*Genet. Res., Camb.* (1997), **69**, *pp.* 173–181. *With 2 figures.* Printed in the United Kingdom © 1997 Cambridge University Press 

# Evidence for clonal structure of natural populations of free-living amoebae of the genus *Naegleria*

PIERRE PERNIN[1] AND MARIE LOUISE CARIOU[2]*

[1] *Laboratoire de Biologie Cellulaire, Faculté de Médecine, Université de Lyon I, 8 avenue Rockefeller, 69373 Lyon cedex 08, France*

[2] *UPR 9034-Populations, Génétique et Evolution, CNRS, 91198 Gif-sur-Yvette cedex, France*

(*Received* 12 *July* 1996 *and in revised form* 6 *February* 1997)

## Summary

Genetic diversity of natural populations of the two species *Naegleria gruberi* and *N. australiensis* collected at the same time in the same area was studied using multilocus enzyme electrophoresis (MLEE analysis). Reference strains and related species were also analysed for comparison. Five loci were found to be polymorphic and allowed identification of 19 different multilocus genotypes among 67 isolates – eight for *N. gruberi* and 11 for *N. australiensis* – none being common to the two species. Large departures from Hardy–Weinberg equilibrium were found at all the loci, with significant heterozygote deficiencies in most cases. The two species exhibited strong linkage disequilibrium and the predominance of a few multilocus genotypes within each species was observed. These lines of evidence strongly suggest non-random association of alleles, leading to the conclusion that *N. gruberi* and *N. australiensis* have predominantly clonal genetic structures in the wild. Populations consist of a few major clones with some closely related ones – a structure found for several parasitic protozoans and bacteria. This contrasts with the panmictic structure found for *N. lovaniensis*, another species of this genus, and suggests that closely related species within the genus have evolved to have different reproductive strategies.

## 1. Introduction

Free-living amoebae of the genus *Naegleria* are very common in soil and freshwater. They have been extensively studied during the past decades mainly because the species *Naegleria fowleri* is responsible for an acute meningoencephalitis in humans which invariably leads to death. According to the recent review by De Jonckheere (1994), this genus now comprises 11 species. Some of these (e.g. *N. jadini* and *N. italica*) were described a long time ago and have never been found since. In contrast, *N. gruberi*, *N. australiensis*, *N. andersoni*, *N. lovaniensis* and *N. fowleri* were found to be widespread throughout the world. Most of the studies using either biological characteristics (temperature tolerance, immunological characters, pathogenicity, etc.) or genetic markers concentrated on species identification, especially regarding the pathogenic species, *N. fowleri*. The goal was to find species-diagnostic characters. In addition, many studies dealing with allozymes (Pernin *et al.*, 1985; Adams *et al.*, 1989) and DNA variation (De Jonckheere, 1987; Clark *et al.*, 1989) have used laboratory collection strains from generally widely geographically separated locations and times. Thus they were not strictly population samples. In fact it appears that very little has been done to obtain information about genetic diversity in natural populations of amoebae. Furthermore, amoebae were thought to have exclusively asexual reproduction until we demonstrated, on the basis of their genetic structure, that *Naegleria lovaniensis* do have genetic exchanges although the processes are poorly understood (Pernin *et al.*, 1992).

We undertook the present study of isolates of the two species, *N. gruberi* and *N. australiensis*, collected from a single site to test for genetic recombination if they undergo some sexual process, or strict clonal structure if they reproduce asexually. The aim was to determine whether sex among amoebae, and especially the genus *Naegleria*, is exceptional or not.

* Corresponding author. Tel: +33 01 69 82 37 10. Fax: +33 01 69 07 04 21.
e-mail: cariou@pge.cnrs-gif.fr.

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

## 2. Materials and methods

### (i) *Isolation of wild strains*

The 67 non-thermotolerant isolates of *Naegleria* were collected during systematic biological control of water near Pierre Bénite on the river Rhône downstream of the city of Lyon (France). Thirty-six samples were collected at the same place during a 1 month period. Each sample included four serial volumes (0·1, 1, 10 and 100 ml) replicated three times for quantitative evaluation of amoebae by the most probable number method (MPN) (Tyndall *et al*., 1989).

For volumes $\geqslant$ 10 ml the amoebae were isolated by water filtration (1·2 µm pore size cellulose acetate filters). The inverted membranes were incubated on a gelose surface overlaid with *E. coli* (non-nutrient agar: NNA) at 37 °C for 4–8 days. Volumes < 10 ml were processed by direct spreading on the NNA surface. *Naegleria* were identified microscopically according to their usual morphological characters (eruptive pseudopods, double-walled cysts with pores) and confirmed by the ability of trophozoites to transform into flagellates when suspended in distilled water. The isolates were grown axenically on SCGYEM liquid medium or monoxenically on gelose NNA.

