joining trees and the amino-acid maximum parsimony phylogenies, and 100 replicates for the nucleotide maximum likelihood tree and the amino-acid distance-based analyses (Dayhoff PAM matrix) (see Supplementary Information for additional trees and summary of bootstrap support). We performed tests of alternative phylogenetic hypotheses using Kishino–Hasegawa[29] (parsimony and likelihood) and Templeton's non-parametric[30] tests.

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Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants

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Most of the 470-million-year history of plants on land belongs to bryophytes, pteridophytes and gymnosperms, which eventually yielded to the ecological dominance by angiosperms 90 Myr ago[1–3]. Our knowledge of angiosperm phylogeny, particularly the branching order of the earliest lineages, has recently been increased by the concurrence of multigene sequence analyses[4–6]. However, reconstructing relationships for all the main lineages of vascular plants that diverged since the Devonian period has remained a challenge. Here we report phylogenetic analyses of combined data—from morphology and from four genes—for 35 representatives from all the main lineages of land plants. We show that there are three monophyletic groups of extant vascular plants: (1) lycophytes, (2) seed plants and (3) a clade including equisetophytes (horsetails), psilotophytes (whisk ferns) and all eusporangiate and leptosporangiate ferns. Our maximum-likelihood analysis shows unambiguously that horsetails and ferns together are the closest relatives to seed plants. This refutes the prevailing view that horsetails and ferns are transitional evolutionary grades between bryophytes and seed plants[7], and has important implications for our understanding of the development and evolution of plants[8].

Estimates of a phylogeny for the main groups of land plants, each with highly divergent morphologies, have been many, and all have been contested. Bryophytes (liverworts, hornworts and mosses) are consistently shown to be a basal grade, but their relationships to one another and to vascular plants are controversial[1,2,9–13]. Most phylogenetic analyses of vascular plants consistently reconstruct two main lines of evolution: the Lycophytina (clubmosses and relatives), with 1% of extant diversity, and the Euphyllophytina (all other vascular plants)[1,2,10,11,14–17]. Extant Euphyllophytina[1,2] comprises six major monophyletic lineages: Equisetopsida (horsetails), Polypodiidae (leptosporangiate ferns), Spermatophytata (seed plants), Psilotidae (whisk ferns; simple plants regarded by some to be living relicts of the earliest vascular plants[7,18]), Marattiidae and Ophioglossidae (eusporangiate ferns). Phylogenetic assessments based on single genes[10,11,14,15,19] and/or morphology[1,7,12,17,20] have provided only weak and usually contradictory evidence for the relationships among these euphyllophyte lineages. Resolving these relationships would not only stabilize a pivotal region of vascular plant phylogeny but is also key to identifying the most appropriate outgroup for addressing questions related to the evolution of seed plants.

Recent palaeontological studies[1,2,7] attempted to demonstrate that approaches based solely on living species would have difficulties reconstructing relationships among major lineages of vascular plants. Inadequate taxon sampling, rate heterogeneity across DNA nucleotide sites among lineages, and inappropriate algorithms also have been cited as impediments to resolving ancient branching events[21]. However, as predicted by recent theoretical studies[22], combined analysis of DNA sequences from multiple loci proves to

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be very useful in inferring deep phylogenetic patterns[4–6]. With few exceptions[12,20], broad phylogenetic studies rely solely on combined nucleotide sequence data, with authors arguing that morphological character homology assessment among ancient and divergent groups is too challenging. This practice ignores the higher complexity of morphological characters that can conserve character states over time and that have a lower probability of random evolution of similar structures.

