that recrudescence of congenital *Toxoplasma* infection is mainly associated with ocular, rather than brain, lesions. Due to their relative dimensions, minimal inflammatory lesions in the brain may not be discernible clinically; in the retina, however, a similar effect may induce a sight-threatening lesion[13].

It should be noted that acute fatal toxoplasmosis can occur in animals other than the gundi (*Ctenodactylus gundi*; small North African rodents)[14].

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Speciation and Clonality in *Entamoeba histolytica*

In a recent article[1], Michel Tibayrenc concludes that recognition of two cryptic species within *Entamoeba histolytica* is not warranted based on the species' population structure inferred from isoenzyme data. However, his interpretation is dependent on a flawed database and ignores information obtained by methods other than isoenzyme analysis.

Tibayrenc cites the isoenzyme data accumulated by Sargeaunt on 6000 isolates of *E. histolytica* and notes that many possible genotypes are missing. However, Blanc and Sargeaunt have subsequently shown[2] that, although isoenzymes can be used to differentiate the two amoebae, the presence of certain bands crucial for zymodeme assignation is dependent on culture conditions. Thus, Sargeaunt's and certain other data sets cannot be used for Tibayrenc's analyses.

I personally believe that *E. histolytica* is a clonal organism, but I also think that this is irrelevant to the question of whether two species should be recognized. The number of characters that separate the two distinct organisms within what has classically been called *E. histolytica* is considerable. At the latest count there were six isoenzyme, six antigen and seven genetic markers that could be used unambiguously to differentiate the two, and that correlate with clinical and other biological criteria. Not one isolate has been described, to my knowledge, that exhibits a mixture of these characteristics. On the basis of these and other considerations the invasive pathogen *E. histolytica* has recently been redescribed to separate it from the previously cryptic, non-invasive species, *E. dispar*[3]. These 'agamospecies' do have rigorous definitions and boundaries, and their recognition is crucial to a better understanding of *E. histolytica* epidemiology.

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In his comments to my article[1], C. Graham Clark apparently misunderstood my proposals since, for me, we have no serious disagreement. The conclusions reached for *Entamoeba histolytica*[1] were: (1) within this species, there are apparently two genetically distinct groups of organisms[2], which seem to represent actual phylogenetic lineages. (2) Within each of these lineages, strong linkage disequilibrium suggests predominant clonal evolution, which actually reflects Clark's personal views. (3) Inferred clonality within each lineage prevents one from considering them as 'real', biological species, but rather, as 'agamospecies'. (4) Phylogenetic divergence is not sufficient in itself to describe new agamospecies. (5) New agamospecies should be described only on the basis of both phylogenetic divergence and the presence of obvious biological or medical distinctive features. Since all these conditions are fulfilled in the case of pathogenic versus non-pathogenic *Entamoeba histolytica*, I concluded by fully agreeing with the views of Blanc[2] (and, hence, of Clark).

The population genetic analysis[1] based on Sargeaunt's data[3] was criticized by Clark on the basis of the fact that isoenzyme patterns can be modified by cultural conditions and, hence, cannot be considered as reliable genetic markers. Nevertheless, they were apparently good enough to distinguish the two cryptic *Entamoeba* species[4], reliably, long before molecular markers could do it[5]. In his letter, Clark himself relies on six isoenzymes, together with other markers, to discriminate between the two *Entamoeba* species. If enzyme markers are good for species attribution, it means that, overall, they are reliable genetic markers. Hence they are good for population genetics too, even if they can be somewhat modulated by culture conditions.

Nevertheless, I fully acknowledge that the working hypothesis of clonality within each of the *Entamoeba* cryptic species must be verified on more sharply focused data. More generally, population genetics in the case of *Entamoeba* is still in its infancy. One of the main goals of my article was to stimulate this line of research.