Table 1. *Allele frequencies at four polymorphic loci in natural populations of* Naegleria gruberi *and* N. australiensis

| Locus | Allele | *N. gruberi* | *N. australiensis* |
|---|---|---|---|
| *Me* | 3 | 0·031 | — |
| | 4 | 0·031 | 1 |
| | 6 | 0·609 | — |
| | 6′ | 0·266 | — |
| | 7 | 0·063 | — |
| *Hbdh* | 1′ | 0·625 | 0·143 |
| | 1″ | 0·031 | — |
| | 3 | 0·031 | 0·857 |
| | 4 | 0·313 | — |
| *Sod2* | −1 | 0·875 | — |
| | 1 | 0·031 | 0·514 |
| | −2 | 0·031 | 0·243 |
| | 2 | 0·031 | — |
| | 3 | — | 0·243 |
| | 9 | 0·031 | — |
| *Lap2* | 1′ | — | 0·071 |
| | 2 | 0·016 | 0·014 |
| | 2′ | 0·016 | — |
| | 2″ | — | 0·243 |
| | 2‴ | — | 0·057 |
| | 3 | 0·562 | 0·371 |
| | 3′ | 0·031 | — |
| | 3″ | 0·031 | 0·014 |
| | 4 | — | 0·200 |
| | 5 | 0·344 | — |
| | 7 | — | 0·029 |

Each allele number refers to a different electromorph. Primes and minus signs were used for electromorphs with intermediate mobility compared with previously published alleles.

### (ii) *Allozyme analysis*

Both strains were harvested and lysed as previously described by Pernin & Grelaud (1989). Conditions for isofocalization and enzyme activity staining were as in Pernin *et al*. (1985). In all cases an *E. coli* control, prepared in the same manner as the samples, was included to detect possible bacterial isozymes. No differences were found between axenic and monoxenic cultures of the same isolate. Over the seven loci scored, five were found to be polymorphic: $\beta$-hydroxybutyrate dehydrogenase (*Hbdh*), malic enzyme (*Me*), superoxide dismutase ($Sod^2$ and $Sod^3$) and leucine aminopeptidase ($Lap^2$). The $Sod^1$ and $Lap^1$ loci were identically monomorphic in both species. Alleles were numbered from the most cathodic to the most anodic according to their pI after comparison with reference strains and previous studies (see Table 1 for detailed nomenclature).

The mode of reproduction was inferred from population genetics criteria based on two fundamental consequences of sexual reproduction: segregation and recombination. Allelic segregation criteria were based on comparison of observed and expected genotypes at each locus to detect the absence of some possible recombinant genotypes suggesting non-random mating. Departures from expected Hardy–Weinberg genotypic proportions were tested by the exact test proposed by Haldane (1954) using the algorithm of Louis & Dempster (1987) for up to four alleles. For five alleles and more, an unbiased estimate of the exact $P$ value was computed using the Markov chain method (Guo & Thompson, 1992). The Markov chain was set for 10000 iterations and 1000 steps of dememorization.

Recombination criteria were based on the consideration of the entire set of loci defining multilocus genotypes or 'genotypic associations'. For each population, the global disequilibrium between pairs of loci was estimated using the procedure of Weir (1990). The analysis consisted of an unbiased estimate of the exact Fisher test on an $R \times C$ contingency table performed using a Markov chain method. GENEPOP software (v 1.2) was used for all computations (Raymond & Rousset, 1995).

According to the diploid status of the *Naegleria* species (Cariou & Pernin, 1987), allele frequencies at each locus in a single isolate were either 0 or 1 for homozygotes and 0.5 for heterozygous isolates. To describe the population structure, the matrix of allele frequencies was used for multilocus genetic comparisons carried out with a multivariate analysis (centred data analysis; Lefebvre, 1976).

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

Table 2. *Genotypic proportions at individual enzymatic loci for* Naegleria gruberi *and* N. australiensis *populations*

| Loci ($n$)[a] | Genotype[b] Obs. | Genotype[b] Pos. | **Genotypes**[c] Genotypic proportions: observed (expected) numbers[d] | | | | | | | $P$ value[e] | Heterozygote |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***N. gruberi* (32 isolates)** | | | | | | | | | | | |
| *Me* (5) | | | **3/3** | **6/6** | **6′/6′** | **7/7** | **4/6** | **6/6′** | | | |
| | 6 | 15 | 1 (0·02) | 18 (11·8) | 8 (2·2) | 2 (0·01) | 2 (1·2) | 1 (10·5) | | < 0·001 | Deficit |
| *Hbdh* (4) | | | **1′/1′** | **3/3** | **4/4** | **1′/4** | **1″/4** | | | | |
| | 4 | 10 | 20 (12·4) | 1 (0·02) | 9 (3) | 0 (12·7) | 2 (0·6) | | | < 0·001 | Deficit |
| *Sod2* (5) | | | **−1/−1** | **1/1** | **2/2** | **−2/9** | | | | | |
| | 4 | 15 | 28 (24·4) | 1 (0·02) | 1 (0·02) | 2 (0·06) | | | | < 0·001 | Deficit |
| *Lap2* (6) | | | **3/3** | **5/5** | **2/2′** | **3/3″** | **3/5** | | | | |
| | 4 | 21 | 18 (10) | 11 (3·7) | 1 (0·02) | 2 (0·06) | 0 (12·6) | | | < 0·001 | Deficit |
| ***N. australiensis* (35 isolates)** | | | | | | | | | | | |
| *Hbdh* (2) | | | **3/3** | **1′/3** | | | | | | | |
| | 2 | 3 | 25 (25·7) | 10 (8·7) | | | | | | 1·000 | |
| *Sod2* (3) | | | **1/1** | **−2/−2** | **3/3** | **−2/3** | **1/−2** | **1/3** | | | |
| | 2 | 6 | 18 (9·1) | 0 (2) | 0 (2) | 17 (4·2) | 0 (8·9) | 0 (8·9) | | < 0·001 | Deficit |
| *Lap2* (8) | | | **3/3** | **4/4** | **3/4** | **3/3″** | **3/2″** | **2″/2‴** | **2″/1′** | | |
| | 9 | 36 | 2 (4·7) | 1 (1·3) | 12 (5·3) | 1 (0·4) | 9 (6·4) | 3 (1) | 5 (1·2) | < 0·001 | Excess |

