for the separation of the moa, which was consistent with the estimated emu/cassowary split at 30–35 Myr. The analysis was a simple extension of a described method[29] to allow more than four taxa. The assumption of rate constancy among the ratites was tested using a likelihood ratio test of the molecular clock model[30]. With a likelihood ratio of 12.68, rate constancy can be rejected ($P < 0.01$). However, Fig. 2 suggests that the ostrich may have an elevated rate of substitution, so the test was repeated with the ostrich allowed a different rate from those of other ratites. The resulting likelihood ratio of 0.449 ($P = 0.92$) shows that this two-rate model is consistent with clock-like behaviour. The two-rate model has little effect on the divergence estimates (Table 2), with ostrich dates becoming younger by 5% of the largest change.

Received 12 July; accepted 26 October 2000.

1. Cracraft, J. Phylogeny and evolution of the ratite birds. *Ibis* **116,** 494–521 (1974).
2. Sibley, C. G. & Ahlquist, J. E. *Phylogeny and Classification of Birds* (Yale Univ. Press, London, 1990).
3. Cooper, A. *et al.* Independent origins of the New Zealand moas and kiwis. *Proc. Natl Acad. Sci. USA* **89,** 8741–8744 (1992).
4. Cooper, A. & Penny, D. Mass survival of birds across the Cretaceous–Tertiary: Molecular evidence. *Science* **275,** 1109–1113 (1997).
5. Feduccia, A. *The Origin and Evolution of Birds* (Harvard Univ. Press, Cambridge, Massachusetts, 1997).
6. Van Tuinen, M., Sibley, C. & Hedges, S. B. Phylogeny and biogeography of ratite birds inferred from DNA sequences of the mitochondrial ribosomal genes. *Mol. Biol. Evol.* **15,** 370–376 (1998).
7. Olson, S. L. in *Avian Biology* Vol. VIII (eds Farner, D. S., King, J. R. & Parkes, K. C.) 79–238 (Academic, Orlando, 1985).
8. Lee, K., Feinstein, J. & Cracraft, J. in *Avian Molecular Evolution and Molecular Systematics* (ed. Mindell, D.) 173–208 (Academic, New York, 1997).
9. Houde, P. Ostrich ancestors found in the Northern Hemisphere suggest new hypothesis of ratite origins. *Nature* **324,** 563–565 (1986).
10. Bledsoe, A. H. A phylogenetic analysis of postcranial skeletal characters of the ratite birds. *Ann. Carnegie Mus.* **57,** 73–90 (1988).
11. Cooper, A. in *Avian Molecular Evolution and Molecular Systematics* (ed. Mindell, D.) 345–373 (Academic, New York, 1997).
12. Handt, O., Krings, M., Ward, R. H. & Pääbo, S. The retrieval of ancient human DNA sequences. *Am. J. Hum. Genet.* **59,** 368–376 (1996).
13. Krings, M. *et al.* Neandertal DNA sequence and the origin of modern humans. *Cell* **90,** 19–30 (1997).
14. Cooper, A. in *Ancient DNA* (eds Herrmann, B. & Hummel, S.) 149–165 (Springer, New York, 1993).
15. Boles, W. E. Hindlimb proportions and locomotion of *Emuarius gidju* (Patterson & Rich, 1987) (Aves: Casuariidae). *Memoirs of the Queensland Museum* **41,** 235–240 (1997).
16. Lawver, L. A., Royer, J-Y., Sandwell, D. T. & Scotese, C. R. in *Geological Evolution of Antarctica* (eds Thomson, M. R. A., Crame, J. A. & Thomson, J. W.) 533–539 (Cambridge Univ. Press, Cambridge, 1991).
17. Cooper, R. A. & Millener, P. R. The New Zealand biota: Historical background and new research. *Trends Ecol. Evol.* **8,** 429–433 (1993).
18. Fleming, C. A. *The Geological History of New Zealand and its Life* (Univ. Auckland Press, Auckland, 1979).
19. Stevens, G. R. Lands in collision. *N. Z. Dept Sci. Ind. Res. Inf. Serv.* **161** (1985).
20. Storch, G. in *The Africa–South America Connection* (eds George, W. & Lavocat, R.) 76–86 (Clarendon, Oxford, 1993).
21. Martin, P. G. & Dowd, J. M. Using sequences of *rbc*L to study phylogeny and biogeography of *Nothofagus* species. *Aust. Syst. Bot.* **6,** 441–447 (1993).
22. Herzer, R. *et al.* Reinga Basin and its margins. *N. Z. J. Geol. Geophys.* **40,** 425–451 (1997).
23. Sauer, E. G. F. Ratite eggshells and phylogenetic questions. *Bonn Zool. Beitr.* **23,** 3–48 (1972).
24. Krause, D. W., Prasad, G. V. R., von Koenigswald, W., Sahni, A. & Grine, F. E. Cosmopolitanism among Gondwanan Late Cretaceous mammals. *Nature* **390,** 504–507 (1997).
25. Sampson, S. D. *et al.* Predatory dinosaur remains from Madagascar: Implications for the Cretaceous biogeography of Gondwana. *Science,* **280,** 1048–1051 (1998).
26. Cooper, A. & Poinar, H. Ancient DNA: Do it right or not at all. *Science* **289,** 1139 (2000).
27. Swofford, D. L. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)* (Sinauer, Sunderland, Massachusetts, 1999).
28. Huelsenbeck, J. P., Hillis, D. M. & Jones R. in *Molecular Zoology: Strategies and Protocols* (eds Ferraris, J. & Palumbi, S.) 19–45 (Wiley, New York, 1996).
29. Rambaut, A. & Bromham, L. Estimating divergence dates from molecular sequences. *Mol. Biol. Evol.* **15,** 442–448 (1998).
30. Felsenstein, J. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17,** 368–376 (1981).

