Methods

Isolation of *Wnt* genes from *N. vectensis*

Nested PCR was used to amplify 122–144-bp fragments of *Nematostella Wnt* genes. We used degenerate primers aimed to amplify any *Wnt* types. PCR was done on complementary DNAs reverse-transcribed from messenger RNA isolated from 12–120 h *N. vectensis* embryos. Primer combinations were as follows: 5′-TGG(GC)A(AGCT)T GGGG(AGCT)GG(AGCT)TG-3′ as forward primer in both rounds of nested PCR, and 5′-T(CT)(AGCT)CC(AG)TG(AG)CA(CT)TT(AG)CA-3′ as outer and 5′-CC(AGCT)GC (AGCT)(CT)(GCT)(AG)TT(AG)TT(AG)TG-3′ as inner reverse primer. Eleven different *Wnt* genes were thus isolated from *N. vectensis*; *NvWnt8a* was obtained from an EST project (U.T. and T.W.H.). The 3′ and 5′ ends of the corresponding genes were amplified from cDNA libraries by using vector-specific primers and gene-specific (non-degenerate) primers. Primer sequences and experimental conditions are available upon request. PCR products were cloned into the pGEM-T vector (Promega) TOPO-TA or into the pCR2.1-TOPO vector using the TOPO TA Cloning reagents (Invitrogen); all clones were sequenced on an ABI automated sequencer.

Retrieval and alignment of *Wnt* gene sequences

Wnt protein sequences were obtained through the retrieval of Wnt protein sequences listed on R. Nusse's Wnt home page (http://www.stanford.edu/~rnusse/wntwindow.html) or by database searches on NCBI, SWISSPROT as well as Sanger, and by BLASTP search at the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/blast/). All sequences and their accession numbers are available as Supplementary Information (Table 1, Fig. 1). ClustalW was used for the protein alignments (http://www.ebi.ac.uk/clustalw/). Where available, only full-length sequences were used. *PvWntA* and *PdWntA* sequences were included because no other full-length sequences are available; the *NvWnt6* and *NvWnt8a* sequences are not yet completely full-length, but give sufficient sequence information for a reliable phylogenetic analysis. Alignments were subsequently manually improved by using alignments of Wnt domains available in PFAM (http://www.sanger.ac.uk/Software/Pfam/) or SMART (http://smart.embl-heidelberg.de/). The Wnt domain itself contains several regions of high conservation separated by less-conserved stretches of amino acids that are not particularly well aligned. Given that sequence alignment influences phylogenetic reconstruction, we explored alternative alignments of these less-conserved regions by changing the gap penalty of ClustalW. These different alignments gave essentially similar results in the phylogenetic analyses, as well as discarding positions with more than 50% gaps.

Phylogenetic analyses

Bayesian analysis was performed with MrBayes 3.0B4 (http://morphbank.ebc.uu.se/mrbayes/) using the Jones–Taylor–Thornton (JTT) model of protein evolution with invariant sites and four Gamma-distributed rates. Six chains were run for 20,000,000 generations; after a burn-in of 1,000,000 generation every 100th tree was sampled for a 50% majority consensus. In addition, ML analyses were done with TREE-PUZZLE 5.2 (http://www.tree-puzzle.de/), as well as IQPNNI 2.2 (ref. 30) (http://www.bi.uni-duesseldorf.de/software/iqpnni/). Bootstrap support values were constructed using PAUP* 4.0b (http://paup.csit.fsu.edu/), applying the MP criterion. For details see Supplementary Methods.

In situ hybridization

The procedure of the *in situ* hybridization was performed as described[13] with the following changes: Specimens were fixed in 4% MEMPFA containing 0.0625% glutaraldehyde for 3 h, and then stored in methanol at −20 °C. Hybridization of the DIG-labelled RNA probe was carried out at 44–65 °C for at least 36 h, post-hybridization washes were done in 50% formamide/2 × SSC/0.02% TritonX-100 over 8 h by raising the temperature gradually from 47 °C to 56 °C. Visualization of the labelled probe was performed using NBT BCIP (Boehringer) as substrate for the alkaline phosphatase conjugated anti-DIG antibody used in the procedure.