[a] $n$, number of alleles identified.
[b] Genotype: the expected numbers of different genotypes given the number of alleles at each locus (Pos.) and the observed number (Obs.) are given.
[c] Genotypes are given in **bold** type; each number refers to a different allele. The nomenclature is as in Table 1.
[d] Observed and expected genotypic proportions (within parentheses) calculated assuming Hardy–Weinberg equilibrium, are indicated.
[e] $P$ value refers to type I error probability of the Hardy–Weinberg exact probability test or its unbiased estimate (see Section 2).

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

## 3. Results

Because *Naegleria* are diploid, electromorph patterns allow us to infer genotypes for every enzymatic locus in each isolate: single-band phenotypes correspond to homozygotes and multiple-band phenotypes to heterozygotes. In all cases the banding pattern was consistent with the quaternary structure known for these enzymes (Pernin *et al*., 1985, 1989) and, as expected, a few new variants were found. They produce new complex heterozygous phenotypes never recovered before, e.g. the five-banded heterozygotes $Hbdh^{1'/3}$ and $Hbdh^{1''/4}$ and the three-banded $Me^{4/6}$, $Me^{6/6'}$ and $Sod2^{-2/3}$ (Table 2).

### (i) *Segregation at individual loci*

Allele frequencies for each locus and species are given in Table 1. The expected genotype proportions assuming Hardy–Weinberg equilibrium have been calculated (Table 2). On the basis of the number of alleles identified within each species, it appears that only a few of the possible genotypes are observed. In addition, for each genotype the comparison between observed and expected numbers shows large discrepancies in both species. Some genotypes are greatly overrepresented (e.g. $Hbdh^{1'/1'}$, $Lap2^{3/3}$ for *N. gruberi*; $Sod2^{1/1}$, $Sod2^{-2/3}$ for *N. australiensis*) while others are totally missing although they have high expected frequencies considering alleles frequencies (e.g. $Lap2^{3/5}$ for *N. gruberi*; $Sod2^{1/-2}$ or $Sod2^{1/3}$ for *N. australiensis*).

Hardy–Weinberg equilibrium was rejected in all cases except for *Hbdh* in *N. australiensis* (Table 2). However, this result is less conclusive because one of the two alleles ($Hbdh^3$) had a frequency near one (0·9), forcing near identity of observed and expected frequencies. Globally a deficit of heterozygotes is observed for the overall population of *N. gruberi*. There are several reasons for a deficit of heterozygotes. First, the occurrence of a null allele at an enzymatic locus will lead heterozygotes between a functional and the null allele to be scored as homozygotes. This will bias the results towards an observed deficit of heterozygotes. In our case this hypothesis is not likely because no homozygous nulls were observed for the different loci analysed. Furthermore, these loci have never been found to have null alleles in previous studies. However, there is an exception at locus *Sod3*; the active $Sod3^2$ allele is present in both species but the absence of activity in a single isolate of *N. gruberi* (see Table 4) makes the occurrence of a null allele possible. This locus was not considered in Table 2 but, if it is true that a null allele is present, its frequency in the population (0·17) will create a deficit weaker than those observed for the other loci. A second explanation is a Wahlund effect if populations with different allele frequencies are mixed. This is also unlikely as these amoebae were collected at the same place, in the same habitat and over a short period. Amoebae live in a fixed state on substrates such as pilings, dead wood, stones and plants, and they move by crawling along the substrate. They may detach from their substrate but passive migration is probably limited as genetic differentiation occurs between populations over a few kilometres (Pernin *et al*., 1992). Such a rate of migration seems insufficient to explain the large deficit of heterozygotes we observed at most loci. Moreover, *N. australiensis*, collected mixed with *N. gruberi* in the same samples of water, shows an opposite trend: heterozygote excess is observed at locus *Lap2*, and the genotype $Sod2^{-2/3}$ is also in large excess despite a global deficit of heterozygotes at this locus. The data rather suggest the lack of segregation in these populations.

