Archaeal dominance in the mesopelagic zone of the Pacific Ocean

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The ocean's interior is Earth's largest biome. Recently, cultivation-independent ribosomal RNA gene surveys have indicated a potential importance for archaea[1] in the subsurface ocean[2–4]. But quantitative data on the abundance of specific microbial groups in the deep sea are lacking[5,6]. Here we report a year-long study of the abundance of two specific archaeal groups (pelagic euryarchaeota and pelagic crenarchaeota)[2] in one of the ocean's largest habitats. Monthly sampling was conducted throughout the water column (surface to 4,750 m) at the Hawai'i Ocean Time-series station[7]. Below the euphotic zone (> 150 m), pelagic crenarchaeota comprised a large fraction of total marine picoplankton, equivalent in cell numbers to bacteria at depths greater than 1,000 m. The fraction of crenarchaeota increased with depth, reaching 39% of total DNA-containing picoplankton detected. The average sum of archaea plus bacteria detected by rRNA-targeted fluorescent probes ranged from 63 to 90% of total cell numbers at all depths throughout our survey. The high proportion of cells containing significant amounts of rRNA suggests that most pelagic deep-sea microorganisms are metabolically active. Furthermore, our results suggest that the global oceans harbour approximately 1.3×10^{28} archaeal cells, and 3.1×10^{28} bacterial cells. Our data suggest that pelagic crenarchaeota represent one of the ocean's single most abundant cell types.

Sampling was carried out at roughly monthly intervals from September 1997 to December 1998 at the Hawai'i Ocean Time-series station ALOHA[7] (22° 45′ N, 158° 00′ W) in the North Pacific subtropical gyre. We quantified cells belonging in the domain Bacteria, and two specific phylogenetic groups in the domain Archaea, which are prevalent in marine plankton: 'group 1 archaea' (referred to here as pelagic crenarchaeota); and 'group 2 archaea'

