.34E-01                      | no          |
| sp33         | BRC20005 | 9                 | 134               | 0.616            | 1.26E-07                      | 2.90E-06                      | yes         |
| wallacei     | JU1904   | 9                 | 53                | 0.528            | 9.62E-01                      | 9.88E-01                      | no          |
| tropicalis   | JU1373   | 10                | 118.5             | 0.512            | 9.88E-01                      | 9.88E-01                      | no          |
| brenneri     | JU1397   | 5                 | 150               | 0.49             | 9.69E-01                      | 9.88E-01                      | no          |
| doughertyi   | JU1333   | 8                 | 190.5             | 0.558            | 4.87E-04                      | 2.80E-03                      | yes         |
| sp44         | BRC20300 | 10                | 171.5             | 0.57             | 5.69E-05                      | 6.54E-04                      | yes         |
| elegans      | N2       | 8                 | 101.5             | 0.512            | 9.36E-01                      | 9.88E-01                      | no          |
| becei        | QG704    | 15                | 139               | 0.519            | 1.87E-01                      | 3.30E-01                      | no          |
| panamensis   | QG702    | 9                 | 286               | 0.514            | 8.23E-01                      | 9.88E-01                      | no          |
| imperialis   | JU1905   | 10                | 203               | 0.556            | 1.29E-03                      | 4.93E-03                      | yes         |
| virilis      | JU1968   | 9                 | 72                | 0.529            | 8.25E-01                      | 9.88E-01                      | no          |
| vivipara     | NIC1070  | 8                 | 145.5             | 0.561            | 7.31E-03                      | 2.10E-02                      | yes         |
| castelli     | JU1427   | 7                 | 64                | 0.548            | 2.20E-01                      | 3.62E-01                      | no          |
| waitukubuli  | NIC564   | 7                 | 95                | 0.509            | 9.45E-04                      | 4.35E-03                      | yes         |
| portoensis   | EG4788   | 6                 | 171.5             | 0.535            | 1.21E-01                      | 2.52E-01                      | no          |
| sp45         | NIC759   | 7                 | 105               | 0.549            | 1.68E-02                      | 3.87E-02                      | yes         |
Supplementary Figures and Tables

Figure S1. Day-by-day sex ratios of species not included in Figure 2
Table S1. Sex ratios of inter-stain crosses
Table S2. Sex ratios of 31 C. briggsae strains
Table S3. Day-by-day sex ratios by replicates of each species (unlimited mating)
Table S4. Day-by-day sex ratios by replicates of each species (limited mating)

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sex ratio bias in two experiments

- female–bias in both
- non–bias in unlimited mating but female–bias in limited mating
- non–bias in both
- female–bias in unlimited mating but non–bias in limited mating

brood size
- 50
- 100
- 500
Prevalent sex ratio bias in *Caenorhabditis* nematodes

Supplementary Information

Figure S1. Day-by-day sex ratios of species not included in Figure 2
Table S1. Sex ratios of inter-strain crosses