Clonality in *Entamoeba* has the same relevance than for other parasitic protozoa such as *Trypanosoma* and *Leishmania*[6]. The clone concept is not against the notion of species in protozoa. The first simply supplements the second. The newly recognized invasive *Entamoeba histolytica* could be too broad a taxonomic unit for medical purposes, if clones within it differ for relevant medical properties such as virulence or resistance to drugs. This cannot be rejected *a priori*, and should be tested. On the other hand, the clones are inferred to spread unchanged on wide geographical areas and for long periods of time. If they are accurately labelled by appropriate genetic tools, they constitute reliable markers for epidemiological tracking, an application broadly used in bacteriology[7].

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Entamoeba, *Giardia* and *Toxoplasma*: Clones or Cryptic Species?

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The availability of molecular markers has renewed interest in the taxonomy of parasitic protozoa at the subspecific and specific levels. Nevertheless, the conclusions reached are heavily dependent upon the way the data are analyzed. Some authors have emphasized the value of genetic distances to propose the creation of new species, while others have rather favoured a population genetic approach to account for the intraspecific variability of parasites. In this article, using three illustrative cases, Michel Tibayrenc shows that dissimilar taxonomic approaches can be built from quite comparable sets of data.

The biological concept of species relies on the presence or absence of regular interbreeding. This is a difficult criterion where parasitic protozoa are concerned. Indeed, we have proposed that many of them were predominantly clonal, including the three species discussed in this article, ie. *Giardia duodenalis*[1], *Entamœba histolytica*[1] and *Toxoplasma gondii*[2]. The intraspecific variability of these species has been considered by others[3–5]. Although the rough genetic data from these three studies present striking similarities (see below), the conclusions drawn are dissimilar. Andrews, Blanc and colleagues[3,4] propose that both *G. duodenalis* and *E. histolytica* should be subdivided into additional species, while Sibley and Boothroyd[5] do not consider this to be the case for *T. gondii*, and rather, corroborated the clonal model proposed for this species[2], with the help of more extensive data.

In light of the clonal model, these three examples will give us the opportunity to illustrate several major problems concerning parasitic protozoa taxonomy. (1) Are the new species infered within both *Giardia*[3] and *Entamœba*[4] mere clusters of clones, or are they 'real' biological species (an alternative not considered in the works cited). If the second hypothesis is true, could this hidden biological speciation be responsible for some features hitherto wrongly accounted for by clonality? (2) If the species surveyed here are actually clonal, are genetic distances and trees, considered separately, reliable means to subdivide them into new asexual taxa (or 'agamospecies')? (3) In the case of predominantly uniparental organisms, why describe new species?

To approach these problems, it is first necessary to briefly review previous evidence for clonality in these three species (for more details, see Refs 1,2,6).

Arguments for Clonality

Circumstantial evidence for uniparental propagation is based mainly upon departures from Hardy–Weinberg expectations (lack of segregation of alleles at given loci) and linkage disequilibrium (nonrandom association between genotypes at different loci)[1]. More detailed information on this classical population genetics approach, on the statistics used and on the possible biases due to either geographical distance or natural selection (including selection by culture medium), have been detailed elsewhere[1,2,6].

In *Toxoplasma*, the culture forms used for genetic analysis are haploid. For the other two species, the level of ploidy is not fully ascertained; this makes Hardy–Weinberg statistics impracticable. Hence, the main evidence was taken from linkage between loci, since this can be made independently from any consideration on the level of ploidy[1,2]. Linkage generates (separately or together) several features, that can be estimated by various statistical tests (Ref. 1 and see Box 1), all based on the null hypothesis of free recombination. (1) Since the different loci are not shuffled together every generation, some multilocus genotypes are over-represented (tests d1 and d2, Box 1), and persist unchanged over long periods of time and vast geographic areas. (2) Conversely, other genotypes, which should be present if the organism was sexual, are under-represented or absent (test e). (3) The genotypes occuring at different loci are not reassorted at random (test f). Knowing the genotype recorded at a given locus makes it possible to predict, at least statistically, the genotypes that will be present at other loci, which is not the case in a sexual species.

