Genetic evidence for female host-specific races of the common cuckoo

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The common cuckoo *Cuculus canorus* is divided into host-specific races (gentes)[1]. Females of each race lay a distinctive egg type that tends to match the host's eggs, for instance, brown and spotted for meadow pipit hosts or plain blue for redstart hosts[2–4]. The puzzle is how these gentes remain distinct. Here, we provide genetic evidence that gentes are restricted to female lineages, with cross mating by males maintaining the common cuckoo genetically as one species. We show that there is differentiation between gentes in maternally inherited mitochondrial DNA, but not in microsatellite loci of nuclear DNA. This supports recent behavioural evidence that female, but not male, common cuckoos specialize on a particular host[5], and is consistent with the possibility that genes affecting cuckoo egg type are located on the female-specific W sex chromosome[6]. Our results also support the ideas that common cuckoos often switched hosts during evolution[7,8], and that some gentes may have multiple, independent origins, due to colonization by separate ancestral lineages.

We assessed patterns of variation in cuckoo populations in Great Britain[9] and Japan[5] using two types of rapidly-evolving genetic markers with different modes of inheritance. If gentes of the common cuckoo are restricted to female lineages, then we would expect significant differences between their mitochondrial DNA (mtDNA) , as it is transmitted through females only. However, their nuclear microsatellite DNA (nDNA), which segregates through both sexes, would not differ. On the other hand, if male cuckoos mate only with female cuckoos raised by the same host, then the gentes would be genetically isolated (cryptic species) and would differ in both mtDNA and nDNA.

We obtained blood samples from unrelated cuckoo chicks found in nests across Great Britain with three major hosts—the reed warbler (*Acrocephalus scirpaceus*), the meadow pipit (*Anthus pratensis*) and the dunnock (*Prunella modularis*). Average egg colour patterns differ among these gentes[3]. We were unable to document the colour of the eggs from which the chicks hatched and so our analyses refer to cuckoo chicks raised in different host nests and not host-specific egg patterns. We assume that each chick hatched from an egg whose morphology is representative of a particular cuckoo race.

Figure 1a shows an unrooted, maximum parsimony tree of relationships between the 20 mtDNA control region haplotypes (Table 1) from 43 cuckoo chicks in Great Britain. With one exception (GB 5), individual haplotypes are found in only a single type of host (Table 1). Haplotypes within a given gens can be quite different (for example, 'reed warbler' cuckoo haplotypes GB1 and GB3) leading to a pattern in which host-specific haplotypes form multiple clusters of closely-related haplotypes separated by a number of mutational steps (Fig. 1a). The frequencies of different haplotypes are significantly different between cuckoo chicks from different hosts (exact test across all three hosts, $P < 0.001$; all pair-wise exact tests, $P < 0.05$) and values were all significantly different from zero (overall $F_{ST} = 0.18$; $P < 0.001$; pair-wise F_{ST} values = 0.10–0.27; all $P < 0.05$) (see analysis section in methods).

The genetic similarity in haplotypes among chicks from the same host race cannot be explained as a by-product of sampling large numbers of related individuals from the same locality. Four of the seven haplotypes with more than one chick (GB 2, GB 5, GB 8 and GB 17) were represented by samples collected from geographically distinct locations separated by a mean (± s.d.) of 180.8 ± 86.5 km (range, 10–324 km). The three others (GB 1, GB 3 and GB 15) had chicks from more than one site (9, 2 and 2 sites respectively; mean distance apart, 147.8 ± 66.7 km; range, 10–280 km) and small numbers of chicks (≤ 5) from the same site. Multiple chicks from the same site were unrelated on the basis of genetic and spatial criteria[9]. In addition, when analyses were conducted for samples from each race separately to control for the effect of host type, there was no relationship between pair-wise genetic distances between individual sequences and geographical distances between sample locations (Mantel tests, all $P > 0.05$; range of r^2 values, 0.008–0.08).

Japanese cuckoos from a single population near Nagano[5,10] represent an interesting comparison with the British cuckoos in two ways. First, although there is behavioural evidence from genetic analysis of parentage[5] and radio-tracking[10] that females are host specialists, individuals with different hosts lay eggs that are more similar and mimic host eggs less well than those in Great Britain[11]. Second, one of the main hosts of this population, the azure-winged magpie (*Cyanopica cyana*), only became a host about 30 years ago[11].