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Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei

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Arbuscular mycorrhizal fungi (AMF) are ancient asexually reproducing organisms that form symbioses with the majority of plant species, improving plant nutrition and promoting plant diversity[1,2]. Little is known about the evolution or organization of the genomes of any eukaryotic symbiont or ancient asexual organism. Direct evidence shows that one AMF species is heterokaryotic; that is, containing populations of genetically different

nuclei[3]. It has been suggested, however, that the genetic variation passed from generation to generation in AMF is simply due to multiple chromosome sets (that is, high ploidy)[4]. Here we show that previously documented genetic variation in Pol-like sequences, which are passed from generation to generation, cannot be due to either high ploidy or repeated gene duplications. Our results provide the clearest evidence so far for substantial genetic differences among nuclei in AMF. We also show that even AMF with a very large nuclear DNA content are haploid. An underlying principle of evolutionary theory is that an individual passes on one or half of its genome to each of its progeny. The coexistence of a population of many genomes in AMF and their transfer to subsequent generations, therefore, has far-reaching consequences for understanding genome evolution.

Arbuscular mycorrhizal fungi (Glomeromycota) are a basal group of fungi[5,6]. All known members of this group are symbiotic with plants. They are also thought to have been asexual for 400 million years[3,7]. Understanding their genome organization is important for understanding the evolutionary biology of symbiotic eukaryotes and ancient asexual organisms, and also because of their importance for plant growth. Genetic variation within single spores of AMF is well documented for ribosomal DNA and also for protein-coding genes[3,6,8]. There are three possibilities for how this genetic variation is organized: (1) variants of a locus exist in different nuclei (Fig. 1a); (2) the variants are present in each nucleus owing to polyploidy (Fig. 1b); (3) the variants exist in each nucleus owing to duplication events (the variants represent a multiple copy

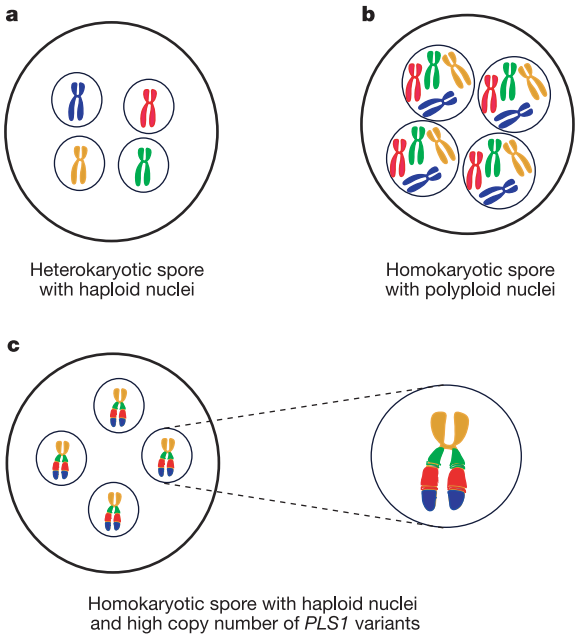

Figure 1 Three possibilities for how the genetic variation of *PLS1* is organized in *G. etunicatum*. **a**–**c**, *PLS1* variants exist in different nuclei (**a**; heterokaryosis), all *PLS1* variants are present in each nucleus owing to polyploidy and nuclei are genetically identical (**b**; homokaryosis), or all *PLS1* variants are present in each nucleus as copies due to duplication events of PLS in a haploid genome (**c**; also homokaryotic). For clarity we used only four colours to represent the 13 different PLS variants and only four nuclei instead of 750 nuclei contained in each *G. etunicatum* spore. For simplicity, haploid nuclei are depicted with one chromosome and polyploid nuclei with four chromosomes, although actual chromosome number in this fungus is unknown. The four colours represent variants of the *PLS1* region, although up to 13 variants have previously been recorded per spore.