Table 3. *Measure of linkage disequilibrium between loci in* Naegleria gruberi *and* N. australiensis

| Species | Loci | Probability ± SE |
|---|---|---|
| *Naegleria gruberi* (32 isolates) | *Me–Hbdh* | $< 10^{-4}$ |
| | *Sod2–Hbdh* | 0·0059 ± 0·0016 |
| | *Sod2–Me* | $< 10^{-4}$ |
| | *Sod3–Hbdh* | 0·0284 ± 0·0022 |
| | *Sod3–Me* | 0·0616 ± 0·0042 |
| | *Sod3–Sod2* | 0·0652 ± 0·0042 |
| | *Lap2–Hbdh* | $< 10^{-4}$ |
| | *Lap2–Me* | $< 10^{-4}$ |
| | *Lap2–Sod2* | 0·0245 ± 0·0051 |
| | *Lap2–Sod3* | 0·0339 ± 0·0027 |
| *Naegleria australiensis* (35 isolates) | *Sod2–hbdh* | $< 10^{-4}$ |
| | *Lap2–Hbdh* | $< 10^{-4}$ |
| | *Lap2–Sod2* | $< 10^{-4}$ |

The statistic is as in Weir (1990). See text for details.

### (ii) *Non-independence between loci and multilocus genotype analysis*

Genotypic association between each pair of loci was measured by the common correlation coefficient. It was tested with an exact test giving the probability of being wrong (*P* value) if independence is rejected when it is true, and the standard error associated with the estimate was calculated (Table 3). Linkage disequilibrium was highly significant ($P < 10^{-4}$) between most of the variable loci, indicating non-random association of genotypes between each pair of loci. Two tests, involving the *Sod3* locus, were close to but did not reach significance ($P > 0·05$, Table 3).

Different modes of reproduction, sexual or not, will have different consequences on the multilocus genotype structure of each isolate. While sexual reproduction will lead to random assortment of genotypes for each isolate, strong genotypic associations would be expected under clonal reproduction, each isolate being reproduced identically. Nineteen different genotypic associations (GAs) were found over the 67 isolates of *Naegleria*: eight among the 32 isolates of

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

Table 4. *Multilocus genotypes identified for natural isolates of* Naegleria gruberi *and* N. australiensis

| Isolate | *n* | Enzymatic loci | | | | |
|---|---|---|---|---|---|---|
| | | *Hbdh* | *Me* | *Sod2* | *Sod3* | *Lap2* |
| *N. gruberi* | | | | | | |
| Ng F | 2 | 1′ | 6 | −1 | 2 | 3′/3″ |
| Ng G | 16 | 1′ | 6 | −1 | 2 | 3 |
| Ng H | 2 | 1′ | 4/6 | −1 | 2 | 3 |
| Ng I | 6 | 4 | 6′ | −1 | 2 | 5 |
| Ng J | 2 | 1″/4 | 6′ | −1 | 2 | 5 |
| Ng 86 | 1 | 3 | 6/6′ | 2 | 0 | 2/2′ |
| Ng L | 2 | 4 | 7 | −2/9 | 2 | 5 |
| Ng 95 | 1 | 4 | 3 | 1 | 2 | 5 |
| *N. australiensis* | | | | | | |
| Na 1 | 1 | 3 | 4 | 1 | 2 | 3 |
| Na 9 | 1 | 3 | 4 | 1 | 2 | 3/3″ |
| Na 11 | 1 | 3 | 4 | 1 | 2 | 4 |
| Na A | 12 | 3 | 4 | 1 | 2 | 3/4 |
| Na C | 2 | 3 | 4 | 1 | 2 | 2″/2‴ |
| Na 89 | 1 | 3 | 4 | 1 | 2 | 7 |
| Na 2 | 1 | 3 | 4 | −2/3 | 2 | 2/2‴ |
| Na 27 | 1 | 1′/3 | 4 | −2/3 | 2 | 2″/2‴ |
| Na D | 9 | 1′/3 | 4 | −2/3 | 2 | 2″/3 |
| Na E | 5 | 3 | 4 | −2/3 | 2 | 1′/2″ |
| Na 54 | 1 | 3 | 4 | −2/3 | 2 | 3 |
| Reference strains | | | | | | |
| Ng 1e (USA) | | 4/5 | 3 | −1 | 2 | 4 |
| Ng R1a (USA) | | 4 | 3/6 | −1 | 2 | 4 |
| Ng 1f (USA) | | 3 | 6 | 0 | 1/2 | 3/3′ |
| Ng 1d (England) | | 4 | 7 | −2 | 2 | 5 |
| Ng Aud1 (France) | | 1′ | 6 | −1 | 2 | 3 |
| *N. jadini* (Belgium) | | 1′ | 6 | 3 | 1 | 3 |
| Na LSR34 (France) | | 3 | 4 | 1 | 2 | 3 |
| Na PP397 (Australia) | | 3 | 4 | 1 | 2 | 2‴/3 |
| Na *italica* (*Italy*) | | 3 | 5 | 2 | 0 | 1/1′ |

Genotype associations (GA) are also given for nine reference strains of the same species and two related ones. *n* indicates the number of isolates with identical multilocus genotypes. Allele nomenclature is as in Table 1. 0 refers to absence of activity involving a putative null allele.

*N. gruberi* and 11 for the 35 isolates of *N. australiensis* (Table 4). Furthermore, in the latter species, more than half the strains (18/35) differ from each other by only one locus, the very polymorphic *Lap2*. Only two isolates among *N. gruberi* and seven for *N. australiensis* are unique (Table 4) and none of them is common to the two species. Interestingly the most frequent multilocus genotypes were regularly recovered during more occasional samplings in the river Rhône (unpublished data). In two cases, GAs found in natural populations were identical to reference strains: isolate Na1 and the reference strain LSR34 for *N. australiensis* and the predominant NgG and the reference strain Aud1 for *N. gruberi* (Table 4).