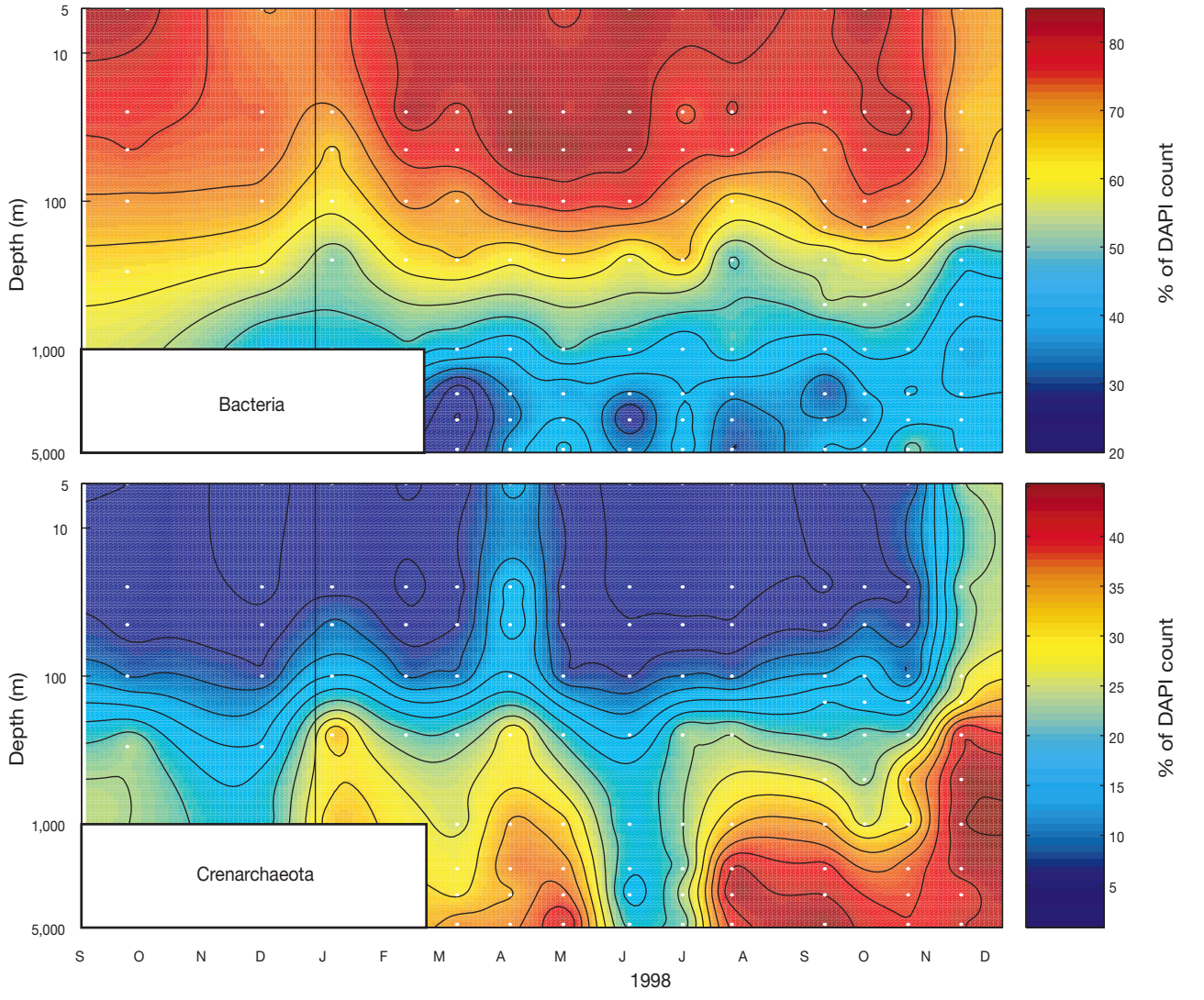


Figure 1 Contour plots of relative abundances with depth of bacteria and pelagic crenarchaeota during a 1-yr sampling effort at the Hawai'i Ocean Time-series station, ALOHA, in the North Pacific subtropical gyre. White dots indicate dates and depths where samples were collected. Contour lines are percentages of bacteria and pelagic crenarchaeota as compared with total microbial abundance at each depth. Total cell abundance was assessed using the DAPI nucleic acid stain. Bacteria and archaea were enumerated using whole-cell rRNA targeted fluorescent *in situ* hybridization with fluorescein-labelled polynucleotide probes. See also Supplementary Information.

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(referred to here as pelagic euryarchaeota)[2]. Ribosomal RNA-targeted polynucleotide probes[6] specific for either pelagic crenarchaeota, euryarchaeota or bacteria were hybridized with all samples collected from September 1997 until December 1998 (one deep sample collected in October 1998 had strong spurious background fluorescence and was omitted from the data set). Probe-binding cells were enumerated relative to total cells stained by 4′,6-diamidino-2-phenylindole (DAPI)[8] double staining[9]. Probe-conferred fluorescence was most intense in samples from shallower depths (0–500 m), and was weaker for both archaea and bacteria at depths greater than 500 m. Negative control counts (hybridization with a nonspecific probe and autofluorescence) were below 5% of total cells in 96% of all samples, hence a high degree of confidence in this method was achieved. (See also Supplementary Information.)

The two archaeal groups surveyed, as well as bacteria collectively, lacked clear seasonal trends in relative cellular abundance (Fig. 1). Therefore, averages were calculated for all samples for each depth layer, yielding the mean annual depth profile for each phylotype (Fig. 2a). Cells hybridizing with the bacterial probe dominated the population in the upper 150 m of the water column, representing up to 90% of all cells. Bacteria decreased in relative abundance with increasing depth (Figs 1, 2a), and below 1,000 m they represented only 35–40% of total cells. By comparison, pelagic crenarchaeota increased sharply in relative abundance at the 250-m depth layer, and below 1,000 m were as common as bacteria (Figs 1, 2a). Samples from 150 m (included in 4 of the 14 sampling dates) indicated that the relative increase in pelagic crenarchaeota occurred between 100 and 150 m, near the depth of the 1% isolume (Figs 1, 2a). In surface layers, pelagic crenarchaeota were only present sporadically, and never abundant numerically. Pelagic euryarchaeota occasionally appeared in the near surface layer, but generally remained at a few per cent of the total count over the entire water column, too close to negative counts to establish a statistically reliable estimate (Fig. 2a). Pelagic crenarchaeota and euryarchaeota thus showed different patterns of abundance in the open sea (Fig. 2a), with low numbers of euryarchaeota in surface waters, in contrast to previous observations in coastal waters[6,10,11].

