Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution

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The origin of the ratites, large flightless birds from the Southern Hemisphere, along with their flighted sister taxa, the South American tinamous, is central to understanding the role of plate tectonics in the distributions of modern birds and mammals. Defining the dates of ratite divergences is also critical for determining the age of modern avian orders[1–6]. To resolve the ratite phylogeny and provide biogeographical data to examine these issues, we have here determined the first complete mitochondrial genome sequences of any extinct taxa— two New Zealand moa genera—along with a 1,000-base-pair sequence from an extinct Madagascan elephant-bird. For comparative data, we also generated 12 kilobases of contiguous sequence from the kiwi, cassowary, emu and two tinamou genera. This large dataset allows statistically precise estimates of molecular divergence dates and these support a Late Cretaceous vicariant speciation of ratite taxa, followed by the subsequent dispersal of the kiwi to New Zealand. This first molecular view of the break-up of Gondwana provides a new temporal framework for speciation events within other Gondwanan biota and can be used to evaluate competing biogeographical hypotheses.

Ratites and tinamous (together described as palaeognathes) share a primitive skull structure (the palaeognathous palate) and are taxonomically separated from all other living birds (neognathes). Ratites include the rhea (*Rhea americana*, *Rhea pennata*, South America), ostrich (*Struthio camelus*, Africa and formerly Eurasia), emu (*Dromaius novaehollandiae*, Australia), cassowary (*Casuarius casuarius*, *Casuarius bennetti*, *Casuarius unappendiculatus*, Australasia) and kiwi (*Apteryx mantelli*, *Apteryx owenii*, *Apteryx haasti*, New Zealand), as well as the recently extinct moa (11 spp., New Zealand) and elephant-bird (~3 spp., Madagascar)[1,2]. Ratite fossils are found on all the Southern continents formed by the Cretaceous break-up of the supercontinent Gondwana[1,7], and are commonly used as an example of vicariant speciation[1,8]. This hypothesis has been challenged by the discovery of small flighted palaeognathe fossils (lithornids) from the early Tertiary of the Northern Hemisphere[9], and suggestions that modern birds represent a post-Cretaceous radiation[5].

Despite much research, the nature of the basal ratite divergences remains unclear, confounding biogeographical interpretations. An influential early morphological study suggested that the New Zealand ratites were monophyletic, and the first to diverge[1] (but see ref. 10). However, initial molecular studies suggested that the kiwi, emu and cassowary were a derived group, with either the rhea or ostrich as the basal divergence[2,3,6]. Ancient DNA methods allowed short mitochondrial DNA (mtDNA) sequences from several moa taxa to be compared; these indicated that the New Zealand ratites were not monophyletic, and that the moa fell between a basal rhea branch and the ostrich[3,11]. This result was challenged by further mtDNA data from living taxa[6,8], and remains unresolved because of the small amounts of sequence data available, particularly for the extinct moa.

To resolve this issue we used ancient DNA techniques to generate contiguous sequences of the complete mitochondrial genomes of two moa genera, *Emeus crassus* and *Dinornis giganteus*, as a series of 400–600 base-pair (bp) amplifications from subfossil bones 1,300–1,500 years old (see Methods). Competitive polymerase chain reaction (PCR) assays indicated that mtDNA was preserved at around 0.3–1.5 million copies per gram of bone in the specimens, roughly three orders of magnitude higher than the Neanderthal (2,500–3,750 copies per g) and Ice Man DNA (8,600 copies per g), but similar to some Hohokam mummies from the US southwest (2 million copies per g)[12,13]. The high concentration of moa mtDNA indicates that amplified sequences are unlikely to be influenced by damage to individual template molecules[12,13]. This was confirmed by cloning experiments (Table 1) where sequencing discrepancies occurred at rates comparable to modern taxa (~2 errors per 1,000 bp) and consisted mainly of singletons. Sequencing experiments replicated in Barcelona and London were identical and cloning error rates were similar to those in Oxford. In the control regions of both moa, a 90-bp section could not be sequenced unambiguously because flanking thymidine homopolymers caused sequencing reactions to stutter. A putative nuclear copy of mitochondrial sequences (numt) was also detected in *Dinornis*. The putative numt featured two indels of 82 bp and 12 bp, but was otherwise identical to the mtDNA sequences.