Supplementary information, including clone and primer sequences is available on *Nature*'s World-Wide Web site (http://www.nature.com), or on http://evolve.zoo.ox.ac.uk/data/Ratites/, or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank W. Boles, R. Cooper, R. Herzer, P. Houde, C. Mourer-Chauviré, D. Penny and T. Worthy for valuable comments, and M. Sorenson for allowing us access to unpublished rhea and ostrich sequences. We are grateful to T. Worthy and the staff of the Museum of New Zealand for the moa samples. Modern samples were kindly provided by A. C. Wilson (deceased), M. Potter and M. Braun, and laboratory space by R. Thomas, J. Bertranpetit and the Oxford University Museum. A.C. was supported by the NERC, the Leverhulme Fund, the New Zealand Marsden Fund and the Royal Society. C.L.F. was supported by the Comissionat per a Universitats i Recerca (Catalan Autonomous Government), and A.R. was supported by the Wellcome Trust.

Correspondence and requests for materials should be addressed to A.C. (e-mail: alan.cooper@zoo.ox.ac.uk). Software is available at http://evolve.zoo.ox.ac.uk/software.

Wolbachia-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*

Seth R. Bordenstein, F. Patrick O'Hara & John H. Werren

Department of Biology, The University of Rochester, Rochester, New York 14627, USA

Wolbachia **are cytoplasmically inherited bacteria that cause a number of reproductive alterations in insects, including cytoplasmic incompatibility[1,2], an incompatibility between sperm and egg that results in loss of sperm chromosomes following fertilization.** ***Wolbachia*** **are estimated to infect 15–20% of all insect species[3], and also are common in arachnids, isopods and nematodes[3,4]. Therefore,** ***Wolbachia*****-induced cytoplasmic incompatibility could be an important factor promoting rapid speciation in invertebrates[5], although this contention is controversial[6,7]. Here we show that high levels of bidirectional cytoplasmic incompatibility between two closely related species of insects (the parasitic wasps** ***Nasonia girault*****i and** ***Nasonia longicornis*****) preceded the evolution of other postmating reproductive barriers. The presence of** ***Wolbachia*** **severely reduces the frequency of hybrid offspring in interspecies crosses. However, antibiotic curing of the insects results in production of hybrids. Furthermore, F_1 and F_2 hybrids are completely viable and fertile, indicating the absence of F_1 and F_2 hybrid breakdown. Partial interspecific sexual isolation occurs, yet it is asymmetric and incomplete. Our results indicate that** ***Wolbachia*****-induced reproductive isolation occurred in the early stages of speciation in this system, before the evolution of other postmating isolating mechanisms (for example, hybrid inviability and hybrid sterility).**