Figure 1b shows an unrooted, maximum parsimony tree of the relationships between the four mtDNA control region haplotypes

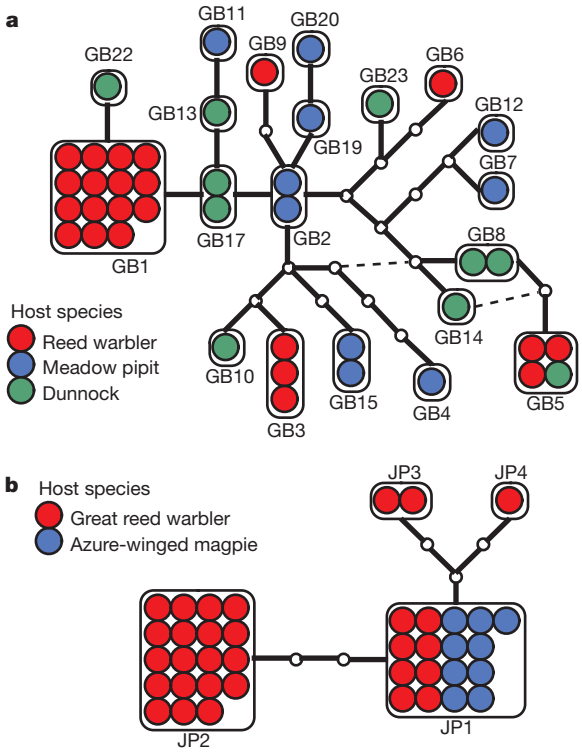

Figure 1 Unrooted, maximum parsimony trees for cuckoo mtDNA haplotypes from Great Britain (**a**) and Japan (**b**). Individual haplotypes are represented by boxes labelled with the name of the haplotype (Great Britain, GB1 to GB23; Japan, JP1 to JP4). The number of coloured circles within each box shows the number and host identity of cuckoos with a particular haplotype. The number of line segments connecting the boxes gives the number of nucleotide differences between two haplotypes. For the GB network, alternative connections defining other equally parsimonious trees are shown by dotted lines.

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found in the 39 female cuckoos of the Japanese population. Two common (JP1 and 2) and two rare (JP3 and 4) haplotypes were present (Table 1). Mean sequence divergence between these haplotypes and those from Great Britain equals 5.5% (range, 4.6–6.3%). The reduced level of mtDNA variation in the Japanese cuckoos may be due to the restricted area from which they were collected as compared with the British birds. As with the British cuckoos, there was a highly significant difference ($P < 0.0001$) in haplotype frequencies of cuckoos from different races; all 'magpie' females had the common haplotype JP1, whereas the 'great reed warbler, (*Acrocephalus arundinaceus*)' females possessed all four haplotypes (Table 1). The pair-wise F_{ST} value was also large and significant ($F_{ST} = 0.39$; $P < 0.001$). This differentiation is not simply due to the presence of related groups of females with the same host. After adjusting for the presence of possible relatives among the sampled birds, a total of 23 'great reed warbler' and eight 'magpie' cuckoos remained. Nonetheless, the pair-wise F_{ST} value remained large and significant ($F_{ST} = 0.30$; $P = 0.0098$) as did the differences in haplotype frequencies ($P = 0.0037$). The limited variation in the 'magpie' cuckoos is consistent with the idea that, although such cuckoos are behaviourally a distinct race, they are recently derived. However, this pattern could also be due to the smaller number of 'magpie' cuckoos sampled. Their distinctiveness, despite their recent origin, is expected if the 'magpie' race in Nagano is composed of colonists from other pre-existing gentes in the area[11] or is made up of females from extant 'magpie' races in other parts of Japan.

Assuming that identical mitochondrial sequences did not originate independently in different gentes by parallel (or convergent) mutations, there has been a minimum of nine host switches by British female cuckoos. Including the host as an equally weighted character lowers this to eight host switches. A single host switch from great reed warblers to azure-winged magpies is consistent with the available mtDNA data for Japanese cuckoos. In both populations, the minimum number of host switches inferred from the mtDNA haplotype tree is far less than that expected under a null model of no association between cuckoo mtDNA lineages and host species ($P \ll 0.001$).