We also used long-range PCR to amplify and sequence all the mitochondrial protein coding genes (apart from ND6) of the emu, cassowary, kiwi and two tinamou genera. The data were aligned

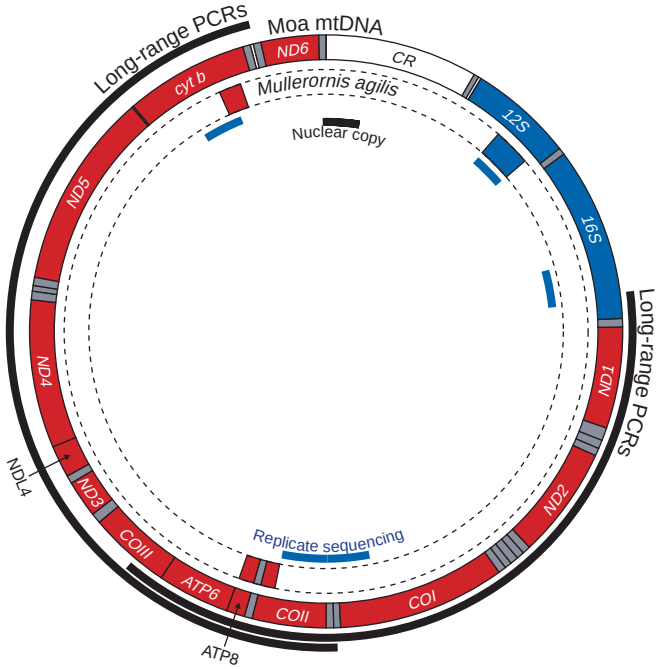

Figure 1 Mitochondrial genome arrangement for *Dinornis giganteus*. The moa mtDNA gene arrangement is similar to published rhea and ostrich sequences, and is 16,997 bp in length. Sequences of *Dinornis* and *Emeus* independently replicated in London and Barcelona, respectively, are indicated and the primers used were 12S (London and Barcelona), 1753FH/2150RH; 16S and COII (Barcelona), 3348Fb/3797RH, 8320Fb/8807Rb; COI and Cyt *b* (London), 7807FH/8325Rb, 15303Fb/15783Rb. Sequences of *Mullerornis* were obtained for 12S (1753FH/1985Rb; 1856Fb/2020Rb; and 1999Fb/2150RH), COII (8861Fb/9036RH), ATP8 (9043Fb/9241RH) and Cyt *b* (15671FH/15902RH), and 1856Fb/2020Rb sequences were replicated in London. Long-range PCR amplifications of extant taxa were used to minimize the risk of numts, and used primers 03725FH-16SFor.2/10218RH-COIII-LR, and 08171FH-tSer-LR/16120Rb-tPro-LR.

with the two moa sequences, and the published rhea, ostrich and chicken sequences (see Methods). After ambiguous sites and gaps were removed, the remaining 10,767 nucleotides were used for phylogenetic analysis. Identical maximum-likelihood trees (Fig. 2) were generated by exhaustive heuristic and constrained searches. These confirmed that the moa and kiwi are not monophyletic, and represent separate ratite invasions of New Zealand[3,11,14]. Although bootstrap values are relatively low, nearest-neighbour interchange values show that the tree is locally stable and that the ostrich is never basal. Parametric bootstrapping rejected the two alternative ratite phylogenetic hypotheses at the $P < 0.001$ significance level (Fig. 2). Maximum-likelihood analyses of a short (1000-bp) dataset featuring the elephant-bird (see Methods and Supplementary Information) placed it among the derived ratite taxa (Fig. 2). Although these data do not allow precise placement or an estimate of divergence date, the elephant-bird is clearly not the result of a recent divergence from the ostrich, or any other ratite lineage.