We obtained DNA sequences (5,072 aligned base pairs) of four genes from two plant genomes: plastid *atpB*, *rbcL* and *rps4*, and nuclear small-subunit ribosomal DNA. We also assembled a congruent data set of 136 vegetative and reproductive morphological/anatomical characters. We sampled 35 representatives from all major monophyletic lineages of land plants. The selection of taxa reflects our focus on basal vascular plants, and all six Euphyllophytina[1] lineages are represented by two or more members. Five bryophytes

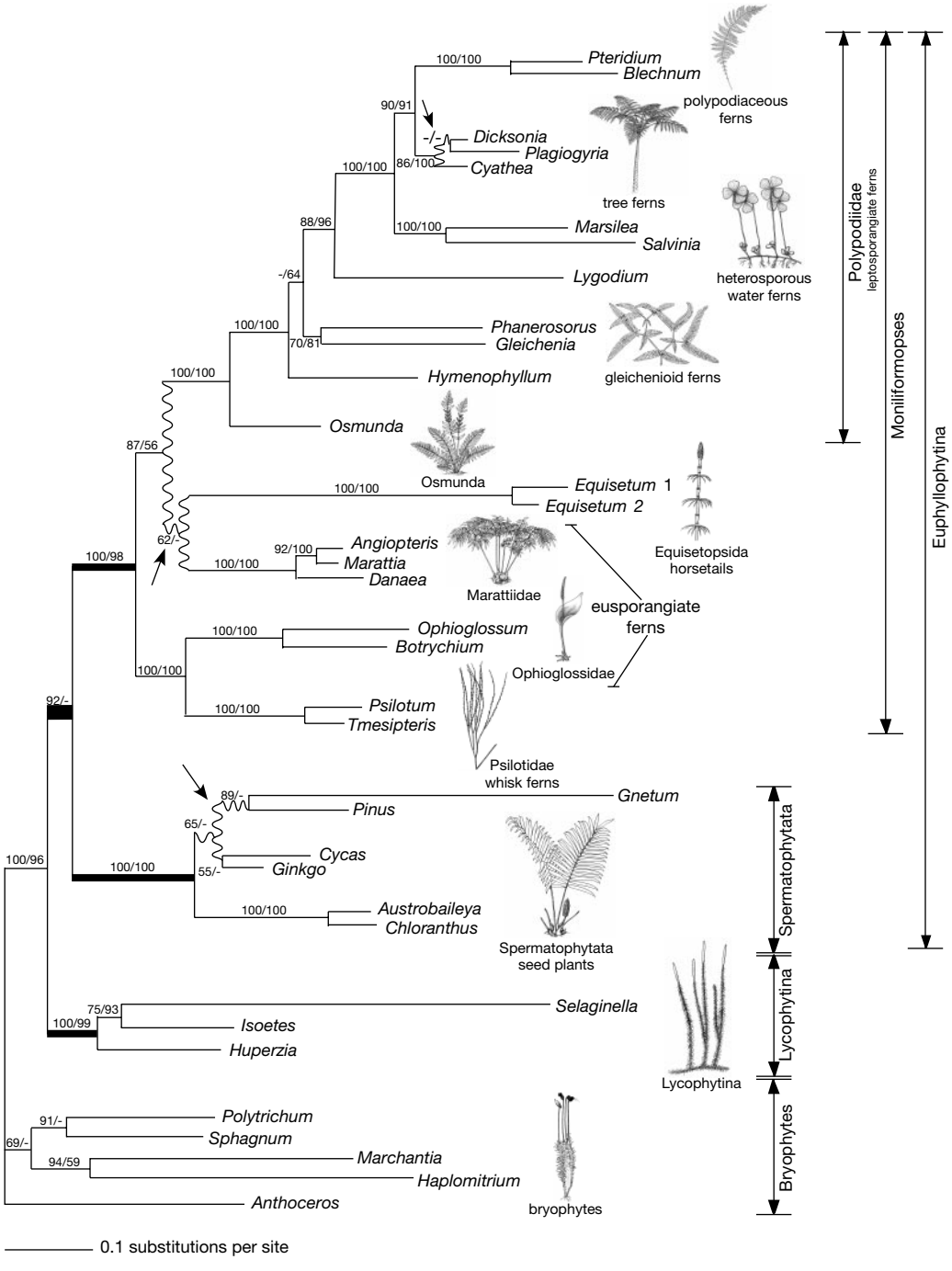

Figure 1 Phylogenetic relationships for all the main lineages of vascular plants inferred from maximum-likelihood (ML) analysis of the combined chloroplast *rbcL*, *atpB*, *rps4* and nuclear small-subunit rDNA data set. Numbers at nodes and before the slash are ML bootstrap values ≥50%; maximum parsimony (MP) bootstrap values ≥50% appear after the slash when these same nodes were supported in the MP unequally weighted analysis of the combined four-genes plus morphology data set (single MP tree = 14165.04 steps). A minus sign indicates a node had less than 50% bootstrap support in one or the other analysis. The topology is rooted by all bryophytes, hence relationships depicted among them are arbitrary. Branches leading to the three monophyletic clades of vascular plants (lycophytes, seed plants and horsetails+ferns) are drawn medium thick. The branch supporting the Euphyllophytina, with horsetails+ferns as sister group to seed plants, is the thickest. Wiggled lines (at straight arrows) indicate three areas of conflict between the ML and MP analyses. Branch lengths are proportional to number of substitutions per site (scale bar). Thumbnail sketches of plant representatives accompany major clades. Taxonomy follows ref. 1.

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were specified as outgroups. We analysed the data sets using both maximum-parsimony (MP) and maximum-likelihood (ML) optimization criteria; bootstrap (BS) analyses were conducted to measure the stability of observed phylogenetic patterns.

Using ML on the combined four-gene data set we recovered one most likely tree (-ln likelihood = 36466.6365) for each of the 100 replicates (Fig. 1). We also observed an essentially identical topology using MP on the combined four-gene and morphology data set (three areas that differ are highlighted on Fig. 1). Regardless of the analytical approach (MP or ML), three major lineages emerged as monophyletic clades with exceptional support (100% BS). The first clade comprises the Lycophytina, increasingly recognized as a distinct group of vascular plants only distantly related to other extant pteridophytes and seed