Symbiotic microorganisms are widespread in nature and often have intimate associations with their hosts, ranging from mutualistic to parasitic relationships. It has been suggested that these associations may act as a source of evolutionary innovation for their hosts, leading to differentiation between host populations and ultimately to the evolution of new species[8]. *Wolbachia* are particularly good candidates for symbiont-induced speciation, because these bacteria can modify compatibility between eggs and sperm of hosts, and thus directly cause reproductive isolation without long-term coevolution of the host and symbiont[5]. There is some empirical evidence for a role of *Wolbachia* in speciation in mushroom-feeding *Drosophila*[9], the flour beetle *Tribolium*[10], and parasitic wasps[11]. However, the view that *Wolbachia* are involved in invertebrate speciation is still controversial[5–7]. Here we present evidence that *Wolbachia*-induced reproductive isolation precedes the evolution of other postmating isolating mechanisms in *Nasonia*. The finding supports the view that *Wolbachia* can play a role in reproductive isolation and speciation.

Nasonia is a complex of three closely related species of haplodiploid parasitic wasps. *Nasonia vitripennis* is found worldwide, and is a generalist that parasitizes a variety of fly species. *Nasonia girault*i occurs in eastern North America and *N. longicornis* in western North America, where they parasitize the pupae of blowflies in birds' nests[12]. Genetic and molecular evidence shows that *N. girault*i and *N. longicornis* are more closely related sister species. Estimates place the divergence of these two species at around 0.250 Myr ago and their divergence from *N. vitripennis* at around 0.800 Myr[13].

All three species are infected with *Wolbachia*, and individuals of each species are typically infected with two different bacterial types, each belonging to the two major subgroups of arthropod *Wolbachia* (A and B)[14]. Furthermore, phylogenetic analysis (data not shown) indicates that the A group bacteria of each species are not closely

related to each other, and therefore have been independently acquired by horizontal transmission. Thus, the *Nasonia* system appears to be prone to the acquisition of *Wolbachia*, and is a promising system for studying the role of these bacteria in reproductive isolation.

Previous studies have shown *Wolbachia*-induced bidirectional incompatibility between two diverged species, *N. vitripennis* and *N. giraulti*[11]. F_1 hybrids are not formed unless *Wolbachia* are removed by antibiotic curing. However, several other isolating barriers exist between these species, including high levels of F_2 hybrid lethality, abnormal courtship behaviours in F_2 hybrid males (behavioural sterility), and partial premating (sexual) isolation (refs 15, 16, and F.P.O'H., A C. Chawla and J.H.W., manuscript in preparation). It is therefore unclear whether *Wolbachia*-induced cytoplasmic incompatibility (CI) evolved before the evolution of other isolating barriers or after the divergence of the species. If *Wolbachia* play a causal role in speciation, cases where *Wolbachia*-induced CI evolved before other mechanisms of reproductive isolation should exist.

Here we investigate the role of *Wolbachia* in reproductive incompatibility in a younger species pair, using the more closely related species *N. giraulti* and *N. longicornis*. First, we screened field-collected insects to determine the frequencies of infections in natural populations of the three species. A polymerase chain reaction (PCR) method was employed using previously published specific primers[17]. In all three species, 100% of the individuals from various geographical areas were found to be infected (*N. giraulti*, $n = 29$; *N. longicornis*, $n = 31$; *N. vitripennis*, $n = 31$). All samples were doubly infected with A and B, except for one *N. longicornis* strain with a single A infection. Sequence analysis of PCR-amplified products of the *wsp* gene[18] from a subset confirms that the species are infected with species-specific *Wolbachia*, and that the *Wolbachia* from different intraspecific strains form monophyletic groups (data not shown), with little sequence variation within a host species.

We undertook experiments to determine whether *Wolbachia* cause reproductive incompatibility between the 'young' species pair, *N. giraulti* and *N. longicornis*. Wild-type infected strains and antibiotically cured strains derived from those infected strains were crossed in all pairwise combinations. Results show that bidirectional CI occurs between infected *N. giraulti* and *N. longicornis* (Fig. 1). When *Wolbachia* are present, no F_1 hybrid (female) offspring are produced in the *N. giraulti* male × *N. longicornis* female cross and 29.7 ± 2.6 (mean ± s.e., and hereafter) hybrid offspring are produced in the reciprocal *N. longicornis* male × *N. giraulti* female cross. In contrast, crosses using antibiotically cured strains produce 63.9 ± 4.1 hybrid offspring and 82.9 ± 5.1 hybrid offspring, respectively. Thus, presence of *Wolbachia* causes a 100% reduction in F_1 hybrids in one direction and 62.8% reduction in the other direction. In *N. giraulti* and *N. longicornis*, CI results in both a paternal genome loss[19] and offspring lethality (data not shown). These results show that *Wolbachia*-induced CI is a significant component of reproductive incompatibility between *N. giraulti* and *N. longicornis*.