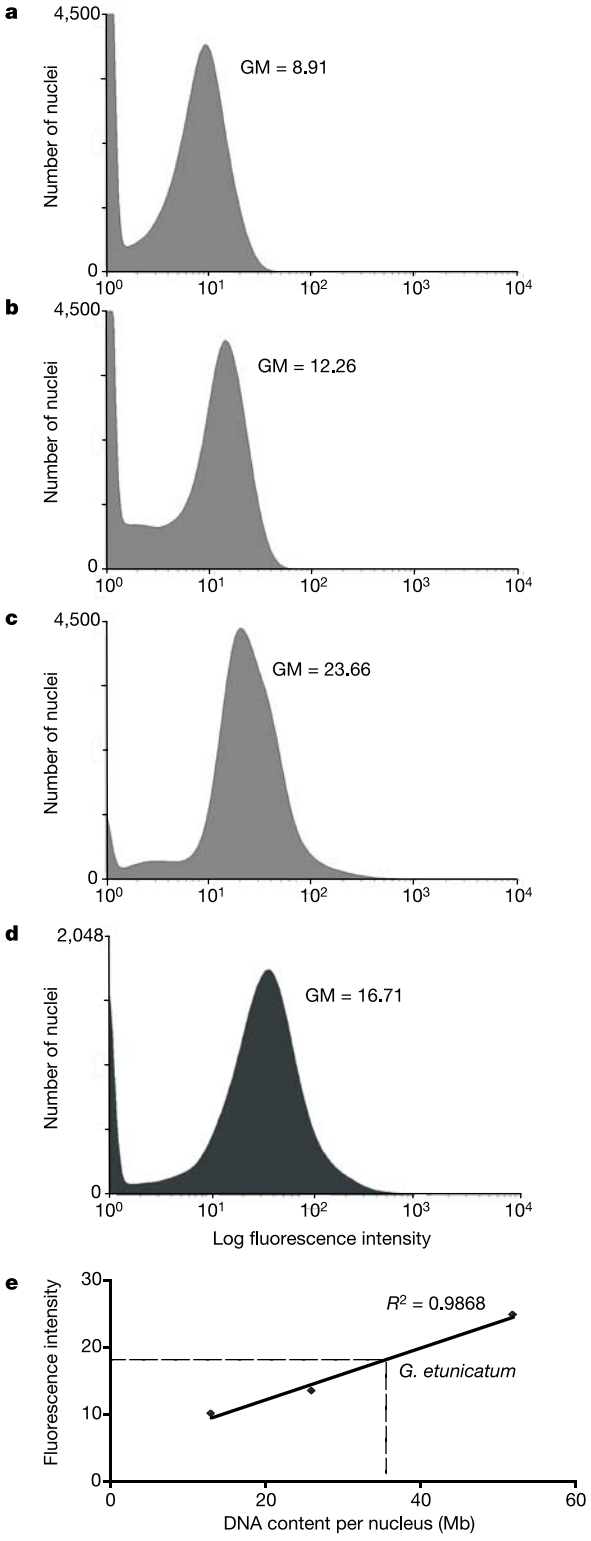

Figure 2 Measurements of fluorescence intensity for estimating the nuclear DNA content of *G. etunicatum*. **a**–**c**, Histograms from one of the three replicate experiments showing fluorescence intensities obtained by flow cytometry for propidium-iodide-stained nuclei of haploid (**a**), diploid (**b**) and tetraploid (**c**) *S. cerevisiae*, and of *G. etunicatum* (**d**). **e**, Linear regression relating the geometric mean of fluorescence intensity (GM) to DNA content per cell of haploid, diploid and tetraploid *S. cerevisiae*, used to estimate the DNA content per nucleus of *G. etunicatum*. The dotted line shows the geometric mean of fluorescence of *G. etunicatum* nuclei and the corresponding DNA content.