plants[1,16]. The second diverging lineage corresponds to seed plants. The third, novel, clade includes all non-seed-producing lineages of Euphyllophytina, including horsetails (Equisetopsida), leptosporangiate ferns (Polypodiidae), eusporangiate ferns (Marattiidae, Ophioglossidae) and whisk ferns (Psilotidae). Seed plants, ferns and horsetails are united as a monophyletic group, to the exclusion of lycopods, in both the ML (92% BS) and MP (<50% BS) analyses.

We observed one unambiguous length discrepancy in *rps4* that can be interpreted as a molecular 'signature' providing additional support for horsetail–fern monophyly. A portion of the *rps4* alignment is shown for base pairs 646–696 (Fig. 2), which includes 27 ambiguously aligned base pairs (658–684) flanked by unambiguously aligned regions. The ambiguously aligned region was excluded entirely from the ML analysis. In the MP analysis, the same region was recoded simply as a single absence/presence character for the observed length increase. This multi-residue length increase in horsetails and ferns is not as likely to be a random convergence as is a single point mutation and provides further evidence for this clade.

Within the horsetail–fern lineage, Psilotidae is most closely related to Ophioglossidae (100% BS). Although this association was only weakly suggested in recent single-gene analyses[11,19,20], the current evidence unambiguously invalidates the traditional morphological and palaeobotanical view that Psilotidae are relatively unaltered descendants of the psilotophytes, among the earliest vascular plant fossils[7,18]. Ophioglossidae and Psilotidae differ so radically in phenotype that this close relationship, implying a shared origin of phenotypic simplification, was never before explicitly considered. All other ferns and horsetails make up its sister clade (87% BS). The relationships of horsetails also have been controversial: sister to seed plants[7], sister to leptosporangiate (Polypodiidae) and eusporangiate (Ophioglossidae and Marattiidae) ferns[1], or as a basal grade euphyllophyte lineage[17]. Our analysis clearly (100% BS) places *Equisetum* within the non-lycophyte pteridophyte clade, although its exact relationships within this clade are not yet well resolved. In the ML analysis, *Equisetum* is sister to Marattiidae (62% BS), whereas in the MP analysis, it is sister to leptosporangiate ferns (<50% BS). This study also confirms a sister relationship between tree ferns and the more derived 'polypodiaceous' leptosporangiate ferns (90% BS), and places the heterosporous water ferns as sister to this clade (100% BS) (Fig. 1). Relationships among these groups were equivocal in earlier studies[17,20].