A centred data analysis has been performed on allele frequencies of each of the distinct multilocus genotypes for natural populations and the reference strains (Fig. 1). The plot of the GAs on the first three axes explains 62% of the total variability and shows several clear-cut groups. The two species appear to be genetically distinct as they have distant positions on the first axis. Among *N. australiensis*, the first axis again discriminates two subgroups. The first one comprises one of the major clones (NaA = 12 isolates) found in the Pierre Bénite population, several related ones (NaC, 9 and 11) and the two reference strains. The second one corresponds to the other natural isolates (NaD, E, 2, 27 and 54). The species *N. gruberi* also splits into two major groups mainly because of a clear discrimination on axis 2. The first group comprises the reference strain 1518/1f and the most predominant multilocus genotype, NgG, which is identical to the reference strain NgAud1. The second group includes the other predominant GA, NgI, and the other reference strains.

Regarding the two additional species, included in this study because of their possible affinities with the two main species, *N. jadini* is plotted within the first subgroup of *N. gruberi*. The species *N. italica*, formerly considered a subspecies of *N. australiensis* (De

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

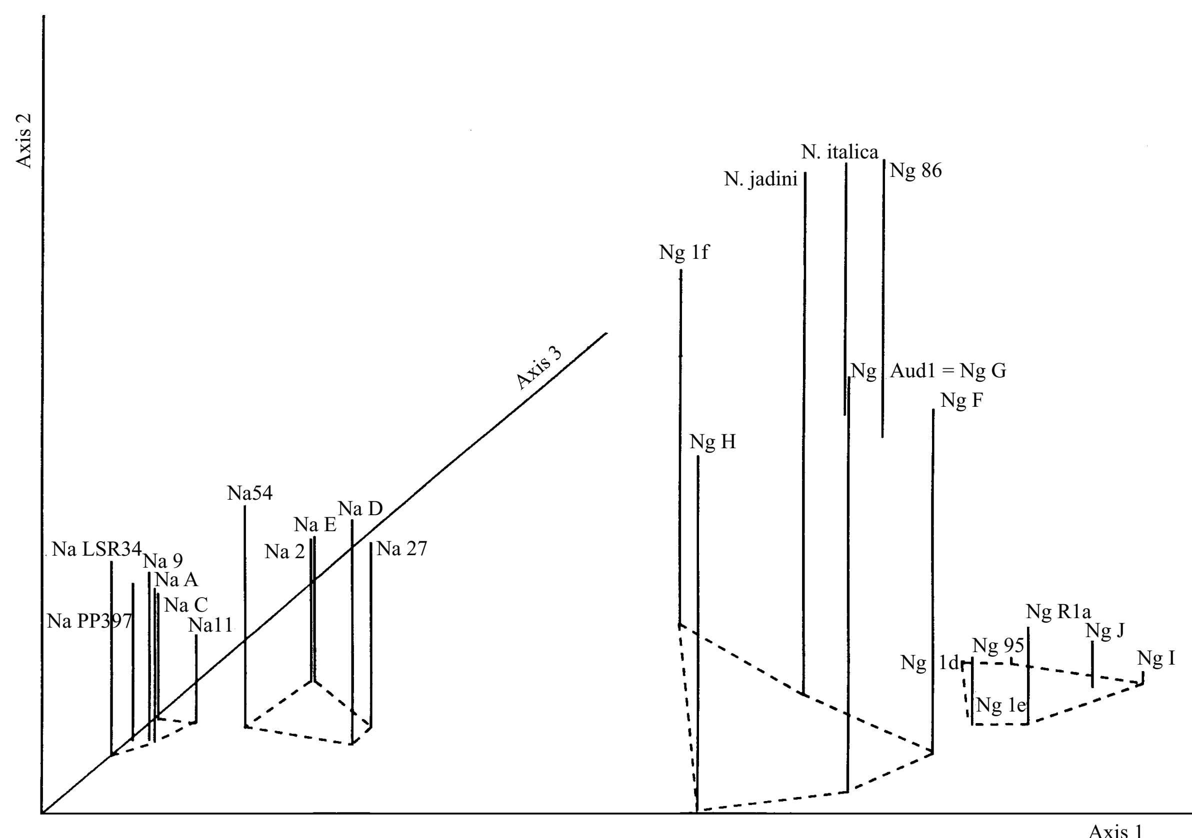


Fig. 1. Centred data analysis based on allele frequencies for each multilocus genotype of *Naegleria gruberi* and *N. australiensis*. Isolates (as in Table 4) plotted on the first three axes show clear differentiation between the two species and subgroups within each.

Jonckheere, 1994), shows no similarity with our local sample of that species. This confirms the results of Adams *et al.* (1989). In contrast, it is close to a single isolate identified as *N. gruberi*. All this suggests that the status of these entities remains uncertain and deserves further investigation.