The sum of relative abundances of bacteria, pelagic euryarchaeota and pelagic crenarchaeota (subtracting negative controls) shows the total fraction of probe-binding cells over cells stained by DAPI (Fig. 2b). Although our deep-sea data on relative abundance of pelagic crenarchaeota (Fig. 2a) are globally higher than those recently reported in coastal waters[6], our cumulative relative abundance data on total probe-positive cells (Fig. 2b) are lower. This may be due to the oligotrophic nature of the study site. Total cell abundance patterns reflected the higher total cell numbers in surface waters (Fig. 3). Total cell abundance declined by an order of magnitude between 150 and 1,000 m. Thus, the total number of pelagic crenarchaeotal cells peaked between 150 and 500 m, but remained at high relative numbers below this depth to the ocean floor.

In recent years cultivation-independent rRNA gene surveys have revealed new types of archaea in virtually every ecosystem examined[12,13]. Yet marine planktonic archaeal types have eluded cultivation to date, and few quantitative data exist on their cellular distribution and abundance. Most previous studies have relied on the retrieval of rRNA-gene-containing clones to qualitatively describe naturally occurring picoplankton populations[2–4]. A number of studies using quantitative rRNA hybridization techniques[2,10,11,14,15] have suggested a widespread occurrence and high relative cell abundance of archaea in the upper water column. Unfortunately, these earlier studies do not allow for quantification of actual cell numbers. Quantitative microscopic surveys using single-cell, fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes, by contrast, allow direct cell enumeration[16,17]. A few investigations using oligonucleotide probes to enumerate marine picoplankton[5,18–20] have succeeded in detecting

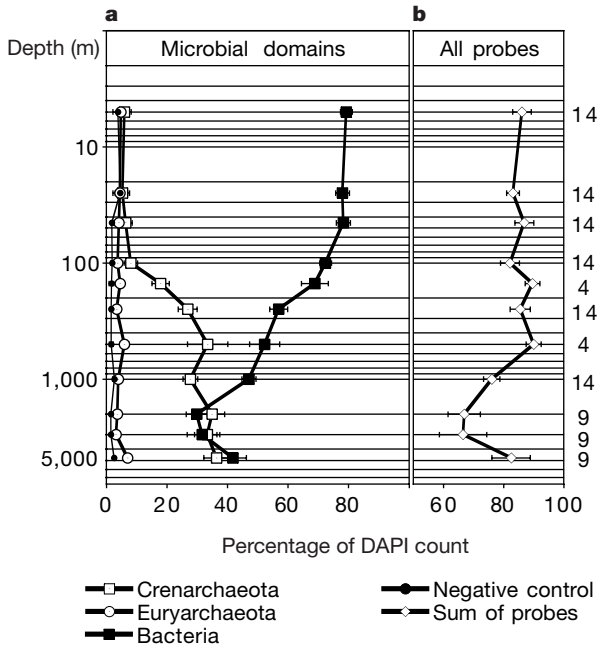


Figure 2 Mean annual depth profiles of microbial domains in the North Pacific subtropical gyre. Numbers are percentages of bacteria and archaea as compared to total microbial abundance at each depth. Total cell abundance was assessed using the DAPI nucleic acid stain. Bacteria and archaea were enumerated using whole-cell rRNA targeted fluorescent *in situ* hybridization with fluorescein-labelled polynucleotide probes. Data are averages of up to 14 roughly monthly samplings over a 1-yr period at the Hawai'i Ocean Time-series station, ALOHA. Error bars show standard error of mean; note column for total sample size at each depth. See also Supplementary Information. **a**, Depth profiles for bacteria (solid squares), pelagic crenarchaeota (open squares), pelagic euryarchaeota (open circles), and a non-specific control probe ('negative', solid circles). **b**, Depth profile of the sum of relative abundances of bacteria, pelagic crenarchaeota and pelagic euryarchaeota (open diamonds). Relative abundances of bacteria and both archaeal groups were summated at each depth and negative control data were subtracted.