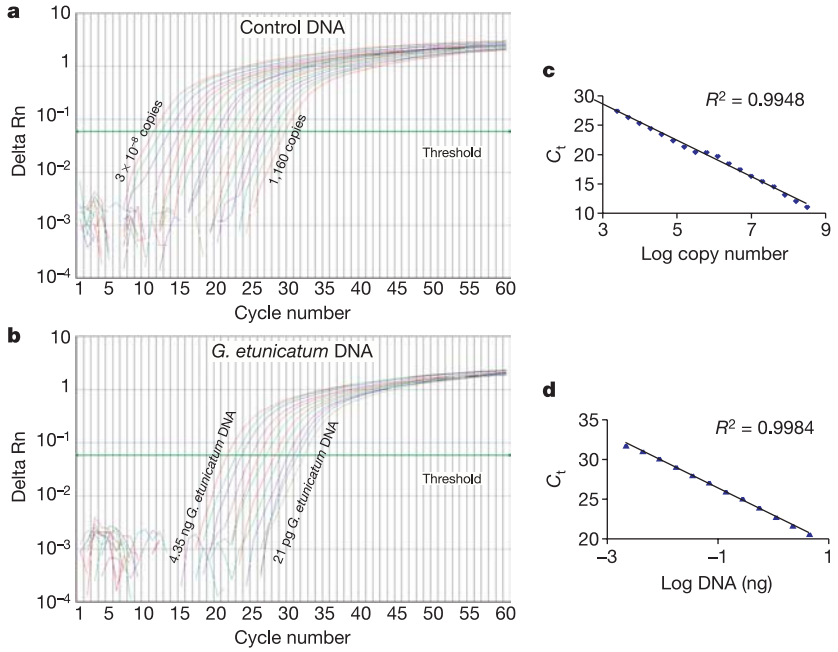

Figure 3 Amplification plots and regressions obtained by real-time PCR. **a**, Amplification plot of twofold serial dilutions (from 3×10^8 to 1,160 copies) of plasmid DNA containing the PLS insert, where 'delta Rn' is the fluorescence intensity. **b**, Amplification plot of twofold serial dilutions (from 4.35 ng to 21 pg) of the *G. etunicatum* genomic DNA. **c**, Linear regression relating the cycle threshold parameter values (C_t values) and log copy number of the plasmid DNA. **d**, Linear regression relating C_t values and log DNA concentration of *G. etunicatum* DNA.

region of DNA, even in the haploid genome; Fig. 1c). In addition, the three hypotheses are not mutually exclusive and variation could represent a combination of these three possibilities. Using DNA–DNA fluorescent *in situ* hybridization, different variants of the *ITS2* region have been shown to be located in different nuclei in single spores of the AMF *Scutellospora castanea*, supporting the first of these hypotheses[3]. However, the existence of genetic differences among nuclei has been questioned[4]. Large variation in a *POL1*-like sequence (*PLS1*) has been observed in the AMF *Glomus etunicatum*. Thirteen variants of *PLS1* were shown to be passed from the mother spore to its clonal progeny in five single-spore isolates of this AMF[4]. A mathematical model, based on the random inheritance of nuclei to each clonally produced offspring, predicted that if the 13 variants of *PLS1* existed in genetically different nuclei then the loss of some variants would almost certainly occur after one generation. The variation was assumed to be due to polyploidy because all 13 variants existed in each clonally produced spore and the possibility of the *PLS1* region being multi-copy was ruled out because it is single copy in other eukaryotes[4]. This conclusion would mean that the fungus in question is polyploid to, at least, 13N.