Molecular clock tests revealed that the sequences have evolved in a clock-like manner, with the exception of the ostrich, which has an elevated rate. When the ostrich branch was allowed a different rate, the likelihood ratio of 0.449 is consistent with rate constancy for the other taxa ($P = 0.92$). The large sequence dataset, and clock-like behaviour of the ratite lineages, provides an opportunity to integrate statistically precise divergence time estimates with the geological history of Gondwana. There are two potential calibration points: first, Mid to Late Oligocene fossils which show that the emu and cassowary lineages had clearly separated sometime before 25 Myr ago (ref. 15), probably around 30–35 Myr (W. Boles, personal communication); second, the geological split between New Zealand and Australia/Antarctica around 82–85 Myr (refs 16, 17), which has been related to the basal separation of the moa from the other ratite taxa[14,18,19]. Unfortunately, like many terrestrial avian groups the rest of the ratite fossil record is relatively poor, with only the rhea and ostrich known from the early Tertiary[4,9,20]. These fossils can provide only a minimum age for these long unbranched lineages (Fig. 2).

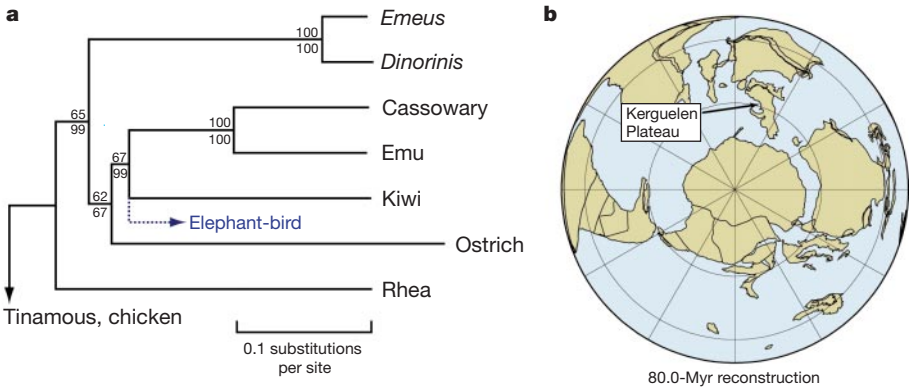

Figure 2 Ratite phylogeography. **a**, Unconstrained maximum-likelihood tree of ratite taxa, rooted with two tinamou and the chicken, using 10,767 bp of mtDNA protein coding sequence. Bootstrap values from 1,000 replications are given above the lines, with nearest-neighbour values[4] underneath. The maximum-likelihood position of the elephant-bird calculated with the short dataset is indicated, but the data prevent an accurate estimate of branch length. Alternative phylogenetic hypotheses for ratites (New Zealand ratites as monophyletic outgroup[1,8] or ostrich as outgroup[2,6]) both fit the data significantly worse than Fig. 2a ($P < 0.001$). **b**, Tectonic reconstruction of Gondwana at 80 Myr BP. The ostrich (and elephant-bird) are proposed to have crossed the Kerguelen Plateau (indicated) to Indo-Madagascar[24,25] and eventually Eurasia. Reconstruction map from www.odsn.de/odsn/services/paleomap/paleomap.html.