The only noteworthy disagreement between our ML and MP analyses is localized within seed-plant relationships, a subject of much current controversy[21,23,24]. Our ML analysis resolved gymnosperms as monophyletic (65% BS) and *Gnetum* as sister to *Pinus* (89% BS). Our MP analysis supports *Gnetum* as basal among seed plants (87% BS), and all other gymnosperms as monophyletic (67% BS) and sister to angiosperms.

In the ML analysis of the combined four-gene data set, there is persuasive support for the Euphyllophytina (92% BS), with a basal dichotomy indicating that the horsetail–fern clade (100% BS) is the closest relative to seed plants (100% BS). To the best of our knowledge, this relationship has been proposed only once previously[1], as a tentative hypothesis on the evidence of a single anatomical character (protoxylem distribution). This led to the provisional classification of the horsetail–fern clade as infradivision Moniliformopses (moniliforms); Psilotidae, however, was not included in that study[1]. Although this same deep dichotomy is also robustly resolved in the MP analysis of the combined four-genes plus morphology data set, the Euphyllophytina node is weakly supported (<50% BS). Exceptionally long branches in each of the three main clades (Fig. 1: *Selaginella*, *Gnetum* and *Equisetum*) and the greater sensitivity of MP over ML to long-branch attraction (statistical inconsistency) effects[21,25] probably explain why parsimony bootstrapping failed to recover this clade with high confidence. When these long-branch taxa were removed and the combined four-genes plus morphology data set was re-analysed with MP, this same basal Euphyllophytina node was highly supported (83% BS, results not shown). Each of our separate single-gene analyses, with the exception of *rps4*, did not resolve the horsetail–fern clade, and none was able to determine confidently the closest relatives to seed plants. Only our morphological data set, when analysed alone with MP, provided the same conclusions