Without measuring the ploidy level or copy number of the *PLS1* region in this fungus, the suggestion that the genetic variation in AMF is due to high ploidy is surprising. So far there is only one published measurement of ploidy in AMF. Nuclei of *Glomus intraradices*, a species related to *G. etunicatum*, were clearly shown to be haploid, with a nuclear DNA content on the lower limit for eukaryotes[9]. We therefore measured the nuclear DNA content of *G. etunicatum* in order to be able to calculate the size of the haploid genome if the nuclei were 13N. Once the amount of DNA per nucleus was known, we were then also able to measure the number of copies of *PLS1* per amount of DNA contained in a nucleus. Flow cytometry was performed on nuclei of *G. etunicatum*, as well as with standards of haploid, diploid and tetraploid *Saccharomyces cerevisiae* (Fig. 2). Nuclear DNA content of *G. etunicatum* nuclei was only 37.45 megabases (Mb) (±3.9 Mb, standard error, $n = 3$). As with previous flow cytometry measurements on the nuclei of 14 other AMF species[9–11], only one peak of fluorescence was obtained. Therefore, there is no evidence for the coexistence of nuclei in different states of ploidy. If *G. etunicatum* nuclei were 13N then the genome size of this fungus would be 2.88 Mb. Such a small genome

Table 1 **Re-association kinetics of genomic DNA from *S. castanea***

Genome component	Fraction*	k†	$C_0t_{1/2}$‡	k_{pure}§	Copy no.‖	Complexity (bp)¶	Size (bp)#
Fold-back	0.0412	–	–	–	–	–	0.33×10^8
Repetitive 1	0.064	0.139	0.366	2.1718	184	27.71×10^4	0.51×10^8
Repetitive 2	0.2389	0.0384	8.123	0.1607	51	3.72×10^6	1.90×10^8
Repetitive 3	0.2361	0.0023	235.294	0.0097	3	62.66×10^6	1.88×10^8
Single copy	0.4196	0.00077	560.342	0.0018	1	3.33×10^8	3.33×10^8
Genome size☆	–	–	–	–	–	–	7.95×10^8

*Fraction of each genome component.
†Re-association rate expressed as $M^{-1}\,s^{-1}$.
‡Product of the molar double-stranded DNA concentration (in nucleotides) and the time (s) for half of re-association completion ($C_0t_{1/2} = 1/k$).
§Modified second-order rate constant for each component, if pure.
‖Copy number of repetitive fraction related to the genome size, complexity and fraction: $(k_{pure} \times \text{fraction}_{\text{repetitive component}})/(k_{pure} \times \text{fraction}_{\text{single copy component}})$.
¶Size of component.
#Total size of each component (copy number × complexity).
☆Genome size calculated relative to the genome size of *E. coli* (4,639,221 bp) and $C_0t_{1/2}$ ($C = (C_0t_{1/2\ S.\ castanea}/C_0t_{1/2\ E.\ coli}) \times C_{E.\ coli}$, where C is the genome size).

size in a eukaryote is highly unlikely given that it is much smaller than that of any other eukaryote, and smaller than that of *E. coli* and most other bacteria[12]. However, our estimation of nuclear DNA content in this fungus does not rule out the possibility of other, lower ploidy levels.

Once the amount of DNA per nucleus is known then elucidating the arrangement of the variation in PLS is relatively simple using real-time polymerase chain reaction (PCR) to estimate PLS copy number. Estimates of the copy number of *PLS1* per 37.45 Mb of *G. etunicatum* genomic DNA, using real-time PCR, showed that the mean number of *PLS1* copies is 1.88 ± 0.055 (s.e.m., $n = 5$) per nucleus (Fig. 3). There was strong support for this figure from regressions of both the amplification of *PLS1* from plasmid DNA ($r^2 = 0.9948$) and from genomic DNA ($r^2 = 0.9984$). Given that a maximum of only two copies of PLS exist per nucleus, there are few remaining possibilities for the organization of the variants, and all possibilities must include considerable genetic differences among nuclei. The first is that *G. etunicatum* nuclei are haploid and that each nucleus contains two copies of PLS. Given that the primers used in real-time PCR also amplify variants of *PLS2* (another group of PLS variants that have also been described[4]), it is likely that each nucleus contains one copy of a *PLS1* variant and one copy of a *PLS2* variant. In this case, the considerable variation of the 13 *PLS1* types and two *PLS2* types must be arranged in different nuclei. The second possibility is that *G. etunicatum* is diploid and that at least 11 of the 13 variants of *PLS1* are segregated among nuclei. Any further increases in ploidy would simply increase the genetic differences among nuclei, given that only two copies of PLS exist per nucleus. Although we used a different isolate of *G. etunicatum* to that used in the previously published study of PLS variation[4], it is improbable that a 12-fold difference in ploidy occurs between these strains, and if that is the case, then it would additionally mean that the variation in the PLS region is organized in a completely different way in the two isolates of the same species. In our opinion, both of these differences together are highly unlikely.