In contrast to the mtDNA results, no significant differences were observed in allele frequencies (all $P > 0.12$ for exact tests) nor was the overall value (overall value 0.0; $P = 0.53$; range of locus-specific R_{ST} values, 0.0–0.05) significantly different from zero for any of eight nuclear microsatellite loci between 'magpie' or 'great reed warbler' cuckoos in Japan (Table 2). For the British cuckoo chicks, only 1 of 30 host-race-by-locus exact tests (cuckoo-specific microsatellite locus Ccμ) 02 for the dunnock versus reed warbler comparison) was significant ($P < 0.01$) after adjusting the critical P value for judging significance using a sequential Bonferroni procedure. Host-race-by-locus R_{ST} values ranged from 0.0 to 0.19, though the overall R_{ST} value (0.023; $P = 0.12$) (Table 2) and all pair-wise values were small and not significantly different from zero (dunnock versus meadow pipit, $R_{ST} = 0.0012$; $P = 0.53$; dunnock versus reed warbler, $R_{ST} = 0.021$; $P = 0.22$; meadow pipit versus reed warbler, $R_{ST} = 0.038$; $P = 0.07$). Using an analysis of molecular variance (AMOVA) to compare directly the results for each class of marker, both populations show a highly significant variation in mtDNA between cuckoos associated with different hosts (GB 19% and Japan 40% of the total variation; both $P \leq 0.002$), but no significant differentiation in nuclear microsatellite loci (0% total variation for both; $P \geq 0.73$). These sex-dependent patterns of genetic differentiation demonstrate the presence of female-specific gentes in cuckoos.

Sequence divergence between mtDNA haplotypes within Great Britain and Japan was low, indicating that these gentes are young in age. The divergence in sequence between the British haplotypes ranges from 2.2% to 0.24% (mean, 1.26%). For Japan, the range is 1.46–0.73% (mean, 1.02%) (Table 1). To estimate time to the most recent common ancestor for all haplotypes for each region, we calculated the average sequence divergence and its standard deviation across the basal node for each region (that is, Great Britain and Japan) in a rooted haplotype tree[12]. Haplotypes from the two regions were used as reciprocal outgroups in this analysis. Mean (± 2 s.d.) sequence divergence across the basal node for each region in the haplotype tree was 1.6% (± 1.0%) for Great Britain and 1.3% (± 0.9%) for Japan. Assuming a molecular clock calibration of 20%

Table 1 mtDNA haplotype variation and distribution among hosts

Haplotype	Position and base composition of variable sites 1111111111111111122233334 11122336666788900000111223334446747933880 24902050234245301457234260671694578079367	Number of each haplotype associated with specific hosts Host 1	Host 2	Host 3
GB1	tcctgtcttccacaatagatagcgcctccgaataggaaact	15	0	0
GB2	.....g.....a.....	0	2	0
GB3	..t.....g.g....a.....a.....	3	0	0
GB4	...c.....g....a...t.....aa....c	0	1	0
GB5	..tc...c.....a.....t.....a.....	3	0	1
GB6	.....a.....ct.....a.....	1	0	0
GB7	...c..t...t.t...ga.....a.....	0	1	0
GB8	..tc.....ga.....t.....a.....	0	0	2
GB9	.....g..c.....a...g..	1	0	0
GB10	..t.....g....a..t.....a.....	0	0	1
GB11	.....g.....c.....a.....	0	1	0
GB12	...ca.t...t.....ga.....a.....	0	1	0
GB13	.....g.....a.....	0	0	1
GB14	...c...c.....ga.....t.....a.....	0	0	1
GB15	.....t.....g....at.....a.....	0	2	0
GB17	.....a.....	0	0	2
GB19	.....g.....a....t.	0	1	0
GB20	.....g.....t.....a....t.	0	1	0
GB22	.....t.....	0	0	1
GB23	.....c.....ga.....t.....a.....	0	0	1
JP1	cttc.c..cttg.....g..att..tagg..a.ggg.c	8	9	–
JP2	cttc.c..cttg.....a..g..att..ta.gc.a.ggg.c	19	0	–
JP3	ctt.....cttg...c....g..att..tagg..a.ggg.c	2	0	–
JP4	cttc....cttg.g.....g..att..tagg.ga.ggg.c	1	0	–

Positions of variable sites refer to nucleotide positions relative to the beginning of the 411-bp mtDNA sequence. Dots represent identical bases to the first sequence. For GB haplotypes, Hosts 1, 2 and 3 refer to reed warbler, meadow pipit and dunnock hosts respectively, while for JP haplotypes, Hosts 1 and 2 refer to great reed warbler and magpie hosts, respectively. These sequences have been deposited in GenBank under accession nos. AF282694–AF282717.

divergence per Myr for this portion of the control region[13], the most recent common ancestor of British haplotypes occurred 80,000 (± 50,000) yr ago and the most recent common ancestor of Japanese haplotypes occurred 65,000 (± 45,000) yr ago. Although these estimates are subject to error associated with assumptions about mutation rate, the time of the most recent mitochondrial ancestor provides an upper limit to the age of extant gentes.