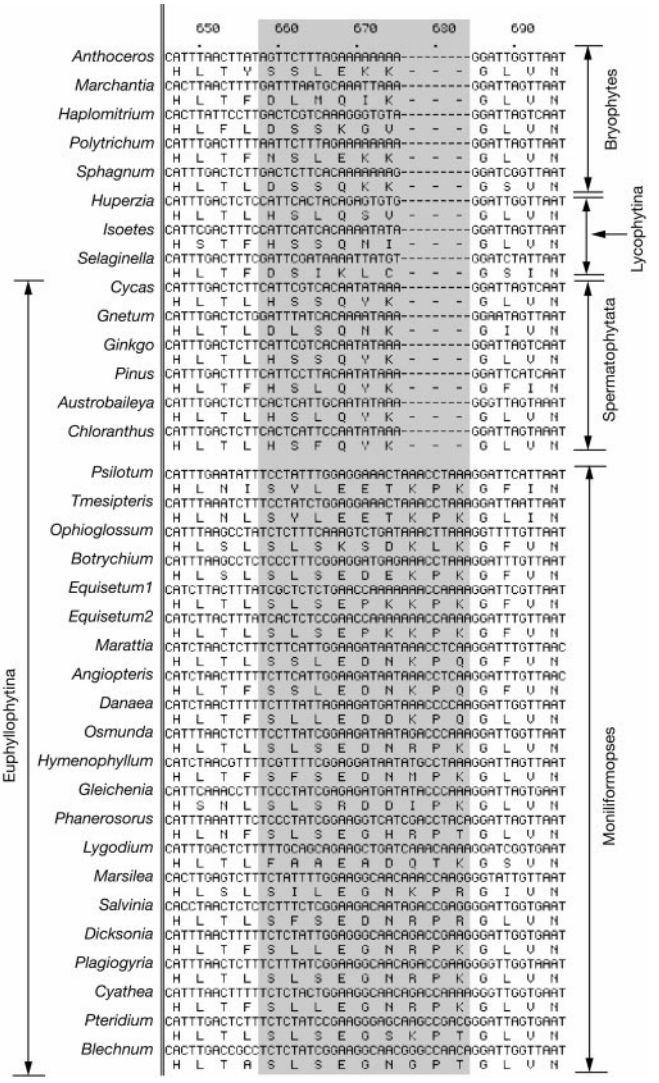

Figure 2 A portion of the chloroplast *rps4* alignment. An ambiguously aligned region (grey box) containing a 9-base-pair length difference distinguishes horsetails and ferns (bottom block) from bryophytes, lycophytes and seed plants (top block). Amino-acid translations are interleaved below each DNA sequence. Dashes indicate gaps.

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regarding the Euphyllophytina as when the four genes were analysed simultaneously with ML. A study using mitochondrial small-subunit rDNA sequence data[10] with a smaller selection of taxa suggested support for this hypothesis; however, critical euphyllophyte taxa (Psilotidae and Marattiidae) were not included. A more recent study[26] that combines data from two genes (nuclear and mitochondrial small-subunit rDNA) strongly corroborates a horsetail–fern clade as sister to seed plants, despite a limited sampling of only seven euphyllophyte taxa from all pertinent lineages.

Our report of a basal dichotomy in the Euphyllophytina, a split that occurred in the early–mid Devonian (about 400 Myr ago), necessitates abandonment of the prevailing view that ferns and horsetails represent paraphyletic successive grades of increasing complexity in early vascular plant evolution, which eventually led to the more complex seed plants, and ultimately to angiosperms. A parallel deep reorganization of metazoan phylogeny has recently been proposed[27], with 'simple' bilaterian taxa (for example, platyhelminths and nemerteans) being displaced from the base of the metazoan tree to within the large lophotrochozoan clade.

A corollary of the demise of the paraphyletic interpretation of early vascular plant evolution is that it is now necessary to confront the many recurring models that derive morphological, developmental and physiological conditions in seed plants from an 'intermediate' or 'primitive' pteridophyte ancestor. We predict that this will require a significant revision in the interpretation of the underlying processes of vascular plant evolution. For example, a number of homeotic genes, such as the MADS-box genes that encode transcription factors critical for regulating physiological and developmental processes, especially flower development, have been well studied in angiosperms[28]. Clarifying the origin of these genes has been hampered by the few reports of homologues from non-seed plants, and therefore it is not known to what extent changes in number, regulation and function of these and other homeotic genes may have driven land plant evolution. The study of these genes from across a stable phylogenetic framework is critical. We note that all the main plant model organisms (for example, *Arabidopsis*, *Glycine*, *Lycopersicon*, *Oryza*, *Petunia* and *Zea*) are recently evolved angiosperms. Efforts to promote developmental and genomic research on model systems in the horsetail–fern clade (for example, *Ceratopteris*[29]), will probably lead to an improved understanding of fundamental aspects of vascular plant development and evolution[8]. □

Methods

Taxon sampling and morphological data set

We selected 35 taxa to sample explicitly at least two members of each major monophyletic group of land plants. The various groups were determined from recent broad-scale phylogenetic analyses[1,12,17,20], and we specified the bryophytes *Anthoceros*, *Haplomitrium*, *Marchantia*, *Polytrichum* and *Sphagnum* as outgroups. Our morphological data set comprised 136 parsimony-informative characters (H.S. *et al.*, manuscript in preparation), which we, for the most part, adopted or modifed from recent studies[1,7,12,17,20].