Table 1 Differences between cloned PCR products and direct sequences of moa, *Mullerornis* and extant taxa

Taxons	Gene	Positions	*N*	Total (bp)	Ts	Tv	Substitutions		Error per 1,000	Insert (bp)
							Single	Multi		
Extant										
Tataupa	ND1	4,747–5,201 (456)	24	7,308	12	3	15	0	2.05	–
Kiwi	ND4L	11,120–11,532 (390)	11	3,672	13	0	11	1	3.54	–
Cassowary	COIII	10,161–10,743 (593)	16	4,513	10	3	13	0	2.88	–
Extinct										
Oxford										
Dinornis	CR	16,733–00441 (600)	17	10,012	13	0	13	0	1.30	–
	ND1	4,747–5,201 (456)	10	4,560	6	1	7	0	1.54	–
	COII/ATP6	8,861–9,349 (492)	10	4,310	5	1	6	0	1.39	–
	ND4	11,120–11,598 (478)	10	4,560	7	4	7	2	2.41	–
Emeus	16S/ND1	3,787–4,311 (519)	10	5,190	12	0	12	0	2.31	–
	COI	7,807–8,328 (521)	7	3,353	4	0	4	0	1.19	–
	COIII	10,161–10,743 (581)	19	9,411	16	2	18	0	1.91	–
	ND4/ND5	12,788–13,200 (415)	10	3,640	9	0	9	0	2.47	1 (7)
Mullerornis	12S	1,856–2,020 (163)	9	1,467	5	1	6	0	4.08	–
London										
Dinornis	12S	1,753–2,148 (396)	9	3,168	5	0	5	0	1.58	–
	COI	7,807–8,325 (513)	9	4,615	7	1	8	0	1.73	–
	Cytb	15,303–15,783 (479)	5	2,395	5	0	2	1	2.09	–
Barcelona										
Emeus	COII	8,320–8,807 (485)	11	5,335	14	3	13	1	3.19	–

More than 50 kb of moa mtDNA sequences and 15 kb of the extant taxa were cloned and sequenced. Substitutions for the direct sequence were scored as errors, and a rate per 1,000 bp calculated (error per 1,000), ignoring inserts. Positions are numbered relative to the chicken mtDNA genome [illegible] the cloned sequence is given in parentheses. *N* = number of clones, total bp = total number of positions cloned, Ts = transition, Tv = transversion, single = singleton substitution (appearing only in one clone), multi = substitution common to more than one clone. Insert, insert relative to the direct sequence, with the number of base pairs in parentheses.

Table 2 Estimated divergence dates of the ratite lineages

Calibration	Divergence	Estimated divergence date, with 95% CI (Myr)
Moa-O,E,C,K - 82 Myr	Rhea–other taxa	89.1 (84.4–94.3)
	Moa–O,E,C,K	*82*
	Ostrich–E,C,K	75.5 (72.5–78.4)
	Kiwi–E,C	68.0 (64.5–71.6)
	Emu–cassowary	35.4 (33.1–38.6)
	Emeus–Dinornis	13.2 (11.9–14.6)
		Estimated evolutionary rate, 0.27% per Myr

Estimated divergence dates, with 95% confidence intervals (CI), using a geological calibration (italics) and 10,767 bp of mtDNA protein coding sequence. Notably, the predicted emu–cassowary split is consistent with palaeontological data. The two-parameter molecular-clock model was used, with the ostrich allowed a separate rate (although the estimated dates are similar whether or not the ostrich is allowed a separate rate estimate). The estimated evolutionary rate per site per Myr is given. O, ostrich; K, kiwi; E, emu; C, cassowary.

Minimal divergence dates for the ratite lineages were estimated using the geological calibration point (Table 2). These molecular estimates predict an emu/cassowary split at 33–39 Myr, encouragingly consistent with the palaeontological data. The concordance between the molecular estimates and the two calibration points give strong support for a Late Cretaceous origin for ratites and, by implication, for other modern avian orders. These divergence dates are compatible with vicariance events during the break-up of Gondwana, apart from the kiwi, for which a subsequent dispersal event is required. The tight clustering of dates and lack of phylogenetic resolution, despite the long sequences, also suggests that the derived ostrich, kiwi and emu/cassowary taxa speciated rapidly, possibly in the remnant Gondwanan landmass of Antarctica/Australia.