## 4. Discussion

### (i) *Genetic coherence of the* Naegleria *species*

A clear discrimination between the two species is found from the MLEE analysis at seven loci (invariant and polymorphic loci) as shown in Fig. 1. This is consistent with the difference we found in thermotolerance between the two species: all the isolates referred to as *N. gruberi* are sensitive to high temperature while those identified as *N. australiensis* are able to grow at 42 °C. Indeed, the latter species has been described as thermotolerant (De Jonckheere, 1981). The present data on natural populations further support the hypothesis that *N. australiensis* is a different species. This species has been reported to be heterogeneous (Clark *et al.*, 1989; Adams *et al.*, 1989). We have demonstrated that significant heterogeneity is also found in samples taken from a restricted location, as two subgroups are identified. They are strictly correlated with the occurrence of either homozygotes for allele *l* at locus *Sod2* (subgroup NaA) or heterozygotes $Sod2^{-2/3}$ (subgroup NaD). At locus *Lap*2, seven alleles are segregating and genotype 3/4 is predominant in the NaA subgroup while genotype 2″/3 is predominant in the NaD subgroup. This situation might be similar to that found for the human parasite *Trypanosoma cruzi*, for which only two major clones have been identified despite extensive analyses of natural populations all over Bolivia, one of its main endemic geographic areas (Tibayrenc, 1995).

The second species, *N. gruberi*, reveals more genetic heterogeneity. Again two main subgroups are identified – the NgG and the NgI subgroups – but within each the related isolates are more dispersed, as shown in Fig. 1. Heterogeneity within *N. gruberi* has been demonstrated several times using techniques as diverse as isoenzymes (De Jonckheere, 1982; Daggett & Nerad, 1983; Pernin *et al.*, 1985; Pernin & Cariou, 1989; Adams *et al.*, 1989; Robinson *et al.*, 1992), genomic DNA restriction fragment length polymorphism (RFLP) analysis (De Jonckheere, 1987) and ribosomal RNA (Clark *et al.*, 1989). Our results provide new information on the genetic structure of natural populations in that a few multilocus genotypes account for most of the isolates present in the population. For example, isolates NgG represent about 50 % of the total population. Interestingly, this isolate is identical to the reference strain NgAud1 also collected in France from a nearby locality 13 years ago. Moreover, during this water survey the same

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

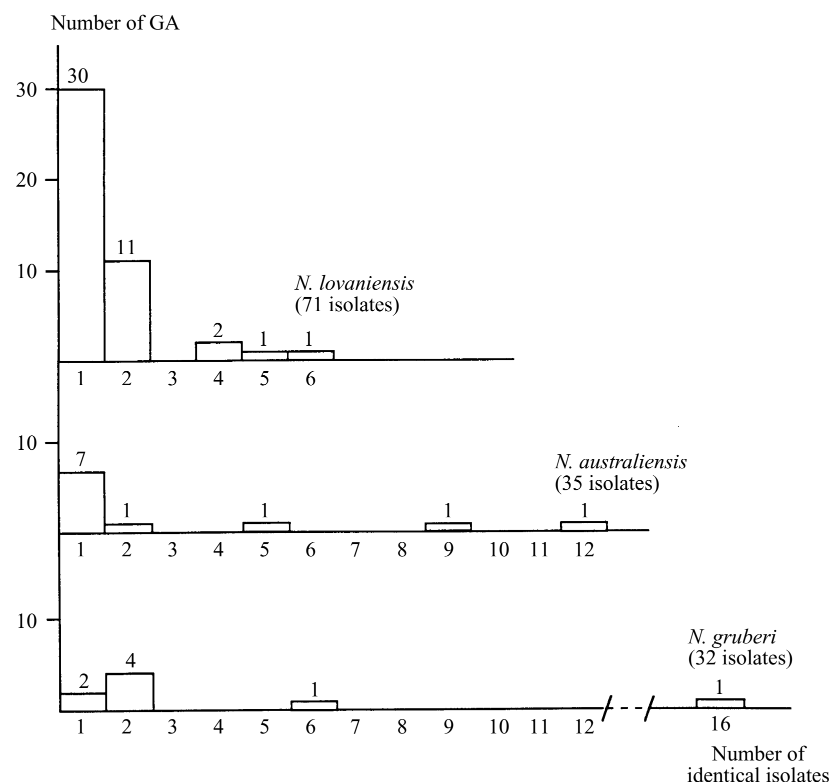


Fig. 2. Distribution of genotype associations (GA) among natural isolates of *Naegleria gruberi* and *N. australiensis*. The data for *N. lovaniensis* are from Pernin *et al*. (1992).

major genotypic associations have been recovered over 2 years (data not shown). The repeated recovery of isolates of the same multilocus genotype at different localities and at different times strongly suggests that the genetic structure might be relatively stable at a local scale. Furthermore, two reference strains were identical to major clones of the natural population, suggesting that while one might expect selection among isolates to promote different clones according to their local selective advantage, some major clones might be widespread all over the world.

### (ii) *Clonal structure of* Naegleria gruberi *and* N. australiensis *populations*

The overall data show marked and repeated deviations from Hardy–Weinberg equilibrium at individual loci and strong genotypic associations at multilocus genotypes, predominantly suggesting that both *Naegleria gruberi* and *N. australiensis* have clonal genetic structures in natural populations.