To assess whether *Wolbachia*-induced incompatibility between *N. giraulti* and *N. longicornis* is one of the first incompatibilities to evolve in the divergence of these species, we tested for several other hybrid incompatibilities. Specifically, we investigated (1) interspecific sperm–egg compatibility, (2) inviability and sterility among F_1 hybrid females and (3) inviability and sterility of F_2 hybrid males. Both spermatogenic and behavioural sterility of F_2 males was examined. All the experiments described below were performed with uninfected individuals to exclude the effects of *Wolbachia* on compatibility and viability.

To investigate viability of F_1 females, we compared the number of progeny produced by females mated to intra- and interspecific males. Crosses with uninfected females show that they produce the same number of F_1 progeny whether they mate with males of their own species or males of the other species (*N. giraulti* female × *N. giraulti* male, 100.2 ± 5.6 versus *N. giraulti* female × *N. longicornis* male, 99.0 ± 4.0; and *N. longicornis* female × *N. longicornis* male, 68.5 ± 4.1 versus *N. longicornis* female × *N. giraulti* male, 78.5 ± 4.8). As only females are hybrids in a haplodiploid insect, we also compared the number of female offspring produced in intra- and interspecific crosses (Fig. 1). There was no reduction in the number of F_1 hybrid females relative to intraspecific controls. Finally, we compared the number of eggs laid during a 6-h oviposition period to the number of adult offspring emerging in hybrid crosses. No significant differences were found (*N. giraulti* female × *N. longicornis* male, 22.5 ± 6.4 eggs versus 20.7 ± 7.1 adults; reciprocal cross, 25.0 ± 9.2 eggs versus 24.3 ± 3.2 adults). Therefore, results clearly indicate that there is no significant F_1 hybrid inviability. They also show that there is no reduction in fertilization of eggs based on whether the sperm came from heterospecific or homospecific males, indicating no incompatibilities in the fertilization mechanism between these species.

The level of F_1 hybrid female fertility was measured by counting eggs laid by females during a time-limited oviposition period. F_1 hybrid females did not show reduced fertility relative to non-hybrid control females (Fig. 2). In fact, hybrid females with the *N. longicornis* cytoplasm laid significantly more eggs than non-hybrid *N. longicornis* females (Mann–Whitney *U*-test (*U*), $P < 0.001$).

Sterility and/or mortality of F_2 progeny (hybrid breakdown) is one of the earlier manifestations of genetic incompatibility between recently evolved species[20,21]. This is believed to be due to the general recessivity of genes involved in hybrid inviability and infertility[20–22]. The haploidy of males in *Nasonia* offers an advantage to the study of recessive incompatibility factors, as such factors will be readily expressed in haploid males[15,22]. We investigated inviability by comparing the number of F_2 eggs laid by F_1 virgin females to the

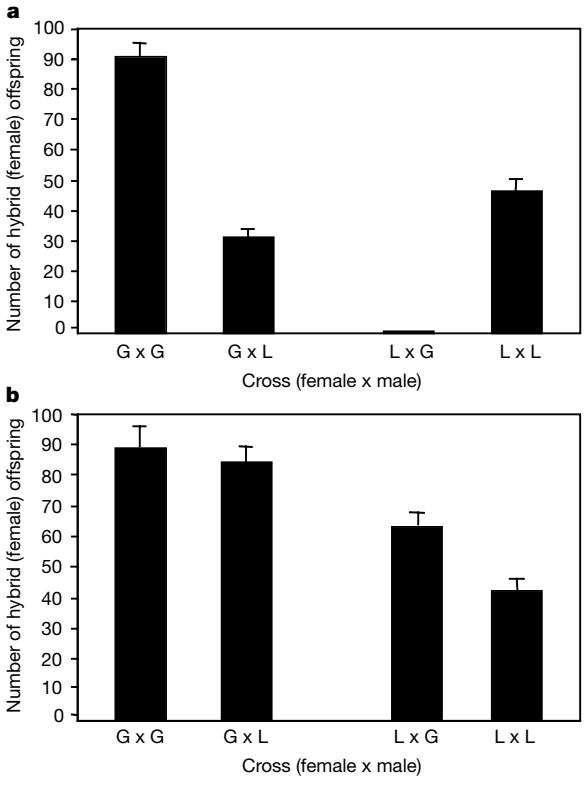

Figure 1 Number of hybrid (female) offspring produced from intra- and interspecific crosses. Results are shown for infected individuals (**a**) and uninfected individuals (**b**). Data are the mean number ± s.e. of F_1 progeny. G and L denote *N. giraulti* and *N. longicornis*, respectively.