The molecular phylogeny in Fig. 2 can be used to provide a temporal framework for biogeographical events during the Late Cretaceous. Thus, the 65–72-Myr divergence of the kiwi provides a provisional estimate for dating limited land-based dispersals between Australia, New Caledonia and New Zealand, presumably along the Norfolk Ridge or Lord Howe Rise, consistent with biogeographic evidence[17,21,22]. More importantly, the ostrich divergence date of 73–78 Myr (Table 2) clearly post-dates the separation of South America and Africa around 90 Myr, suggesting an alternative biogeographic origin. The earliest ostrich fossils (skeletal and putative eggshell) are from early to mid Tertiary deposits in Europe, India and Mongolia, leading to hypotheses that the ostrich evolved in Eurasia before entering Africa[7,23]. Reports of Late Cretaceous connections (~80 Myr) between Australia/Antarctica and Indo-Madagascar through the Kerguelen Plateau[24,25] (Fig. 2b) suggest an alternative hypothesis, more consistent with the molecular phylogeny. Such a land link may have allowed both the elephant-bird and ostrich to enter Indo-Madagascar, with the ostrich eventually arriving in Eurasia via the northerly movement of India. If correct, this hypothesis provides a temporal framework for biogeographic dispersals of other land-based taxa between South America/Antarctica and Eurasia/Africa around this time[20,24,25].

By providing a new perspective on Cretaceous biogeography, this ratite molecular phylogeny can be used to discriminate among competing hypotheses about the origin of taxa that arose during the break-up of Gondwana. It also indicates the potential value of using ancient DNA techniques and recently extinct taxa to resolve critical biogeographical issues.

Methods

Ancient DNA studies are extremely susceptible to contamination with extraneous DNA, and must demonstrate adequate experimental and authentication procedures[11,13,14,26]. Consequently, appropriate negative extraction and amplification controls were used throughout, along with rigorous authentication procedures such as quantitation and cloning[13,26] (see Supplementary Information). Two divergent moa taxa were sequenced to allow reciprocal authentication, and results were independently replicated in two laboratories. Overlapping regions of sequence matched identically, and different DNA extractions from the same individual always gave identical sequences. The study was principally performed in Oxford, where all experiments involving ancient specimens before PCR were performed in the Museum of Natural History, a building free of other molecular research.

DNA extraction

DNA was extracted from 0.1-g samples of cortical bone removed from three moa and one elephant-bird specimen: *Emeus crassus*, Museum of New Zealand (MNZ) S91, tibiotarsus from Castle Rocks, Otago; *Dinornis giganteus*, MNZ S34094, phalange from Hodge Ck, Takaka; *Megalapteryx didinus*, MNZ S23808, tibiotarsus from Mt Owen, Takaka; and *Mullerornis agilis*, MNZ S38300, tibiotarsus from Beloha, Madagascar. Accelerator mass spectrometry carbon dating of bone collagen at the Rafter Laboratory, Lower Hutt, New Zealand, showed that the *Emeus* specimen was 1,330 to 1,160 yr BP (95% CI, NZA 9516), and the *Dinornis* specimen was 703 to 523 yr BP (95% CI, NZA 9517). Amino-acid analysis showed that both specimens were moderately/well preserved (Stafford class III), with a C:N ratio of 2.8. The *M. didinus* specimen has been previously dated at 3,350 ± 70 yr BP (see ref. 3). Whole genomic DNA was extracted from tissue samples from the collection of A. Wilson (now held by S. Pääbo, Max Planck Institute for Molecular Anthropology, Leipzig) of an Elegant Crested tinamou (*Eudromia elegans,* breast muscle), Tataupa tinamou (*Crypturellus tataupa,* toe) and cassowary (*C. casuaris,* muscle). Other samples were kiwi (erythrocytes, supplied by M. Potter, Massey University, New Zealand, from specimen K86, Northland, New Zealand) and emu tissue (Louisiana State University Museum of Zoology B-8607, from San Diego Zoo).