| species   | rplc | paternal_grandma | paternal_grandpa | maternal_stain | total | proportion_female | p.binomial | p.adjusted |
|-----------|------|------------------|------------------|----------------|-------|------------------|------------|------------|
| nigoni    | NN   | BRC20235         | BRC20079         | BRC20235       | 410   | 0.495            | 0.598      | 0.598      |
| nigoni    | OO   | BRC20235         | BRC20079         | BRC20235       | 296   | 0.551            | 0.046      | 0.069      |
| nigoni    | PP   | BRC20235         | BRC20079         | BRC20235       | 321   | 0.564            | 0.013      | 0.025      |
| nigoni    | KK   | BRC20079         | BRC20235         | BRC20079       | 523   | 0.528            | 0.110      | 0.132      |
| nigoni    | LL   | BRC20079         | BRC20235         | BRC20079       | 565   | 0.577            | 0.000      | 0.000      |
| nigoni    | HH   | BRC20079         | BRC20235         | BRC20079       | 572   | 0.601            | 0.000      | 0.000      |
| latens    | E    | NIC1201          | NIC1207          | NIC1200        | 95    | 0.589            | 0.050      | 0.060      |
| latens    | D    | NIC1201          | NIC1207          | NIC1200        | 482   | 0.541            | 0.038      | 0.057      |
| latens    | F    | NIC1201          | NIC1207          | NIC1200        | 469   | 0.563            | 0.004      | 0.007      |
| latens    | A    | NIC1207          | NIC1201          | NIC1200        | 303   | 0.584            | 0.002      | 0.006      |
| latens    | B    | NIC1207          | NIC1201          | NIC1200        | 590   | 0.603            | 0.000      | 0.000      |
| latens    | C    | NIC1207          | NIC1201          | NIC1200        | 33    | 0.485            | 0.636      | 0.636      |
| sp33      | A    | BRC20258         | BRC20005         | BRC20273       | 237   | 0.599            | 0.001      | 0.003      |
| sp33      | D    | BRC20258         | BRC20005         | BRC20273       | 112   | 0.669            | 0.000      | 0.001      |
| sp33      | E    | BRC20005         | BRC20258         | BRC20273       | 90    | 0.611            | 0.022      | 0.033      |
| sp33      | F    | BRC20005         | BRC20258         | BRC20273       | 284   | 0.679            | 0.000      | 0.000      |
| remanei   | A    | my31             | BRC20108         | JU2084         | 417   | 0.535            | 0.085      | 0.102      |
| remanei   | B    | my31             | BRC20108         | JU2084         | 570   | 0.565            | 0.001      | 0.002      |
| remanei   | GG   | my31             | BRC20108         | JU2084         | 656   | 0.550            | 0.006      | 0.008      |
| remanei   | JJ   | BRC20108         | my31             | JU2084         | 250   | 0.656            | 0.000      | 0.000      |
| remanei   | KK   | BRC20108         | my31             | JU2084         | 346   | 0.604            | 0.000      | 0.000      |
Table S2. Sex ratios of 31 *C. briggsae* strains

| strain  | no.rplc | median brood size | median sex ratio | p.combined | p.adjusted |
|---------|---------|------------------|------------------|------------|------------|
| BRC20069 | 5       | 145              | 0.592            | 0.000      | 0.001      |
| BRC20076 | 6       | 108.5            | 0.529            | 0.000      | 0.000      |
| BRC20085 | 4       | 71               | 0.594            | 0.015      | 0.024      |
| BRC20088 | 3       | 117              | 0.548            | 0.045      | 0.061      |
| BRC20092 | 4       | 122              | 0.542            | 0.213      | 0.236      |
| BRC20093 | 4       | 156              | 0.589            | 0.000      | 0.001      |
| BRC20095 | 4       | 102.5            | 0.545            | 0.396      | 0.423      |
| BRC20096 | 4       | 78               | 0.618            | 0.044      | 0.061      |
| BRC20099 | 3       | 81               | 0.636            | 0.000      | 0.001      |
| BRC20102 | 3       | 66               | 0.621            | 0.058      | 0.074      |
| BRC20103 | 6       | 113              | 0.616            | 0.000      | 0.000      |
| BRC20105 | 5       | 146              | 0.607            | 0.000      | 0.000      |
| BRC20115 | 4       | 158.5            | 0.569            | 0.018      | 0.028      |
| BRC20120 | 5       | 160              | 0.539            | 0.101      | 0.120      |
| BRC20228 | 4       | 92.5             | 0.601            | 0.001      | 0.003      |
| BRC20234 | 5       | 100              | 0.505            | 0.659      | 0.659      |
| BRC20242 | 2       | 128              | 0.609            | 0.002      | 0.004      |
| BRC20244 | 5       | 169              | 0.613            | 0.000      | 0.001      |
| BRC20246 | 4       | 95.5             | 0.576            | 0.000      | 0.000      |
| BRC20255 | 4       | 98.5             | 0.584            | 0.013      | 0.022      |
| BRC20269 | 3       | 103              | 0.576            | 0.000      | 0.000      |
| BRC20294 | 5       | 138              | 0.569            | 0.037      | 0.054      |
| BRC20299 | 5       | 200              | 0.542            | 0.105      | 0.120      |
| BRC20312 | 6       | 133              | 0.609            | 0.000      | 0.000      |
| BRC20324 | 5       | 139              | 0.538            | 0.410      | 0.424      |
| BRC20334 | 5       | 219              | 0.544            | 0.072      | 0.089      |
| BRC20339 | 8       | 208              | 0.498            | 0.000      | 0.000      |
| BRC20341 | 5       | 244              | 0.592            | 0.010      | 0.018      |
| BRC20345 | 5       | 193              | 0.597            | 0.001      | 0.002      |
| BRC20347 | 5       | 306              | 0.510            | 0.003      | 0.007      |
| BRC20348 | 5       | 244              | 0.717            | 0.000      | 0.000      |