Several features of that kind are evident in these three species[1,2]. A few examples are cited below:

- In *Giardia duodenalis*, the zymodeme (multilocus isoenzyme genotype) M4 (Ref. 7) appears to be distributed worldwide[1] (in various parts of Australia,

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Papua New Guinea and the USA). The probability of observing it with its actual size (test d1) if *Giardia* was sexual was 10^{-12} (Ref. 1). Concordance between isoenzyme patterns and restriction fragment length polymorphism (RFLP) profiles (a striking case of linkage) led Meloni *et al.* also to conclude that *Giardia* was clonal[8].

- In *Entamœba histolytica*, a sample of 6000 stocks was electrophoretically analyzed[9]. Although lack of detailed information on the origin of the stocks prevents any statistical calculations, it is apparent that many possible genotypes are lacking, since only 20 out of 144 possible genotypes are present[1]. In Canada[10], the result of the e test (Box 1) was 10^{-4} (Ref. 1).
- In *Toxoplasma gondii*, the zymodeme 1 (Ref. 11) was recorded both in the USA and in France, between 1939 and 1985 (Ref. 2). Even with a sample of only 14 stocks[12], the result of the d1 test was only 0.016, while the result of the f test was only 10^{-3} (Ref. 2). Clonality was also proposed with similar arguments taken from molecular data[5]. In this study, a striking association between one of the clonal lineages and virulence in mice was observed.

Hence, it is apparent that, within the three taxa, there exists a considerable linkage disequilibrium, that cannot be parsimoniously* explained either by geographical separation or by selection[2]. Nevertheless, could this result be explained by hidden speciation rather than by clonality? Indeed, if two different biological species are wrongly considered to be a single panmictic unit, drastic departures from panmixia will be recorded, since the two species are genetically isolated from one another.

Box 1. Statistical Tests Used as Circumstantial Evidence for Clonal Propagation

'Clonal' propagation does not amount to 'mitotic' propagation: in population genetics, the term 'clonal' is used in all cases where the progeny is identical to the reproducing individual[6] (this includes several cases of parthenogenesis as well as self-fertilization in haploid organisms). The null hypothesis for all the tests designed to demonstrate clonality is that, in the population under survey, genetic exchanges occur at random ('panmixia'). Significant departures from panmictic expectations, therefore, provide circumstantial evidence that genetic exchanges are inhibited in this population.

One of the main consequences of genetic exchange is recombination of genotypes among different loci. Free recombination would result in the expected probability of a given multilocus genotype being the product of the observed probabilities of the relevant single genotypes (eg. in a panmictic human population, if the frequency of the AB blood group is 0.3, and the frequency of the Rh (+) blood group is 0.7, the frequency of the individuals who have both AB and Rh (+) groups is $0.3 \times 0.7 = 0.21$). Inhibition of recombination leads to linkage disequilibrium, or nonrandom association among loci (the predictions of expected probabilities for multilocus genotypes are no longer satisfied).

Several complementary tests have been proposed elsewhere[1,2] to explore various consequences of linkage disequilibrium. They include:

- d1 = Combinatorial probability of sampling the most common genotype as often as, or more often than, the observed frequency, given by the formula[1]:

$$P = \sum_{i=m}^{n} \frac{n!\,.\,x^{i}\,.\,(1-x)^{n-i}}{i!\,.\,(n-i)!}$$

where x = expected probability of the multilocus genotype (see example above); n = number of individuals sampled; and $i = m$ = number of individuals in the sample with the particular genotype.

- d2 = Probability of observing any genotype as often as or more often than the most common genotype in the sample.
- e = Probability of observing as few or fewer genotypes in the population as are observed in the sample.
- f = Probability of observing a linkage disequilibrium in the population that is as high as that observed in the sample.

[NB d2, e and f are based on computer simulations (Montecarlo tests) with 10^4 runs.]