PCR and sequencing

PCR amplifications of mtDNA 12S sequences revealed that roughly 600-bp PCR amplifications could be obtained from the *Emeus* and *Dinornis* DNA extracts, compared with 500 bp for *Megalapteryx* and ~200 bp for *Mullerornis*. The moa sequences were identical to previous reports[3,14]. For efficiency, it was decided to sequence the entire mtDNA genomes of just *Emeus* and *Dinornis*. Subsequently, a series of primer pairs was designed to amplify the entire moa mtDNA genome in 400–600-bp sections, using aligned sequences of the chicken, ostrich and rhea mitochondrial genomes (GenBank accession nos X52392, NC001953, AF090339). The amplified sections overlapped by 4–212 bp (median 44 bp) to produce a contiguous sequence. At least one primer of each pair was designed to be incompatible with human mtDNA sequences[14] to remove the risk of human contamination.

The *Mullerornis* specimen was poorly preserved, and no attempt was made to sequence the entire genome. Instead, sections of the 12S, COII, ATP8 and Cyt *b* genes were amplified in short 150–200-bp sections (Fig. 1) and the resulting sequence (~1000 bp) was compared with the other taxa. Long-range PCR (GeneAmp XL-PCR, Perkin-Elmer) was used to amplify two overlapping regions of 6–8 kb (16S-COIII and COI/tSer-tPro) of the mitochondrial genomes of the kiwi, emu, cassowary and two tinamou taxa to minimize (or detect) the possibility of amplifying numts (Fig. 1). Products were recovered from agarose gels and 400–500-bp regions re-amplified and sequenced using the moa primers. Sequencing reactions were performed on both strands using an ABI BigDye PRISM kit and ABI 377. Sequences were deposited on GenBank, with accession numbers AY016010–016019.

Quantitation and cloning

The amount of moa mtDNA fragments of length 540 bp was determined using an oligonucleotide construct (08334Fquant) and competitive PCR as described[13] (Fig. 1). To further examine the validity of the direct sequencing results, we carried out PCR amplifications of the four classes of mtDNA genes (rRNA, tRNA, protein coding and control region), and cloned and sequenced the products (Table 1).

Authentication

To replicate the results independently and provide an additional test of authenticity, the extraction, amplification, cloning and direct sequencing experiments were replicated at the University of Barcelona and the Natural History Museum, London, in dedicated ancient DNA laboratories, using separate bone samples and components. Sections of the *Emeus* 12S, 16S and COII genes (Barcelona), *Dinornis* 12S, COI and Cyt *b* genes (London) and *Mullerornis* 12S (London) were sequenced. The *Emeus* COII and *Dinornis* COI, 12S and Cyt *b* products were also cloned (Table 1).

Phylogenetic methods

Maximum-likelihood trees were estimated by both heuristic and constrained exhaustive searches using PAUP 4.0b4a (ref. 27), a general reversible model of substitution and discrete gamma model (see Supplementary Information). Heuristic searches (ten random additions plus branch swapping), and a constrained exhaustive search (where the two moas, the two tinamous and the emu and cassowary were each assumed to form monophyletic pairs and all 105 possible unrooted trees were examined) produced the same maximum-likelihood tree (Fig. 2). Nearest-neighbour interchange values were calculated using bootstrap frequency outputs to determine the local stability of the phylogenetic tree[4]. Alternative phylogenetic hypotheses were tested using a parametric bootstrap[28], and were significantly worse ($P < 0.001$) than the maximum-likelihood tree in Fig. 2. The maximum-likelihood position of *Mullerornis* was calculated using the short (1,000-bp) dataset, with the other taxa constrained to their positions in Fig. 2. The resulting placement (Fig. 2) does not have strong support, although no signal linked *Mullerornis* with any ratite taxon in particular (data not shown).

To estimate the divergence dates of the ratite lineages, a molecular clock approach was used. The tree in Fig. 2 was calibrated using the geological divergence estimate of 82 Myr

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.

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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*.

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

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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