These genetic results confirm inferences from studies of behaviour[5] and egg morphology[3,4] that cuckoo females, but not males, specialize on particular hosts, possibly through some type of imprinting mechanism[14,15], and such female-specific behaviour has continued long enough to result in an unusual pattern of sex-specific genetic differentiation in this parasitic species. Our previous attempt to test for mitochondrial differentiation between cuckoo gentes[9] probably failed because the genetic marker used (an mtDNA control region microsatellite) has an extremely high mutation rate. Phylogenetic reconstruction of repeat number in this microsatellite in the context of the new mtDNA sequence data reveals that this marker retains little historical information.

Other studies have linked egg colour variation to loci on autosomes[16] and the Z sex chromosome[17]. However, our results agree with the proposal that genetic factors controlling the expression of egg colour are present on the female-specific W chromosome[6], as observed for egg patterning in the great tit *Parus major* (A. Gosler, personal communication). This assumes that the patterns of differentiation in cuckoo W chromosomes parallel those observed in mtDNA in the present study. Because we lack direct evidence on the heritability of egg colour from parent–offspring comparisons, we cannot exclude explanations based on autosomal inheritance of cuckoo egg characteristics[5]. If inheritance of these characteristics is female-dependent, then these results would add to the view that sex-determining chromosomes, like W in birds, contain loci that code for products involved in reproductive function, such as the SRY gene in mammals (for review see ref. 18). This female-specific pattern of differentiation also indicates that other possible host-specific adaptations such as matching of host begging calls[19] must have a significant environmental (for example, learned) component as they would be expressed in both male and female cuckoo chicks.

The mtDNA data indicate a rate of host-switching that is very low over ecological time, on the scale of years and decades, such that cuckoo populations associated with different hosts develop significant differences in mtDNA haplotype frequencies. Over longer time periods, however, this low rate of host switching prevents the differentiation of cuckoo gentes into distinct mtDNA clades. Our results support the ideas that gentes are, in an evolutionary sense, relatively young, that host switching has occurred frequently[7,11] and that, at least in Great Britain, each 'host-specific race' may have multiple, independent origins due to colonizations by separate ancestral lineages at different times[8,20]. □

Table 2 Locus-specific and overall R_{ST} values for nuclear microsatellite DNA variation

	R_{ST} values	
Locus	Great Britain	Japan
Ccμ 02	0.0	0.0
Ccμ 13	0.13	0.06
Ccμ 60	0.0	0.0
Ccμ 88	0.04	0.05
Ccμ 100	0.05	0.0
Ccμ 108	0.01	0.0
Ccμ 119	0.01	0.0
Ccμ 137	0.0	0.0
Overall	0.02	0.0
P value	(0.12)	(0.53)

R_{ST} values are for comparisons among cuckoos across all hosts for each population. The *P* value is the probability that the overall R_{ST} value for a particular population is different from zero.

Methods

Cuckoo samples

DNA samples for this study came from previously studied cuckoo populations in Great Britain[9] and Japan[5]. British samples were collected as described[9] from 43 unrelated cuckoo chicks found in the nests of three major hosts: reed warbler ($n = 23$), meadow pipit ($n = 10$) and dunnock ($n = 10$). For Japan, DNA samples were analysed from 39 adult female cuckoos from a single marked population in Nagano whose laying histories had been determined using DNA-based parentage analyses as described[5]. As these earlier results indicate that females in this population are largely host specific, we assumed that females identified as laying only a single egg in a particular host were specialists on that host. Overall 30 females were classified as specializing on great reed warblers and nine females were classified as azure-winged magpie specialists. These samples include a female who laid two eggs in magpie nests and one egg in a warbler nest (classified as a 'magpie' specialist) and exclude a female identified as laying one egg in the nest of each host.