Gene sequencing

We amplified chloroplast *rbcL*, *atpB*, *rps4*, and nuclear small-subunit rDNA genes for all 35 taxa from total cellular DNA by polymerase chain reaction (PCR) and sequenced them using an ABI 377 automated DNA sequencer (PE Applied Biosystems). Details of taxon sampling, DNA isolation, PCR amplification, sequencing, sequence alignment, exclusion and recoding of ambiguously aligned regions, data set combinability testing, and phylogenetic analysis will be published elsewhere (K.M.P. *et al.*, manuscript in preparation). Most *atpB*, *rps4*, nuclear small-subunit rDNA, and some *rbcL* sequences were generated as part of this study. For voucher information, GenBank numbers and the aligned data matrices, see Supplementary Information and http://www.fmnh.org/research_collections/botany/botany_sites/ferns/publications.html; data matrices are also available in TreeBASE, accession number S543, at http://www.herbaria.harvard.edu/treebase/.

Phylogenetic analyses

We conducted heuristic MP (unequal weighting schemes, 1,000 random-addition replicates, tree bisection-reconnection (TBR) branch swapping) and ML (general time-reversible model, accommodating unequal nucleotide frequencies and different-probabilities for each of six substitution types, plus three heterogeneous rate categories across sites following a discrete approximation of the gamma distribution, 100 random-addition replicates) analyses using PAUP* version 4.0b2a[30]. The ML analysis was restricted to the combined four-gene data set because it is not possible to simultaneously implement two models of evolution, one for morphology and one for DNA sequence data, in any currently available computer programs. We further performed both parsimony bootstrap (unequal weighting schemes, 1,000 replicates, each with 10 random-addition replicates and TBR branch swapping) and likelihood bootstrap analyses (212 replicates, using identical parameters to those used to find the most likely tree).

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Export by red blood cells of nitric oxide bioactivity

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Previous studies support a model in which the physiological O_2 gradient is transduced by haemoglobin into the coordinate release from red blood cells of O_2 and nitric oxide (NO)-derived vasoactivity to optimize oxygen delivery in the arterial periphery[1,2]. But whereas both O_2 and NO diffuse into red blood cells, only O_2 can diffuse out[3–5]. Thus, for the dilation of blood vessels by red blood cells, there must be a mechanism to export NO-related vasoactivity, and current models of NO-mediated intercellular communication should be revised. Here we show that in human erythrocytes haemoglobin-derived *S*-nitrosothiol (SNO), generated from imported NO, is associated predominantly with the red blood cell membrane, and principally with cysteine residues in the haemoglobin-binding cytoplasmic domain of the anion exchanger AE1. Interaction with AE1 promotes the deoxygenated structure in SNO–haemoglobin, which subserves NO group transfer to the membrane. Furthermore, we show that vasodilatory activity is released from this membrane precinct by deoxygenation. Thus, the oxygen-regulated cellular mechanism that couples the synthesis and export of haemoglobin-derived NO bioactivity operates, at least in part, through formation of AE1–SNO at the membrane–cytosol interface.

As the first step in analysing the fate of haemoglobin (Hb)-derived NO *in situ*, we determined the disposition of NO groups transfered physiologically from the haems of Hb to β-chain Cys 93 in intact human erythrocytes[3,4]. Red blood cells (RBCs) held at less than 1% O_2 were exposed for 5 min to physiological amounts of NO (100 nM to 1 μM; NO:haem ratios 1:1,000 to 1:100) followed by reoxygenation (21% O_2), and membrane and cytosolic fractions were prepared. Fractions were solubilized with Triton X-100 (TX100), and the NO content of extracts was measured by photolysis/chemiluminescence[3,4]. At the lower NO:haem ratios, which produced intracellular NO concentrations matching those found *in vivo* (100–800 nM), recovery of NO was essentially complete, that is, none was lost to nitrate (Fig. 1a). In this model system, about 15–20% of NO incorporated by RBCs was present as SNO; the remainder was ascribed largely to iron nitrosyl haem (FeNO)[1,3,4,6]. Most iron nitrosyl Hb was recovered with the cytosolic fraction (Fig. 1b). In contrast, SNO was associated predominantly with the membrane fraction (Fig. 1c). These results confirm that, in intact RBCs[7] as with isolated reactants[3,4], Hb will efficiently capture and preserve NO, and form SNO, under physiological conditions. Unexpectedly, however, the formation of SNO is compartmentalized within the RBC.