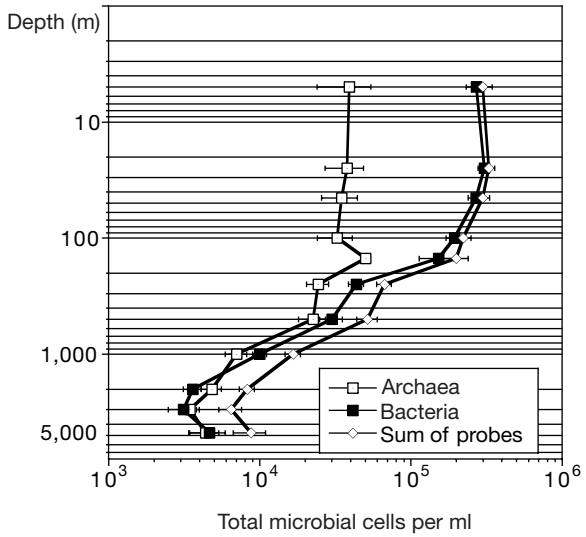


Figure 3 Mean annual depth profiles of microbial domains in the North Pacific subtropical gyre. Numbers are total cell abundances of bacteria and archaea (pelagic crenarchaeota and euryarchaeota combined). Bacteria and archaea were enumerated using whole-cell rRNA targeted fluorescent *in situ* hybridization with fluorescein-labelled polynucleotide probes. Data are averages of up to 14 roughly monthly samplings over a 1-yr period at the Hawai'i Ocean Time-series station, ALOHA. See also Supplementary Information.

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naturally occurring bacterial and archaeal cells. One study[5] using image intensification to enhance the fluorescence signal reported high percentages of Archaea (up to 60%) in coastal temperate waters and the Mediterranean Sea. Several studies, using an oligonucleotide probe targeting the domain Archaea, detected few or no archaeal cells in the upper water column[19,20].

None of these earlier studies, however, examined depths below 600 m, and only one[18] identified specific archaeal cell types beyond the domain level. Further, single-cell hybridization studies have been hampered by the small size and apparent low rRNA content of marine picoplankton[5,6,18]. Multiply labelled, rRNA-targeted polynucleotide probes[6] allow more systematic enumeration of specific groups of planktonic microbes. But this approach has so far been demonstrated only at a coastal ocean site[6], prompting our study at the Hawaii Ocean Time-series station.

In this report, we conclusively show that pelagic crenarchaeota were equivalent in numbers to bacteria throughout the entire meso- and bathypelagic zones in a major ocean gyre, over a 1-yr time-series survey. Two main points arise from our observations. First, on average, the fraction of pelagic crenarchaeota relative to DNA-containing microorganisms equals or exceeds the bacterial fraction below 1,000 m (Fig. 2a). This result suggests that pelagic crenarchaeota are a consistent and significant component of deep sea microbiota, and that they may rival bacterial abundances in the meso- and bathypelagic zones. Further, the standing stocks of bacteria and pelagic crenorchaeota were inversely proportional. Second, the total proportion of cells (bacterial and archaeal) detected with polynucleotide probes (Fig. 2b) remained fairly constant in surface waters to depths of 1,000 m, roughly 80% of the total DNA-containing cells[8]. At depths between 2,000 and 3,000 m, this number decreased to about 60% before increasing again to more than 70% close to the sea floor (Fig. 2b), similar to previously reported microbial cell numbers and metabolic activity profiles in the deep Pacific[21]. DNA-containing cells unlabelled by polynucleotide probes might include cells with low rRNA content, cells not recognized by the probes (for example, unusually small *Eucarya* in the 1-μm range[22]) or dead cells (ghosts)[23]. Our results place an upper limit on the sum of the above categories. We conclude that the use of polynucleotide probes provides a robust approach for identification of deep-sea microbes, and that the fraction of remaining unlabelled cells remains below 20–30% of the total cells.