number of F_2 males that survived to adulthood. (Virgin females produce haploid male progeny from unfertilized eggs in this haplodiploid insect.) There are no significant differences in mortality levels among the F_2 hybrid males relative to the non-hybrid controls (Fig. 2). Mortality was found among F_2 males of hybrid females from the *N. longicornis* male × *N. giraulti* female cross (mean ± s.d. = 19.3 ± 0.4% (ref. 23) mortality; *U*, egg versus adult number, $p = 0.002$). However, a similar level of mortality was also observed among non-hybrid *N. giraulti* males (F_2 males from the *N. giraulti* male × *N. giraulti* female cross; 14.3 ± 0.3% mortality, $P = 0.009$). No significant differences were found between these crosses in the number of F_2 eggs (*U*, $P = 0.595$) or F_2 surviving adults (*U*, $P = 0.862$). Therefore, there is not elevated mortality among hybrids. This finding is quite different from what is found in the older species pair (*N. giraulti* × *N. vitripennis*), which has high levels (70–85%) of F_2 hybrid male mortality[15]. Such recessive genetic incompatibilities have apparently not yet evolved between *N. giraulti* and *N. longicornis*.

We assessed the fertility of F_2 hybrid and non-hybrid males by dissecting testes and categorizing sperm motility into three groups: normal, reduced or absent. All males possessed some motile sperm. The percentage of males with normal quantities of motile sperm was 94.7% ($n = 19$) and 95.0% ($n = 20$) for the two hybrid genotypes and 95.0% ($n = 20$) and 100% ($n = 19$) for non-hybrids. Additionally, we tested the ability of hybrid and non-hybrid sperm to fertilize both *N. giraulti* and *N. longicornis* eggs. Of 69 males that copulated, only one failed to produce female offspring, but this occurred in an intraspecific cross. Thus, hybrid sperm is completely functional. This contrasts to many studies in *Drosophila*, which indicate a prevalence of hybrid male sterility loci[24,25] and that spermiogenic sterility evolves rapidly in the divergence between species[20,21].

F_2 hybrid breakdown can also affect courtship behaviour, due to a general 'sickness' of hybrid males or to specific negative interactions in genes involved in courtship behaviour[26]. We assessed the ability of hybrid and non-hybrid males to (1) locate and mount females, (2) perform the ritualized courtship display, and (3) copulate with females. The type of female did not influence probabilities of initiating courtship and no differences were found among males in their ability to locate and mount females (hybrids, 93.7% ($n = 187$); *N. longicornis*, 97.8% ($n = 46$); *N. giraulti*, 95.7% ($n = 46$); $X^2 = 1.42$, 2 degrees of freedom (d.f.), $P = 0.49$). Among males who successfully mount females, there was a small and nearly significant difference in the proportion of males performing the courtship display (hybrids, 94.2% ($n = 172$); *N. longicornis*, 100% ($n = 45$); *N. giraulti*, 100% ($n = 45$); $X^2 = 5.44$, 2 d.f., $P = 0.07$). Among those males who courted *N. giraulti* females, no differences were found in the proportion of males copulating (hybrids, 91.5% ($n = 94$); *N. longicornis*, 95.8% ($n = 24$); *N. giraulti*, 96.0% ($n = 25$); $X^2 = 0.97$, 2 d.f., $P = 0.62$). However, males did differ in their ability to copulate with *N. longicornis* females (hybrids, 52.9% ($n = 68$); *N. longicornis*, 95.2% ($n = 21$); *N. giraulti*, 21.2% ($n = 19$); $X^2 = 22.74$, 2 d.f., $P < 0.001$). This difference cannot be attributed to hybrid breakdown, because hybrid males with *N. longicornis* females copulate at significantly higher rates than do *N. giraulti* males ($X^2 = 6.08$, 1 d.f., $P = 0.014$).