For the two species the distribution of multilocus genotypes was compared to that of *N. lovaniensis* from a previous study (Pernin *et al*., 1992). In all cases the populations consisted of isolates collected from a very restricted area in similar conditions, although in different rivers (Moselle for *N. lovaniensis*, Rhône for *N. gruberi* and *N. australiensis*). The comparison strongly suggests that they have contrasting genetic structures (Fig. 2). For both *N. gruberi* and *N. australiensis* a few predominant clones are present and very few are unique, while most of the multilocus genotypes were unique for *N. lovaniensis*. The frequencies of the most common clone are 8% in *N. lovaniensis* and 50% and 35% respectively for *N. gruberi* and *N. australiensis*. The frequencies of all repeated clones are 58% for *N. lovaniensis* and 94% and 80% respectively for *N. gruberi* and *N. australiensis*. In addition, the multivariate analysis stresses contrasting distribution patterns of the isolates: subgroups are discriminated among *N. gruberi* and *N. australiensis* while *N. lovaniensis* had a wide panmictic distribution without any clear-cut group (Pernin *et al*., 1992). All these opposite trends strongly suggest that the two groups of species have different reproductive modes. Genetic recombination has been postulated for *N. lovaniensis*, although the processes are still poorly understood (Pernin *et al*., 1992). In contrast, natural populations of *N. gruberi* and *N. australiensis* appear to be basically clonal, i.e. they consist of a collection of independently evolving clones related to a few major ones.

Clonal structures have often been reported for protozoa. The data are especially convincing for the parasite *Trypanosoma cruzi* (Tibayrenc *et al*., 1990, 1991). Many authors have also considered that bacterial populations have a clonal structure (Selander & Levin, 1980; Ochman & Selander, 1984; Selander *et al*., 1987; Whittam *et al*., 1983). However, parasexual mechanisms such as transformation, conjugation or transduction are known to occur in bacteria and comparisons of sequences of individual bacterial genes

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

have shown a mosaic structure for these genes that could be explained only by recombination (Maynard Smith *et al*., 1993).

However, despite recombination a clonal population structure might be maintained, as shown recently in *Escherichia coli* (Desjardin *et al*., 1995). Large spatial scale comparisons may lead to such structures as are shown by isolates of the bacteria *Rhizobium* (Souza *et al*., 1992). In that case the authors concluded that geographic separation between isolates contributed substantially to the observed linkage disequilibrium, leaving open the possibility of frequent recombination in local populations. Such an argument cannot, however, explain the linkage disequilibrium observed for *Naegleria* samples since they were collected from a single site.

The explosive spread of a single type, leading to what Maynard Smith *et al*. (1993) called an 'epidemic population structure', is another possible explanation for our observations. This was demonstrated, for example, in the parasite *Trypanosoma brucei* (Mihok *et al*., 1990). However, such epidemic multiplications have never been reported for *Naegleria* populations except in the case of an abnormal and pronounced elevation of water temperature. In such conditions of thermal pollution, never observed in the wild, the total number of amoebae can increase by a factor of 1000, favouring exclusively thermotolerant species such as *N. lovaniensis* and in particular the pathogenic *N. fowleri*. Interestingly, these species were not found in our water samples and the quantitative estimation of *Naegleria* by MPN methods (data not shown) show that the observed concentrations remain within the limits usually reported in similar environments. All these arguments further support the idea that the two species *Naegleria gruberi* and *N. australiensis* are basically clonal.

Maynard Smith *et al*. (1993) concluded that bacterial populations are not invariably clonal but, according to the species, might range from fully sexual species such as *Neisseria gonorrhoeae* to almost strictly clonal species such as *Salmonella*. It seems that evolution among *Naegleria* has also retained different reproductive strategies: genetic recombination even in a 'covert' manner, as suggested by Hurst *et al*. (1992) for some species, and asexual reproduction for others. This leaves open the question of a possible long-term disadvantage for clonal species compared with sexual ones. If clonality is the condition in *Naegleria gruberi* and *N. australiensis*, new gene arrangements will occur only through point mutations. Evolution will proceed through selection among clones without access to the large genotypic variation otherwise available through recombination.

We are grateful to the two anonymous referees, whose comments contributed greatly to improving the manuscript. This work was supported in part by grants from Electricité de France (EDF) and from the Ministère de l'Environnement (MEV).

## References

Adams, M., Andrews, R. M., Robinson, B., Christy, P., Baverstock, P. R., Dobson, P. J. & Blackler, S. J. (1989). A genetic approach to species criteria in the amoeba genus *Naegleria* using allozyme electrophoresis. *International Journal of Parasitology* **19**, 823–834.

Cariou, M. L. & Pernin, P. (1987). First evidence for diploidy and genetic recombination of free-living amoeba of the genus *Naegleria* on the basis of electrophoretic variation. *Genetics* **115**, 265–270.

Clark, C. G., Cross, G. A. M. & De Jonckheere, J. F. (1989). Evaluation of evolutionary divergence in the genus *Naegleria* by analysis of ribosomal DNA plasmid restriction patterns. *Molecular and Biochemical Parasitology* **34**, 281–296.

Daggett, P. M. & Nerad, T. A. (1983). The biochemical identification of vahlkampfiid amoebae. *Journal of Protozoology* **30**, 126–128.