Clonality Versus Hidden Biological Species

Although both clonality and biological speciation can generate linkage disequilibrium, it is generally possible to decide between the two hypotheses on the basis of different patterns. Considering a given taxon, if the linkage within it is due to the presence of two or more hidden species, this linkage will no longer be observed if each putative species is separately considered. Conversely, if the taxon is predominantly clonal, the linkage will be apparent even when separately considering lower subdividions of the taxon. Of course, in this last case, if the subdivisions each exhibit little or no genetic variability, it will become impossible to look for any linkage between loci. Now the observance of such repeatedly sampled genotypes that are totally monomorphic for a large range of loci is better explained by clonal propagation than by speciation.

Giardia duodenalis would be an illustrative example of this last case. It has been proposed that this taxon should be divided into additional species, for several genetically dissimilar clusters are apparent within it from multilocus enzyme electrophoresis (MLEE)[3]. When considering separately each cluster described, statistical tests are impossible, for each cluster is monomorphic or almost monomorphic. Now, if each of the clusters should be considered as a distinct biological species, the species corresponding to cluster I, as an example, is totally monomorphic for 26 different loci. Moreover, it can be inferred from the stocks considered in both studies that the zymodeme M4 (Ref. 7) is included in cluster I (Ref. 3), and hence, would be included in the same putative cryptic species. As stated above, stocks included in this zymodeme persist unchanged not only for the 13 enzyme loci but also for RFLP patterns from the USA to Papua New Guinea and Australia. Therefore, although it is not possible definitely to rule out the hypothesis that the main clusters described by Andrews and colleagues are each a potentially panmictic unit (biological species), they are more parsimoniously explained by clonal propagation.

Entamœba histolytica was similarly subdivided into two cryptic species, corresponding to two main clusters on the basis of enzyme electrophoresis[4]. Interestingly, all zymodemes previously described as pathogenic[9] fell into the first cluster. The hypothesis that the two clusters correspond to two biological species is not corroborated by population genetic analysis of each cluster considered separately. For example, in Canada[10], when analyzing separately the nonpathogenic zymodemes, the results of the d2 and e tests are

* Law of parsimony, that no more causes or forces should be assumed than are necessary to account for the facts.

3×10^{-4} and $<10^{-4}$, respectively. Similarly, in South Africa[13], within the nonpathogenic group of zymodemes, the result of the d1 test is 10^{-3}. Lastly, in the huge sample of 6000 stocks electrophoretically characterized[9], even if pathogenic and nonpathogenic zymodemes are treated separately, it is apparent that many genotypes are missing. Within the pathogenic group, ten different genotype combinations (zymodemes) were observed, while 28 would be possible. For the nonpathogenic group, the figures are 12 and 24, respectively. This is evidence of strong linkage disequilibrium, not only in the whole taxon *E. histolytica*, but also within the two groups (pathogenic and nonpathogenic) considered separately.

In contrast to *Giardia* and *Entamœba*, in *T. gondii*, a species status was not proposed for the two discrete groups revealed by molecular data[5], which correspond, respectively, to those strains that are virulent and avirulent in mice. Rather, clonal propagation was considered responsible for the existence of these two discrete groups. Nevertheless, population genetic analysis was performed only on the whole *T. gondii* taxon[2,5], and not on the two groups considered separately. Could these two groups be hidden biological species, and would the linkage disequilibrium in the whole taxon be due to the presence of hidden biological species? For the first 'virulent' group, the same reasoning can be made as for *Giardia* cluster I (Ref. 3): a subset of this group involving seven stocks from either USA or Brazil appears quite homogeneous for the 12 molecular markers being studied[5]. Moreover, within this group, two stocks isolated in the USA in 1939 and in France in 1985, respectively, are identical for five molecular markers[5] and for six enzyme loci[11]. Although the sample still is limited, this 'virulent' group, even analyzed separately, is better equated to a clonal lineage[5] than to a cryptic, genetically homogeneous species. The other subset (the avirulent stocks) shows more variability. Even analyzed separately, this group exhibits significant linkage: the results of the d1, d2 and e tests are 5.7×10^{-4}, 1.1×10^{-3}, and 1.8×10^{-4}, respectively. So this subset of 'avirulent' stocks also shows drastic departures from panmixia, which does not favour the hypothesis that it represents a hidden biological species.