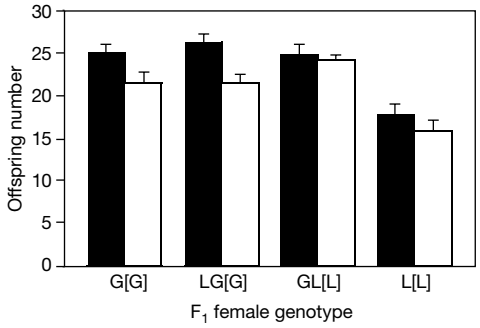

Figure 2 F_2 egg and adult offspring number produced from F_1 hybrid and non-hybrid females. Data are the mean number ± s.e. of eggs (black bar) and surviving adults (white bar). The term in brackets denotes the cytotype, while the term before the brackets denotes nuclear genotype. For instance, LG[G] hybrid females are derived from the cross, L male × G female.

The above results are therefore best explained as mate discrimination of *N. longicornis* females against F_2 hybrid males, rather than to F_2 hybrid 'sickness'. In contrast, our findings with F_2 hybrid males from the older species pair (*N. giraulti* and *N. vitripennis*) indicate high levels of reproductive incompetence throughout the various stages of courtship and mating. For example in the older species cross, 27.6% of F_2 hybrid males failed to locate and mount females, and of those that did mount females, 26.8% failed to perform the ritualized courtship display. As a result, a total of 53.2% of F_2 hybrid males in the older species cross fail to successfully mount females and perform the courtship display (compared to only 13.8% who fail to do so in the younger species cross, not significantly different from controls). We conclude that the genetic incompatibilities responsible for these problems have not arisen since the more recent divergence of *N. giraulti* and *N. longicornis*.

Finally, we investigated the level of premating isolation between the two species in single pair-mating situations. During a 30-min mating period, *N. giraulti* females show no mate discrimination towards *N. longicornis* males, mating at similar frequencies as they do to homospecific males (94.5% mating, $n = 200$ versus 95.6%, $n = 159$, $P = 0.32$). In contrast, *N. longicornis* females show partial mate discrimination towards *N. giraulti* males relative to homospecific males (46.9% mating, $n = 113$ versus 89.9%, $n = 178$, $P < 0.0001$).

The experiments presented here clearly indicate that the species pair *N. giraulti* and *N. longicornis* do not show significant levels of F_1 or F_2 lethality, F_1 or F_2 reproductive sterility, or F_2 'hybrid sickness' as manifested by competence in courtship behaviour. In contrast, high levels of *Wolbachia*-induced reproductive incompatibility are present in this species pair. Therefore, we conclude that interspecies bidirectional CI has preceded the evolution of these other isolating mechanisms in this system. In addition to *Wolbachia*-induced reproductive incompatibility, there is partial premating isolation in one direction between these species. The strength of premating isolation, at least under the conditions tested here, is weaker than the postmating reproductive incompatibilities caused by *Wolbachia*.

The role of *Wolbachia* in speciation is a matter of current debate[5–7] and so far, there is limited empirical support for it[9–11]. We do not claim that *Wolbachia* are currently causing reproductive isolation between *N. giraulti* and *N. longicornis* in nature. Other factors, such as geographical isolation (allopatry) are likely to be more important. However, our results do show that *Wolbachia*-induced bidirectional CI has preceded the evolution of other intrinsic, postmating reproductive isolation barriers in these newly evolving species. The present work therefore provides further support for the argument that the cytoplasmic bacterium *Wolbachia* could promote host speciation. □

Methods

Crosses for assay of CI and copulation frequencies

Single pairs of male and female virgins were observed for 30 min in a 12 × 75-mm vial. We only collected data on incompatibility relationships from crosses with an observed copulation. After 24 h, the male was discarded from the vial, and each mated female was hosted with two *Sarcophaga bullata* blowfly pupal hosts for egg laying. F_1 progeny were scored for sex ratio and family size upon death. RV2, RV2R, IV7 and IV7R2 are the *N. giraulti* and *N. longicornis* infected and uninfected strains, respectively. Uninfected strains were generated from the corresponding infected strains in 1996 through antibiotic treatment of 1% Rifadin (10% sugar water) for three successive generations. Infection status of these strains was confirmed by PCR before the experiments.