Genetic data

For mtDNA analyses, we determined sequence variation in a 411-base-pair portion of the left-hand hypervariable region of the mtDNA control region using two *Cuculus*-specific primers (CCRL1A, 5′-CAT GAT ACA TTA CAT GTA TGC CTG-3′ and CCRH1, 5′-CTG AAA TAG TAT GAA TGT ATC TGT G-3′). To assess nuclear microsatellite variation in the Japanese samples, we analysed unpublished data on allelic variation in the eight cuckoo-specific loci (Ccμ 02, 13, 60, 88, 100, 108, 119 and 137)[21] which had been used for the parentage analyses[5]. For the British samples, we genotyped the samples using the cuckoo-specific loci described above (except for Ccμ 13) and combined these data with genotypes for the three previously presented loci (Ccμ 01, 09, and 13)[9] for an overall data set for the British samples based on 10 loci.

Analyses

Phylogenetic relationships among unique mtDNA haplotypes were inferred using maximum parsimony as implemented in PAUP[22]. We used ARLEQUIN[23] to conduct pair-wise comparisons of mtDNA haplotype and microsatellite allele frequencies between putative gentes. Differentiation was assessed using exact tests[24] for differences in haplotype or allele frequencies and in terms of F_{ST}[25] values for mtDNA and R_{ST}[26] values for microsatellite variation. For F_{ST} values, similarity between haplotypes was weighted on the basis of the magnitude of gamma-corrected ($\alpha = 0.08$) Kimura 2-parameter values between haplotypes. We also used this program for AMOVA[27] to partition total variance in both mtDNA and microsatellite variation into within versus between putative host race components. Significance levels for particular tests or values were assessed using permutation procedures implemented in the respective programs.

To identify possible relatives among 'magpie' and 'great reed warbler' females from Japan, we used the microsatellite allele frequencies for the overall population[21] with the program KINSHIP[28] to calculate 95% confidence intervals for the expected *r* values[29] for pair-wise comparisons of unrelated individuals. Any pair of females with the same mtDNA haplotype and an *r* value greater than 0.33 (the upper bound of the interval) were identified (1/30 and 8/420 possible pairs of 'magpie' and 'great reed warbler' females, respectively) and a single randomly chosen member of pair was deleted from the data set. We then repeated our analysis of differentiation in mtDNA using this modified data set.

To estimate the minimum or most parsimonious number of host switches that could have occurred given the evolutionary history of these populations, the number of steps in the character 'host' on the shortest mtDNA trees was determined using MacClade[30] with the assumption of soft polytomies. To evaluate if the observed number of host switches was smaller than expected under a null model of no association between 'host' and cuckoo mtDNA haplotype, 1,000 randomized characters were generated by reassigning individual cuckoos to hosts and calculating the minimum number of host switches for the randomized characters on the shortest mtDNA trees.

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1. Newton, A. *A Dictionary of Birds*. (Black, London, 1893).
2. Baker, E. C. S. *Cuckoo Problems*. (Witherby, London, 1942).
3. Brooke, M. de L. & Davies, N. B. Egg mimicry by cuckoos, *Cuculus canorus*, in relation to discrimination by hosts. *Nature* **335,** 630–632 (1988).
4. Moksnes, A. & Røskaft, E. Egg-morphs and host preference in the common cuckoo (*Cuculus canorus*): an analysis of cuckoo and host eggs from European museum collections. *J. Zool.* **236,** 625–648 (1995).
5. Marchetti, K., Nakamura, H. & Gibbs, H. L. Host race formation in the Common Cuckoo. *Science* **282,** 471–472 (1998).
6. Punnett, R. C. Inheritance of egg-colour in the parasitic cuckoos. *Nature* **132,** 892 (1933).
7. Davies, N. B. & Brooke, M. de L. An experimental study of co-evolution between the cuckoo, *Cuculus canorus*, and its hosts. II. Host egg markings, chick discrimination, and general discussion. *J. Anim. Ecol.* **58,** 225–236 (1989).
8. Davies, N. B. *Cuckoos, Cowbirds and Other Cheats* (Poyser, London, 2000).
9. Gibbs, H. L., Brooke, M. de L., & Davies, N. B. Analysis of genetic differentiation of host races of the common cuckoo *Cuculus canorus* using mitochondrial and microsatellite DNA variation. *Proc. R. Soc. Lond. B* **263,** 89–96 (1996).
10. Nakamura, H. & Miyazawa, Y. Movements, space use and social organization of radio-tracked cuckoos during the breeding season in Japan. *Jap. J. Ornithol.* **46,** 23–53 (1997).
11. Nakamura, H., Kubota, S. & Suzuki, R. in *Parasitic Birds and Their Hosts* (eds Rothstein, S. I. & Robinson, S. K.) 94–112 (Oxford Univ. Press, Oxford, 1998).
12. Steel, M. A., Cooper, A. C. & Penny, D. Confidence intervals for the divergence time of two clades. *System. Biol.* **45,** 127–134 (1996).
13. Avise, J. C. & Walker, D. Pleistocene phylogeographic effects on avian populations and the speciation process. *Proc. R. Soc. Lond. B* **265,** 457–463 (1998).