De Jonckheere, J. F. (1982). Isozyme patterns of pathogenic and non-pathogenic *Naegleria* sp. using agarose isoelectric focusing. *Annals of Microbiology* **133**, 319–342.

De Jonckheere, J. F. (1981). *Naegleria australiensis* sp. nov., another pathogenic *Naegleria* from water. *Protistologica* **17**, 423–429.

De Jonckheere, J. F. (1987). Characterization of *Naegleria* species by restriction endonuclease digestion of whole cell DNA. *Molecular and Biochemical Parasitology* **24**, 55–66.

De Jonckheere, J. F. (1994). Comparison of partial SSUrDNA sequences suggests revisions of species names in the genus *Naegleria*. *European Journal of Protistology* **30**, 333–341.

Desjardin, P., Picard, B., Kaltenböck, B., Elion, J. & Denamur, E. (1995). Sex in *Escherichia coli* does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism. *Journal of Molecular Evolution* **41**, 440–448.

Guo, S. W. & Thompson, E. (1992). Performing the exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics* **48**, 361–372.

Haldane, J. B. S. (1954). An exact test for randomness of mating. *Genetics* **52**, 631–635.

Hurst, L. D., Hamilton, W. D. & Ladle, R. J. (1992). Covert sex. *Trends in Ecology and Evolution* **7**, 144–145.

Lefebvre, J. (1976). *Introduction aux analyses statistiques multidimensionnelles*. Masson, Paris.

Louis, E. J. & Dempster, E. R. (1987). An exact test for Hardy–Weinberg and multiple alleles. *Biometrics* **43**, 805–811.

Maynard Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993). How clonal are bacteria? *Proceedings of the National Academy of Sciences of the USA* **90**, 4384–4388.

Mihok, S., Otieno, L. H. & Darji, N. (1990). Population genetics of *Trypanosoma brucei* and the epidemiology of human sleeping sickness in the Lambwe valley, Kenya. *Parasitology*, **100**, 219–233.

Ochman, H. & Selander, R. K. (1984). Evidence for clonal population structure in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the USA* **81**, 198–201.

Pernin, P. & Cariou, M. L. (1989). Large genetic heterogeneity within amoebae of the species *Naegleria gruberi* and evolutionary affinities to the other species of the genus. *Journal of Protozoology* **36**, 179–181.

Pernin, P. & Grelaud, G. (1989). Application of isoenzymatic typing to the identification of nonaxenic strains of *Naegleria* (Protozoa, Rhizopoda). *Parasitology Research* **75**, 595–598.

Pernin, P., Ataya, A. & Cariou, M. L. (1992). Genetic structure of a natural population of the free-living

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press

amoeba, *Naegleria lovaniensis*: evidence for sexual reproduction. *Heredity* **68**, 173–181.

Pernin, P., Cariou, M. L. & Jacquier, A. (1985). Biochemical identification and phylogenetic relationships in free-living amoebae of the genus *Naegleria*. *Journal of Protozoology* **32**, 592–603.

Raymond, M. & Rousset, F. (1995). GENEPOP (version 1.2): a population genetics software for exact tests and ecumenism. *Journal of Heredity* **86**, 248–249.

Robinson, B. S., Christy, P., Hayes, S. J. & Dobson, P. J. (1992). Discontinuous genetic variation among mesophilic *Naegleria* isolates: further evidence that *N. gruberi* is not a single species. *Journal of Protozoology* **39**, 702–712.

Selander, R. K. & Levin, B. (1980). Genetic diversity and structure in *Escherichia coli* populations. *Science* **210**, 245–247.

Selander, R. K., Musser, J. M., Caugant, D. A., Gilmour, M. N. & Whittam, T. S. (1987). Population genetics of pathogenic bacteria. *Microbial Pathogenesis* **3**, 1–7.

Souza, V., Nguyen, T. T., Hudson, R. R., Pinero, D. & Lenski, R. (1992). Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: evidence for sex?. *Proceedings of the National Academy of Sciences of the USA*. **89**, 8389–8393.

Tibayrenc, M. (1995). Population genetics of parasitic Protozoa and other microorganisms. *Advances in Parasitology* **36**, 47–115.

Tibayrenc, M., Kjellberg, F. & Ayala, F. J. (1990). A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences of the USA*. **87**, 2414–2418.

Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Breniere, S. F., Darde, M. L. & Ayala, F. J. (1991). Are eukaryotic microorganisms clonal or sexual? A population vantage. *Proceedings of the National Academy of Sciences of the USA*. **88**, 5129–5133.

Tyndall, R. L., Ironside, K. S., Melter, P. L., Tan, E. L., Hazen, T. C. & Fliermans, C. B. (1989). Effect of thermal additions on the density and distribution of thermophilic amoebae and pathogenic *Naegleria fowleri* in a newly created cooling lake. *Applied and Environmental Microbiology* **55**, 722–732.

Weir, B. (1990). *Genetic Data Analysis*. Sunderland, MA, USA: Sinauer Associates.

Whittam, T. S., Ochman, H. and Selander, R. K. (1983). Multilocus genetic structure in natural populations of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the USA*. **80**, 1751–1755.

https://doi.org/10.1017/S0016672397002723 Published online by Cambridge University Press