F_2 hybrid viability

F_1 hybrid and non-hybrid virgin adult females (1–2-d old) were placed on four hosts for roughly 48 h for host feeding and egg laying. Females were immediately transferred to one host for a 6-h laying period, after which females were removed from the vial. We limited the ovipositioning period to prevent wasps from becoming resource-limited. Half of these replicates were immediately scored for the number of F_2 eggs laid in 6 h and the remaining half were scored later for the number of adults.

Dissections for sperm motility assay

Testes and seminal vesicles were viewed under a microscope at ×400 magnification for the presence of motile sperm. Tested males were dissected on the day they emerged in a drop of phosphate-buffered saline. At least one testis and one seminal vesicle from each male were viewed. Males were scored as fully fertile if motile sperm were observed in all testes and seminal vesicles observed. Males were scored as partially fertile if a reduced number of motile sperm were observed in any organs viewed.

F_2 hybrid male behavioural and spermiogenic fertility

Single males, aged 18–48 h, were placed in clear 12 × 75-mm vials with five virgin females, no more than four days old. Behaviour of each male was observed for 15 min. After courtship observation, males were left in the vial with females for an additional 105 min (2 h total) and then removed. Females were then given five hosts for feeding and egg laying. On death of their F_1 progeny, each vial was inspected for the presence of female offspring, indicating successful fertilization of at least one female by the tester male. Behavioural fertility data were not significantly different for F_2 hybrid males from the two reciprocal crosses (F_2 males from *N. girault*i males × *N. longicornis* females and from *N. girault*i females × *N. longicornis* males), and therefore the data were pooled for statistical analysis.

Received 22 September; accepted 30 November 2000.

1. Werren, J. H. Biology of *Wolbachia*. *Annu. Rev. Entomol.* **423,** 587–609 (1997).
2. Hoffmann, A. A. & Turelli, M. in *Influential Passengers* (eds O'Neill, S. L. Hoffmann, A. A. & Werren, J. H.) (Oxford Univ. Press, New York, 1997).
3. Werren, J. H. & Windsor, D. M. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium. *Proc. R. Soc. Lond. B* **267,** 1277–1285 (2000).
4. Werren, J. H., Windsor, D. & Guo, L. R. Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B* **262,** 197–204 (2000).
5. Werren, J. H. in *Endless Forms: Species and Speciation* (eds Howard, D. J. & Berlocher, S. L.) (Oxford Univ. Press, Oxford, 1998).
6. Hurst, G. D. D. & Schilthuizen, M. Selfish genetic elements and speciation. *Heredity* **80,** 2–8 (1998).
7. Stouthamer, R., Breeuwer, J. A. J. & Hurst, G. D. D. *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* **53,** 71–102 (1999).
8. Margulis, L. & Fester, R. *Symbiosis as a Source of Evolutionary Innovation* (MIT Press, Cambridge, Massachusetts, 1991).
9. Shoemaker, D. D., Katju, V. & Jaenike, J. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. *Evolution* **53,** 1157–1164 (1999).
10. Wade, M. J., Chang, N. W. & McNaughton, M. Incipient speciation in the flour beetle *Tribolium confusum*: partial reproductive isolation between populations. *Heredity* **75,** 453–459 (1995).
11. Breeuwer, J. A. J. & Werren, J. H. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346,** 558–560 (1990).
12. Darling, C. D. & Werren, J. H. Biosystematics of two new species of *Nasonia* (Hymenoptera: Pteromalidae) reared from birds' nests in North America. *Annals Enton. Soc. Amer.* **83,** 352–370 (1990).
13. Campbell, B. C., Steffen-Campbell, J. D. & Werren, J. H. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an rDNA internal transcribed spacer (ITS2). *Insect Mol. Biol.* **2,** 255–237 (1993).
14. Werren, J. H., Zhang, W. & Guo, L. R. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc. R. Soc. Lond. B* **251,** 55–71 (1995).
15. Breeuwer, J. A. J. & Werren, J. H. Hybrid breakdown between two haplodiploid species: the role of nuclear and cytoplasmic genes. *Evolution* **49,** 705–717 (1995).
16. Bordenstein, S. R. & Werren, J. H. Effects of A and B *Wolbachia* and host genotype on interspecies cytoplasmic incompatibility in *Nasonia*. *Genetics* **148,** 1833–1844 (1998).
17. Perrot-Minnot, M. J., Guo, L. R. & Werren, J. H. Single and double infections with *Wolbachia* in the parasitic wasp *Nasonia vitripennis*: effects on compatibility. *Genetics* **143,** 961–972 (1996)
18. Zhou, W. G., Rousset, F. & O'Neill, S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. Lond. B* **265,** 509–515 (1998).
19. Reed, K. M. & Werren, J. H. Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Mol. Reprod. Dev.* **40,** 408–418 (1995).
20. Wu, C. I. & Palopoli, M. F. Genetics of postmating reproductive isolation in animals. *Annu. Rev. Genet.* **28,** 283–308 (1994).
21. Orr, H. A. Haldane's rule. *Annu. Rev. Ecol. Syst.* **28,** 195–218 (1997).
22. Gadau, J., Page R. E. & Werren, J. H. Mapping of hybrid incompatibility loci in *Nasonia*. *Genetics* **153,** 1731–1741 (1999).
23. Mood, A. M., Graybill, F. A. & Boes, D. C. *Introduction to the Theory of Statistics* (McGraw Hill, Singapore, 1974).
24. Palopoli, M. F. & Wu, C. I. Genetics of hybrid male sterility between *Drosophila* sibling species—a complex web of epistasis is revealed in interspecific studies. *Genetics* **138,** 329–341 (1994).
25. True, J. R., Weir, B. S. & Laurie, C. C. A genome-wide survey of hybrid incompatibility factors by the introgression of marked segments of *Drosophila mauritiana* chromosomes into *Drosophila simulans*. *Genetics* **142,** 819–837 (1996).
26. Noor, M. A. F. Genetics of sexual isolation and courtship dysfunction in male hybrids of *Drosophila pseudoobscura* and *Drosophila persimilis*. *Evolution* **51,** 809–815 (1997).