A previous study[24] suggested that the number of cells positive for rRNA hybridization techniques may reflect the number of metabolically active cells. If true, then the total number of probe-binding cells should represent the proportion of metabolizing cells in the water column (60–80% of total cells present). The decrease in probe-positive cells between 2,000 and 3,000 m might reflect decreasing availability or quality of growth substrate, resulting in a metabolically less active population. Close to the sea floor, gravitationally deposited organic matter might trigger the observed increase of the proportion of all probe-positive cells (Fig. 2b). This result is consistent with observed increases in metabolic activity near the sea floor previously found in Pacific bathypelagic microbial populations[21]. Our data indicate that most deep-sea microbes (archaea and bacteria) contain appreciable amounts of rRNA, and so may be active contributors to the ecosystem. Furthermore, given that the total number of microbes strongly decreases with depth in the ocean[21,25], our data lead to an ecologically significant conclusion: growth conditions for pelagic microbes do appear to be accurately reflected by microbial standing stock. There may also be a population shift with increasing depth to microbial groups better adapted to deep sea conditions, a large fraction of which appears to be pelagic crenarchaeota.

Combining our present data set with continental shelf data[6], we can extrapolate total cell abundances for archaea to the global ocean[26]. Such an extrapolation suggests that the world ocean contains approximately 1.3×10^{28} archaeal cells, and 3.1×10^{28} bacterial cells. About 1.0×10^{28} cells, that is, 20% of all the picoplankton cells in the world ocean appear to be represented by one specific clade, the pelagic crenarchaeota. The habitat range for this single microbial group, spanning from mesopelagic to bathypelagic depths, is unusually broad. As a dominant component of the deep ocean, Earth's largest biome, archaea are thus far from confined to extreme niche habitats. Rather, the distribution of these archaea suggests that a common adaptive strategy has allowed them to radiate throughout nearly the entire oceanic water column. □

Methods

Sample preparation

Samples were fixed with 0.2-μm filtered formalin (2% final concentration) and filtered onto 0.2-μm polycarbonate filters. After filtration cells were treated by overlaying the filter for 2 min with a 0.25 M NaCl solution made up in 50% (v/v) ethanol. Samples were stored frozen either before or after filtration, with equal preservation of probing potential.

Whole-cell fluorescent *in situ* hybridization counts

Ribosomal RNA targeted hybridization was carried out on the filter-bound cells using fluorescein isothiocyanate (FITC) multiply labelled probes[6] of more than 100 base pairs in length, targeting crenarchaeota (marine group 1 archaea) or euryarchaeota (marine group 2 archaea)[10], or bacteria. A non-binding probe (consisting of the complementary strand of the pelagic crenarchaeotal probe) served as the negative control, while the summation of archaeal and bacterial fractions (minus negative control) provided a check on how total probe-positive cells compared with total cell counts using the DAPI stain[8]. Probe was added to a hybridization buffer at a final concentration of 2 ng μl^{-1}. The hybridization buffer of 10% (w/v) dextran sulphate, 0.01% poly(A) and 0.1% sodium dodecyl sulphate (SDS) in 5× SET was used with either 50% formamide (bacterial and negative control probes) or 70% formamide (for both archaeal probes, 1× SET is 150 mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris, pH 8.0).

We did not automatically subtract the negative count corresponding to each sample because cells showing a 'negative' image, for example, owing to autofluorescence, could be of any group: crenarchaeota, euryarchaeota or bacteria. When reporting the sum of relative cell numbers from all probes, however, we did subtract the negative count as here we can relate the negative count to all cells, yielding a conservative estimate for total probe positive cells. During April 1998, high negative values of 25% and 36% were observed for the 5-m and the 25-m layers, respectively; for the other 120 samples the negative control averaged 2.0 ± 1.5% (mean ± s.d.). Hybridization temperatures were 65 °C for the two archaeal probes, and 55 °C for bacterial and negative control probes. Hybridization time was 12 h. Post-hybridization wash (2 h) was at 50 °C for archaeal probes, and 45 °C for bacterial/negative control probes in a 0.2× SET solution made up in 50% formamide. Filters were mounted in Citifluor (Ted Pella), and FITC-positive cells were counted in relation to DAPI-positive cells on the same filter[9]. Counting was performed using a Zeiss epifluorescence microscope equipped with a ×100 Neofluar objective and 100-W mercury lamp illumination, as well as the appropriate filter sets for DAPI and FITC (Chroma Technology).