14. Teuschl. Y., Taborsky, B. & Taborsky, M. How do cuckoos find their hosts? The role of habitat imprinting. *Anim. Behav.* **56,** 1425–1433 (1998).
15. Brooke, M. de L. & Davies, N. B. A failure to demonstrate host imprinting in the common cuckoo *Cuculus canorus* and alternative hypotheses for the maintenance of egg mimicry. *Ethology* **89,** 154–166 (1991).
16. Collias, E. C. Inheritance of egg-colour polymorphism in the village weaver (*Ploceus cucullatus*). Auk **110,** 683–693 (1993).
17. Schoffner, R. N. *et al.* The effect of a protoporphyrin mutant on some economic traits of the chicken. *Poultry Sci.* **61,** 817–820 (1982).
18. Roldan, E. R. S. & Gomendio, M. The Y chromosome as a battleground for sexual selection. *Trends Ecol. Evol.* **14,** 58–62 (1999).
19. Kilner, R. M., Noble, D. G. & Davies, N. B. Signals of need in parent-offspring communication and their exploitation by the common cuckoo. *Nature* **397,** 667–672 (1999).
20. Dawkins, R. *Unweaving the Rainbow.* (Allen Lane, London, 1998).
21. Gibbs, H. L., De Sousa, L., Marchetti, K. & Nakamura, H. Isolation and characterization of microsatellite DNA loci for an obligate brood parasitic bird, the common cuckoo (*Cuculus canorus*). *Mol. Ecol.* **7,** 1437–1439 (1998).
22. Swofford, D. PAUP, Ver. 4.0 (Sinauer Associates, 1999).
23. Schneider, S., Kueffer, J. M., Roessli, D. & Excoffier, L. *Arlequin ver. 1. 1: A Software for Population Genetic Data Analysis.* (Genetics and Biometry Laboratory, Univ. Geneva, Switzerland, 1997).
24. Raymond, M. & Rousset, F. An exact test for population differentiation. *Evolution* **49,** 1280–1283 (1995).
25. Hudson, R., Slatkin, M. & Maddison, W. Estimation of levels of gene flow from DNA sequence data. *Genetics* **132,** 583–589 (1992).
26. Slatkin, M. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139,** 457–462 (1995).
27. Excoffier, L., Smouse, P. E. & Quattro, J. M. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA. *Genetics* **131,** 479–491 (1992).
28. Goodnight, K., Queller, D. C. & Poznansky, T. Kinship Ver. 1.2 (1997). Available on http://www.bioc.rice.edu/~kfg/GSoft.html.
29. Queller, D. C. & Goodnight, K. F. Estimating relatedness using genetic markers. *Evolution* **42,** 258–275 (1989).
30. Maddison, W. P. & Maddison, D. R. *MacClade: Analysis of Phylogeny and Character Evolution.* Ver. 3.0. (Sinauer Associates, 1992).

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WNT signalling molecules act in axis formation in the diploblastic metazoan *Hydra*

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Members of the Wnt/wingless family of secreted proteins act as short-range inducers and long-range organizers during axis formation, organogenesis and tumorigenesis in many developing tissues[1]. Wnt signalling pathways are conserved in nematodes, insects and vertebrates[2]. Despite its developmental significance, the evolutionary origin of Wnt signalling is unclear. Here we describe the molecular characterization of members of the Wnt signalling pathway—Wnt, Dishevelled, GSK3, β-Catenin and Tcf/Lef—in *Hydra*, a member of the evolutionarily old metazoan phylum Cnidaria. Wnt and Tcf are expressed in the putative *Hydra* head organizer, the upper part of the hypostome. Wnt, β-Catenin and Tcf are transcriptionally upregulated when head organizers are established early in bud formation and head regeneration. Wnt and Tcf expression domains also define head organizers created by *de novo* pattern formation in aggregates. Our results indicate that Wnt signalling may be involved in axis formation in *Hydra* and support the idea that it was central in the evolution of axial differentiation in early multicellular animals.