The results summarized above suggest that linkage disequilibrium in these three species is due not to cryptic biological speciation, but rather to predominantly clonal propagation. Again, this linkage cannot be explained solely by either geographical separation or selection[2]. Now, even within the framework of a clonal model, delimitating asexual taxa ('agamospecies') could be useful. Are genetic distances in themselves valid bases towards reaching this goal?

Defining New Species by Genetic Distances and Trees

If a species is predominantly clonal, the measure of genetic divergence among the clones could help to discriminate groups of clones that are markedly distinct from one another, and, hence, could be given a species status (asexual species, or 'agamospecies'). This approach is classical in Bacteriology: several people even propose determining fixed limits of genetic divergence under which bacterial populations are considered the same 'genomic species'[14]. Are 'large' genetic distances, therefore, in themselves, a sufficient basis on which to describe new taxa? A problem presents itself immediately: what is the arbitrary limit beyond which a distance becomes a 'large' one? Let us compare the three examples taken here with one distance index, namely Nei's standard genetic distance (ie. the average number of codon differences per gene between two populations)[15]. The values for *E. histolytica* are communicated by Blanc[4], and they can be estimated from the data on *G. duodenalis*[3] and *T. gondii*[5]. For *T. gondii*, distances were inferred by pooling the variability recorded at some stocks from both molecular[5] and isoenzyme data[11]. Maximum distances so recorded within the three species are very close to each other (1.48, 1.54 and 1.38, for *G. duodenalis*, *E. histolytica* and *T. gondii*, respectively, with 26, 14 and 12 loci analyzed, respectively). Although these distances are in the range of the ones usually observed between species[4], they are roughly comparable to the ones found within *Trypanosoma brucei brucei* (maximum: 1.15; F. Mathieu-Daudé and M. Tibayrenc, unpublished), and far lower than the ones recorded within *T. cruzi* (maximum: 2.7; C. Barnabé and M. Tibayrenc, unpublished). This suggests that it is probable that large genetic distances will be commonplace when parasitic protozoa are considered, and therefore it is risky to consider them as definitive evidence for speciation.

Apart from the rough values of genetic distances, it could be tempting to infer speciation when certain clusters of clones are separates by wide gaps, as visualized on trees and dendrograms. Indeed such a result seems obvious in *Giardia*[3] and *Entamœba*[4]. Again, one has to be cautious. As the number of stocks and the number of markers increase, distinction between principal and lesser clusters could become less clear, as has happened in *T. cruzi*[16,17].

Concluding Remarks

It must be stressed that population genetics of parasitic protozoa is still in its infancy. Much refinement is sorely needed before a reliable picture of the intraspecific variability of these organisms can be drawn. This is especially true for the three species considered above. For none of them is an ideal sampling for population genetic purposes available. Such a sample should include: (1) sets of stocks collected in rigorous sympatric conditions, in order to test accurately the problem of the reproductive system of the organism; and (2) sets of stocks collected over large geographical areas and long periods of time, in order to check for the spatial and temporal stability of the clones, which defines their medical relevance.