Nuclear DNA content among 14 AMF species varies enormously and by over 50-fold[9–11], therefore those species with large nuclear DNA contents might be polyploid. In order to address whether AMF with large nuclear DNA contents might potentially be polyploid, we re-calculated and re-evaluated existing re-association kinetics data to obtain the size of the haploid genome of *S. castanea*[13] and compared this with previously published measurements of nuclear DNA content of this fungus. A re-analysis was made on this data using a mathematical calculation of the genome size rather than the original manual estimation[13]. The nuclear DNA content of *S. castanea* was shown to be 802.93 ± 12.77 Mb (±s.d.)[10]. Re-calculation of the genome size and complexity on the basis of the re-association kinetics revealed a haploid genome of 795 Mb (Table 1; see also Supplementary Fig. 1). In contrast to *G. intraradices*, over 58% of the *S. castanea* genome (429 Mb) was shown to be due to repetitive sequences and another 33 Mb due to fold-back DNA. The similarity between the nuclear DNA content and the size of the haploid genome clearly indicates that this fungus is haploid, despite the fact that the nuclei contain a large amount of DNA.

Although variation in ITS regions was shown to exist within single nuclei in *G. etunicatum*[4], previous work had already shown that variation both among and within nuclei exists for this region of the genome in AMF[3]. However, the variation that has been documented for PLS in *G. etunicatum* represents the most extensive and important evidence that AMF have evolved to harbour a population of different genomes. First, this variation exists in a protein-coding gene, rather than in the rDNA family[3]. Second, that these variants have been shown experimentally to be passed from generation to generation through spores[4] means that there is no stage in their life cycle where the fungus inherits only one genome. We propose that probably not all genetically different nuclei are inherited by every spore, and that in this case, frequent anastomosis among hyphae of the same species allows re-establishment of nuclear genome diversity in the fungus. Given that frequent anastomosis among hyphae originating from spores of the same AMF species has been observed, this is indeed a probable mechanism[14]. Another possibility is that maintenance of the multi-genomic state in AMF is so important for fitness that a mechanism has evolved to ensure that spores receive a genetically diverse group of nuclear genotypes. Whatever the mechanism for the maintenance of this highly unusual genomic organization, we now need to understand how this group of different genomes in one fungus contributes to their ecology, evolution and symbiotic efficiency. □

Methods

Fungal material

The AMF *G. etunicatum* (isolate Native Plants Incorporated) was provided by J.C. Dodd. Haploid, diploid and tetraploid strains of *S. cerevisiae* were those used previously[9].

DNA extraction and conventional DNA quantification

Genomic DNA of *G. etunicatum* was isolated from spores using the ENZA Fungal DNA mini kit (PeqLab Biotechnology) following the instructions of the manufacturer. DNA was quantified using the PicoGreen double strand DNA quantitation kit (Molecular Probe)[9].

Measurement of DNA content per nucleus

DNA content per nucleus of *G. etunicatum* was performed using flow cytometry. Cells of haploid, diploid and tetraploid *S. cerevisiae* were used as standards for quantification. All methods concerning extraction of AMF nuclei into a suspension and all flow cytometry methods and calculations follow those described previously[9].

Real-time PCR for quantification of PLS copy number

For details about real-time PCR for the quantification of the copy number of PLS per nucleus of *G. etunicatum* see Supplementary Methods.

Analysis of re-association kinetics data

For details about re-association kinetics see Supplementary Methods.

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