We used polymerase chain reaction (PCR) to identify and clone *Wnt*, *Dishevelled* and *GSK3* orthologues from the fresh water polyp *Hydra* (*HyWnt*, *HyDsh* and *HyGSK3*, respectively). *Hydra Tcf/Lef* (*HyTcf*) was isolated in a yeast two-hybrid screen with a *Hydra β-Catenin*[3] (*Hyβ-Cat*) bait. A *Hydra* member of the Frizzled family of transmembrane receptors has also been identified[4]. HyWnt has a relatively low overall amino-acid identity (about 35%) to other Wnt factors, but exhibits a Wnt-specific pattern of 22 cysteines and a signal peptide sequence indicating that it is secreted (Fig. 1a). The cytoplasmic members of the pathway are highly conserved: HyDsh has DIX, PDZ and DEP domains, HyGSK3 has a conserved catalytic domain, and Hyβ-Cat has 13 armadillo repeats and a presumptive GSK3 phosphorylation site (Fig. 1a and ref. 3). The transcription factor HyTcf contains a high mobility group (HMG) box most closely related to HMG boxes in vertebrate Tcf proteins and a putative β-Catenin binding domain at its amino-terminal end (Fig. 1a). Predicted amino-acid sequence alignments of HyWnt, HyDsh, HyGSK3 and HyTcf are presented in the Supplementary Information.

The high degree of structural conservation in protein–protein interaction domains in the cytoplasmic transducers indicates that a Wnt signalling pathway evolved early in metazoan evolution. To provide evidence for functional conservation, we examined the ability of Hyβ-Cat to induce secondary body axes in *Xenopus* embryos. Ectopic expression of Hyβ-Cat by injection of messenger RNA into ventral blastomeres at the eight-cell stage induced 100% ($n = 20$) complete secondary body axes containing the most anterior structures (such as eyes and cement gland), indistinguishable from those induced by *Xenopus* β-Catenin (Fig. 1b). Hyβ-Cat can therefore interact with endogenous transcription factors, presumably

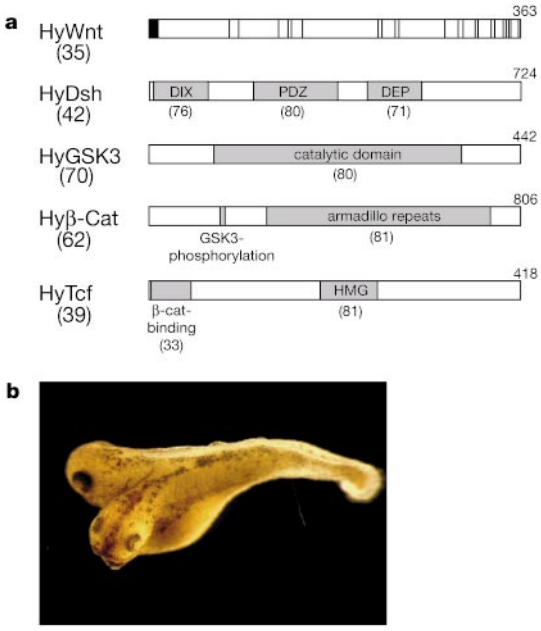

Figure 1 Structural and functional conservation of *Hydra* Wnt signalling molecules. **a**, Schematic representation of the predicted HyWnt, HyDsh, HyGSK3, Hyβ-Cat and HyTcf proteins based on cDNA sequences. HyWnt has a signal peptide sequence (black box) and 22 conserved cysteine residues (vertical lines). Numbers at the ends of the bars represent the number of amino acids in the corresponding open reading frame (ORF). Numbers in parentheses show amino-acid identity between the *Hydra* and human orthologues (Wnt1, Dishevelled1, GSK3β, β-Catenin and Tcf4) throughout the ORF and in protein–protein interaction domains. For details in the predicted Hyβ-Cat structure see ref. 3. **b**, Hyβ-Cat induces complete secondary axes in *Xenopus* embryos.