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1. Woese, C. R., Kandler, O. & Wheelis, M. L. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl Acad. Sci. USA* **87,** 4576–4579 (1990).
2. DeLong, E. F. Archaea in coastal marine environments. *Proc. Natl Acad. Sci. USA* **89,** 5685–5689 (1992).
3. Fuhrman, J. A., McCallum, K. & Davis, A. A. Novel major archaebacterial group from marine plankton. *Nature* **356,** 148–149 (1992).
4. Fuhrman, J. A. & Davis, A. A. Widespread Archaea and novel Bacteria from the deep sea as shown by 16S rRNA gene sequences. *Mar. Ecol. Prog. Ser.* **150,** 275–285 (1997).
5. Fuhrman, J. A. & Ouverney, C. C. Marine microbial diversity studies via 16S rRNA sequences: cloning results from coastal waters and counting of native archaea with fluorescent single probes. *Aquat. Ecol.* **32,** 3–15 (1998).
6. DeLong, E. F., Taylor, L. T., Marsh, T. L. & Preston, C. M. Visualization and enumeration of marine planktonic *Archaea* and *Bacteria* by using polyribonucleotide probes and fluorescent *in situ* hybridization. *Appl. Environ. Microbiol.* **65,** 5554–5563 (1999).
7. Karl, D. M. & Lukas, R. The Hawaii Ocean Time-series (HOT) program: Background, rationale and field implementation. *Deep-Sea Res.* **43,** 129–156 (1996).
8. Porter, K. & Feig, Y. S. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25,** 943–948 (1980).
9. Hicks, R. E., Amann, R. I. & Stahl, D. A. Dual staining of natural bacterioplankton with 4′, 6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. *Appl. Environ. Microbiol.* **58,** 2158–2163 (1992).
10. Massana, R., Murray, A. E., Preston, C. M. & DeLong, E. F. Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara Channel. *Appl. Environ. Microbiol.* **63,** 50–56 (1997).
11. Murray, A. E. *et al.* A time series assessment of planktonic archaeal variability in the Santa Barbara Channel. *Aquat. Microb. Ecol.* **20,** 129–145 (1999).
12. Olsen, G. J. Archaea, archaea everywhere. *Nature* **371,** 657–658 (1994).
13. Stein, J. L. & Simon, M. I. Archaeal ubiquity. *Proc. Natl Acad. Sci. USA* **93,** 6228–6230 (1996).
14. DeLong, E., Wu, K. Y., Prézlin, B. B. & Jovine, R. V. M. High abundance of Archaea in Antarctic marine picoplankton. *Nature* **371,** 695–697 (1994).

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15. Massana, R. *et al.* Vertical distribution and temporal variation of marine planktonic Archaea in the Gerlache strait, Antarctica, during early spring. *Limnol. Oceanogr.* **43,** 607–617 (1998).
16. Amann, R. I., Krumholz, L. & Stahl, D. A. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **172,** 762–770 (1990).
17. Lee, S. & Kemp, P. F. Single-cell RNA content of natural marine planktonic bacteria measured by hybridization with multiple 16S rRNA-targeted fluorescent probes. *Limnol. Oceanogr.* **39,** 869–879 (1994).
18. Murray, A. E. *et al.* Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers island, Antarctica. *Appl. Environ. Microbiol.* **64,** 2585–2595 (1998).
19. Glöckner, F. O., Fuchs, B. M. & Amann, R. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence *in situ* hybridization. *Appl. Environ. Microbiol.* **65,** 3721–3726 (1999).
20. Simon, M., Glöckner, F. O. & Amann, R. Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aquat. Microb. Ecol.* **18,** 275–284 (1999).
21. Nagata, T., Fukuda, H., Fukuda, R. & Koike, I. Bacterioplankton distribution and production in deep Pacific waters: Large-scale geographic variations and possible coupling with sinking particle fluxes. *Limnol. Oceanogr.* **45,** 426–435 (2000).
22. Courties, C. *et al.* Smallest eukaryotic organism. *Nature* **370,** 255 (1994).
23. Zweifel, U. L. & Hagström, Å. Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). *Appl. Environ. Microbiol.* **61,** 2180–2185 (1995).
24. Karner, M. & Fuhrman, J. Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. *Appl. Environ. Microbiol.* **63,** 1208–1213 (1997).
25. Williams, P. M., Carlucci, A. F. & Olson, R. A deep profile of some biologically important properties in the central North Pacific gyre. *Oceanol. Acta* **3,** 471–476 (1980).
26. Menard, H. W. & Smith, S. M. Hypsometry of ocean basin provinces. *J. Geophys. Res.* **71,** 4305–4325 (1966).

Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Testing Hamilton's rule with competition between relatives

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Hamilton's[1,2] theory of kin selection suggests that individuals should show less aggression, and more altruism, towards closer kin. Recent theoretical work has, however, suggested that competition between relatives can counteract kin selection for altruism[3–11]. Unfortunately, factors that tend to increase the average relatedness of interacting individuals—such as limited dispersal—also tend to increase the amount of competition between relatives. Therefore, in most natural systems, the conflicting influences of increased competition and increased relatedness are confounded, limiting attempts to test theory[4,8–10]. Fig wasp taxa exhibit varying levels of aggression among non-dispersing males that show a range of average relatedness levels. Thus, across species, the effects of relatedness and competition between relatives can be separated. Here we report that—contrary to Hamilton's original prediction[1,2,12] but in agreement with recent theory[5–11]—the level of fighting between males shows no correlation with the estimated relatedness of interacting males, but is negatively correlated with future mating opportunities.

Hamilton's rule[1,2] provides a tool for understanding a range of social interactions, including altruism, aggression, selfishness and spite. It states that altruism (or less aggression) is favoured when $rb - c > 0$, where c is the fitness cost to the altruist, b is the fitness benefit to the beneficiary and r is their genetic relatedness. For a given benefit and cost, the evolution of altruism therefore relies upon a sufficiently high relatedness between interacting individuals. Hamilton[2] originally suggested that a high relatedness could arise in two ways: (1) behaviour based upon direct kin recognition between individuals, or (2) limited dispersal (population viscosity).

However, the importance of limited dispersal in increasing the relatedness among interacting individuals and favouring altruism has been controversial[3–11]. Hamilton's original suggestion has been contested because limited dispersal can also increase competition between neighbouring relatives, which opposes the evolution of altruistic behaviour[3–11]. Unfortunately, empirical tests of theory, that determine the relative importance of increases in both relatedness and competition between relatives, have been hindered because both factors are influenced by dispersal, and so their effects are usually confounded[4,8–10].

The variable form of mate competition and population structure across fig wasp species with wingless males offers an opportunity for disentangling the confounded effects of relatedness and competition between relatives in viscous populations[12–18]. Fig wasps are species that develop within the fruit of fig trees, and include mutualistic pollinating species as well as parasitic non-pollinating species[15]. In many species the males are wingless, and mate with the winged females before the females disperse. The level of aggression between these non-dispersing males varies enormously across species[12–15,18]. At one extreme, males of some non-pollinating species are highly modified for combat with armoured bodies and huge mandibles. These mandibles are used to tear soft tissue and sever body parts, including limbs, head and abdomen, and can result in extremely high mortality levels. At the other extreme, males of other non-pollinating and most pollinating species show no modifications for combat or aggression.

Across these species, the average relatedness of competing males varies enormously owing to variation in the number of females that lay eggs in each fruit[12–16]. For example, if only one female lays eggs in a fruit then all the competing males will be brothers; increasing numbers of females laying eggs in a fruit will lead to males

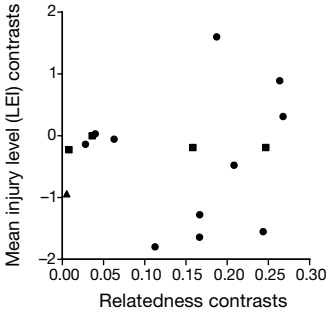


Figure 1 Mean injury level contrasts plotted against estimated relatedness contrasts. Across species, the mean injury level (lifetime extent of injury, LEI) and the proportion of individuals severely injured (SI) showed no significant relationship with estimated relatedness (LEI: all contrasts, $F(1,15) = 1.01$, $r^2 = 0.06$, $p = 0.33$; not including contrasts within the pollinator lineage, $F(1,11) = 0.72$, $r^2 = 0.06$, $P = 0.42$; SI: all contrasts, $F(1,15) = 0.04$, $r^2 < 0.01$, $p = 0.84$; not including contrasts within the pollinator lineage, $F(1,11) = 0.05$, $r^2 < 0.01$, $p = 0.83$). Circles, contrasts between the non-pollinating species; squares, contrasts between the pollinator species; triangle, the contrast between pollinators and non-pollinators.

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