Acknowledgements

We thank R. Billings, M. Vaughn and B. J. Velthuis for technical assistance, and J. Bartos, A. Betancourt, J. Jaenike, J. P. Masly, T. van Opijnen and D. Presgraves for critical reading of the manuscript. This work was supported by the NSF (J.H.W.).

Correspondence and requests for materials should be addressed to S.R.B. (e-mail: sbst@troi.cc.rochester.edu).

Evolutionary radiations and convergences in the structural organization of mammalian brains

Willem de Winter & Charles E. Oxnard

Department of Anatomy and Human Biology, The University of Western Australia, Perth, Western Australia 6907, Australia

The sizes of mammalian brain components seem to be mostly related to the sizes of the whole brain (and body), suggesting a one-dimensional scale of encephalization[1–3]. Previous multivariate study of such data concludes that evolutionary selection for enlargement of any one brain part is constrained to selection for a concerted enlargement of the whole brain[4]. However, interactions between structurally related pairs of brain parts[5] confirm reports of differential change in brain nuclei[6], and imply mosaic rather than concerted evolution. Here we analyse a large number of variables simultaneously using multi-dimensional methods[7]. We show that the relative proportions of different systems of functionally integrated brain structures vary independently between different mammalian orders, demonstrating separate evolutionary radiations in mammalian brain organization[8]. Within each major order we identify clusters of unrelated species that occupy similar behavioural niches and have convergently evolved similar brain proportions. We conclude that within orders, mosaic brain organization is caused by selective adaptation, whereas between orders it suggests an interplay between selection and constraints.

We use data from the same source[9,10] as the previous studies[4,5]. In ref. 4 a small subset of these data was analysed multivariately to study species separations, but in a context where size outweighed most other information; an even smaller subset of the same data was used to study bivariate relationships between pairs of brain parts in ref. 5. Here we examine the complete set of specimen measurements underlying these data by relating the various brain structures in proportion to two reference structures. We explore the detailed structure of the resulting 19-dimensional data space in two stages, and combine the strategies of the previous studies[4,5] by looking for species relationships as well as associations between variables.

The first stage in this study explores species separations in the subspace spanned by the first three principal components (85% of the information). Figures 1 and 2 show that all orders are clearly differentiated; this shows that they differ uniquely in their internal brain proportions. The three large orders—primates, insectivores and bats—are especially different, being dispersed in nearly orthogonal directions (although not along the orthogonal principal components of the analysis). The primate and insectivore dispersions are separate, but are linked together via some bats. The two