As recalled elsewhere[1,2,6,17], the clonal theory proposed for parasitic protozoa[1] does not imply that recombination is totally absent in the populations under survey, but only that it is not frequent enough to break the prevalent pattern of clonal population structure. This model is therefore compatible with the experimental obtaining of recombinants in the laboratory[18,19]. Although the clonal model still needs refining[6], it is most probable that the biological concept of species is invalid for many parasitic protozoa. Two major problems with 'agamospecies' are (1) that they have no rigorous definition, and (2) that their boundaries are arbitrary. In my opinion (which is shared by many others), genetic phylogenies are not sufficient in themselves to build new species in uniparental organisms, but they can be a basis for it, if they are corroborated by notable biological differences[20,21]. Where parasitic protozoa are concerned, a major criterion could be the medical relevance of the new species. We have seen that, on similar genetic bases, new species have been proposed for *Giardia*[3] and *Entamœba*[4], but not for

Toxoplasma[5]. The two clusters distinguished in *E. histolytica*[4], which correspond to the pathogenic and nonpathogenic zymodemes[13], present an obvious medical relevance, and hence, would deserve a specific status. Similarly, the 'virulent' clonal lineage found in *T. gondii*[5] could also be made a species, when its individuality and medical relevance are confirmed, and if specialists of the study of this parasite consider it desirable. Lastly, the striking genetic dissimilarities shown within *G. duodenalis*[3] could serve as a guide to look for significant biological and medical differences among the putative species, which would confirm their value as separate taxa.

The new Linnean taxa built in this way could still be too broad for medical purposes. They can be usefully supplemented with the notion of 'natural clones'[1,17] brought by the clonal model. If the clones, in certain cases, have to be taken as taxonomic units, instead of the species, two problems appear: (1) the number of clones that comprise a given species is potentially unlimited; and (2) the clonal variability recorded in a given study is highly dependent upon the level of resolution of the genetic markers employed. To solve these problems, the notion of 'clonet' (all the isolates in a clonal species that appear to be genetically identical to one another on the basis of a particular set of markers)[6,20] can be used as a taxonomic unit. Widespread clonets (major clones)[17] call for priority studies to deal with issues such as virulence, resistance to drugs and host specificity. The ubiquitous *Giardia* genotype referred to as zymodeme M4 (Ref. 7), or as zymodeme I (Ref. 3), as well as the 'virulent' ubiquitous *T. gondii* clone[5] are probably analogous to the major clonets identified in *T. cruzi*[17].

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Epidemiological Evidence for an Association Between Chloroquine Resistance of *Plasmodium falciparum* and its Immunological Properties

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The appearance of chloroquine-resistant genotypes of Plasmodium falciparum *has thwarted the goal of global eradication of malaria. Although much effort has been put into understanding the molecular mechanisms of chloroquine resistance, many questions about its distribution remain open: Why, some 30 years after the emergence of chloroquine resistance, have resistant genotypes not taken over the population? Why have many parasites remained sensitive? Why, after its first appearance in Africa, has chloroquine resistance spread so rapidly through sub-Saharan Africa? In this paper Jacob Koella reviews epidemiological data that suggest that an answer to these questions may involve an association between chloroquine resistance and immunological properties of malaria parasites.*

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Chloroquine resistance first appeared in Thailand and South America in the late 1950s (Ref. 1; T. Harinasuta, S. Migasen and D. Boonag, abstract*), and has since spread to most parts of the world where malaria is endemic[2,3]. Several observations on the distribution of resistance suggest that selection on resistance is frequency dependent, so that when resistant parasites are rare they have a large advantage over sensitive parasites, but as they become more common, they have a disadvantage.

Such frequency-dependent selection would arise if chloroquine resistance of a parasite is associated with its immunological properties, as has been suggested by Clyde[4] and Peters[5]. This idea relies on the generally accepted assumption[6,7] that immunity against malaria is strain specific. Then, if resistance is associated with immunological properties, resistant parasites will encounter little immunity and thus be favoured shortly after invading a population. As resistance becomes more common, the advantage of resistance will be balanced by high levels of immunity, so that sensitive parasites will be maintained in the population. Thus, different immunological properties of resistant and sensitive parasites would explain why it is that, in areas with intense transmission, resistance initially spreads very rapidly, but then remains fairly stable[8].

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