Haemoglobin associates with the cytoplasmic face of the RBC membrane through specific protein–protein interactions[8–10]. To determine the disposition of Hb-derived membrane SNO, we examined the interaction of SNO–Hb[5,6] with inside-out vesicles (IOVs) prepared by everting RBC membrane ghosts[11]. IOVs incubated with SNO–Hb and washed at pH 8 to remove bound Hb incorporated about 450 pmol NO per mg of TX100-extracted IOV protein (Fig. 1d). All the incorporated NO was present in complex with thiol, that is, as SNO. It is important to note that SNO was not detected in extracts of IOVs exposed to NO in the absence of Hb (data not shown).

To rule out the possibility that apparent NO group transfer to IOVs was an artefact of residual membrane-bound SNO–Hb, we incubated IOVs with SNO–Hb immobilized on Sephadex beads. After centrifugal separation, washes at pH 7 and solubilization in TX100, extracts of IOVs were free of Hb as assessed by spectrophotometric detection of haem. SNO was present in those extracts at somewhat higher levels than in extracts derived from IOVs incubated with free SNO–Hb (suggesting a greater loss of

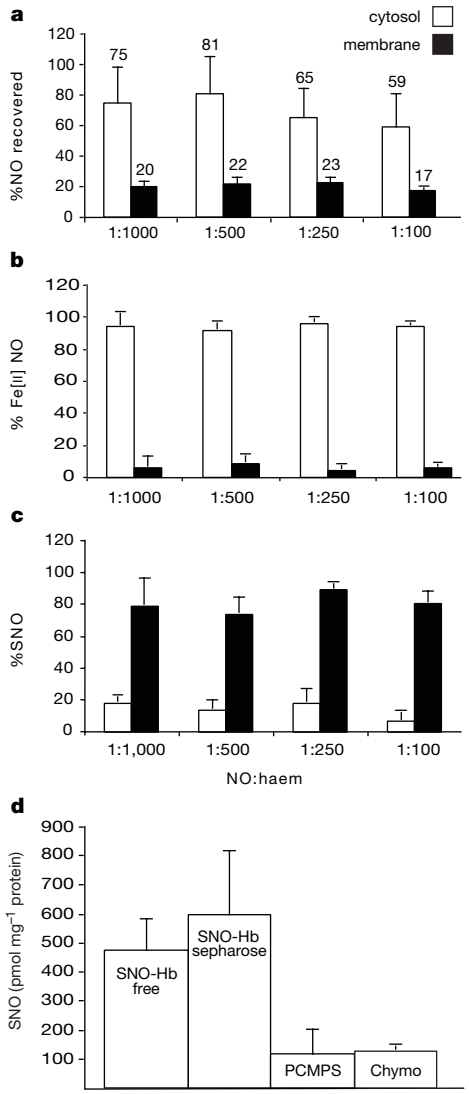

Figure 1 Haemoglobin-derived SNO is associated with cysteine thiols of RBC membrane proteins. **a**–**c**, Distribution in cytosolic and membrane fractions of NO groups after exposure of intact RBCs to NO. Recovery of NO is essentially complete at low, physiological NO:haem ratios (**a**), which yield 100–800 nM intracellular NO; FeNO is predominantly cytosolic (**b**), whereas SNO is largely membrane associated (**c**) ($P < 0.05$ for all pairwise comparisons). **d**, SNO content of IOVs exposed to free or Sepharose-bound SNO–Hb (50 nmol SNO–Hb per mg IOV protein). Transfer of NO groups to the membrane is greatly reduced ($P < 0.05$) after treatment of IOVs with the thiol-modifying reagent PCMPS and after mild digestion of IOVs with chymotrypsin (chymo). ($n = 3$–7 for **a**–**d**.)

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