Heterozygosity, Heteromorphy, and Phylogenetic Trees in Asexual Eukaryotes

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ABSTRACT

Little attention has been paid to the consequences of long-term asexual reproduction for sequence evolution in diploid or polyploid eukaryotic organisms. Some elementary theory shows that the amount of neutral sequence divergence between two alleles of a protein-coding gene in an asexual individual will be greater than that in a sexual species by a factor of $2tu$, where t is the number of generations since sexual reproduction was lost and u is the mutation rate per generation in the asexual lineage. Phylogenetic trees based on only one allele from each of two or more species will show incorrect divergence times and, more often than not, incorrect topologies. This allele sequence divergence can be stopped temporarily by mitotic gene conversion, mitotic crossing-over, or ploidy reduction. If these convergence events are rare, ancient asexual lineages can be recognized by their high allele sequence divergence. At intermediate frequencies of convergence events, it will be impossible to reconstruct the correct phylogeny of an asexual clade from the sequences of protein coding genes. Convergence may be limited by allele sequence divergence and heterozygous chromosomal rearrangements which reduce the homology needed for recombination and result in aneuploidy after crossing-over or ploidy cycles.

BEGINNING with the pioneering studies of HUBBY and LEWONTIN (1966) and HARRIS (1966), population geneticists have analyzed the diversity of genes at the molecular level in populations, and heterozygosity in individuals, in increasing detail (reviewed by AVISE 1994). These studies initially used protein electrophoresis to distinguish alleles, a method that detects much but not all of the actual sequence differences between alleles. The use of restriction analysis to detect restriction fragment polymorphism allowed the detection of some of the synonymous, as well as nonsynonymous, sequence differences between alleles. The logical extension of these studies was the detection of all sequence variation in a sample of alleles by DNA sequencing (KREITMAN 1983). This approach has been applied to a number of genes in Drosophila, a sexual diploid. Although the two alleles of a nuclear gene in a diploid individual could show moderate sequence differences, no attempt has been made to sequence both alleles from individual organisms, presumably because the difference between alleles in an individual will be similar to that between alleles from any two randomly chosen organisms in a random mating sexual species. Only recently has sequence analysis been extended to diploid or polyploid eukaryotes that reproduce strictly asexually. If asexual reproduction involves only mitotic (equational) divisions, the two (or more) alleles of a gene in an asexual lineage can show extremely high levels of heterozygosity; in addition, homologous chromosomes can acquire different chromosome rearrangements resulting in heteromorphy (WHITE 1973). This prediction has been verified by recent sequence analyses for some asexual species but not others.

Some basic theory presented here shows that neutral allele sequence divergence can accumulate to a high degree in asexual lineages. High levels of divergence in a lineage are evidence that it has been asexual for a very long time, and the number of different alleles can be used to determine the ploidy in eukaryotic microorganisms where conventional cytological methods fail. However, sequence divergence can be reduced or eliminated by gene conversion, mitotic crossing-over, or reduction of ploidy. These convergence processes can be identified if they occur infrequently and sufficiently many genes are examined. Allele sequence divergence confounds phylogenetic analysis, causing gene trees to depart drastically from species trees and making it difficult or impossible to recover the correct tree topology. This has profound implications for the use of protein-coding genes for phylogenetic reconstruction in evolutionary studies or to study epidemics of eukaryotic pathogens. Testing theories about the evolutionary advantages and disadvantages of asexual reproduction often requires knowing how long asexual lineages survive and the extent to which detrimental mutations accumulate in diploids and polyploids, information that can be obtained by studying all alleles in each clone.

ALLELE SEQUENCE DIVERGENCE

Alleles are alternate forms of a gene that differ from each other in base sequence but code for the same polypeptide or RNA product when functional. They

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usually occupy the same locus on a particular chromosome. In diploid (or polyploid) organisms, the two (or more) copies of a gene can be the same or different alleles, referred to as homozygosity and heterozygosity. Organisms that reproduce sexually at least occasionally can produce offspring with new combinations of alleles, so no one lineage "owns" any pair of alleles (except in the case of obligate selfers). Although the alleles in a single individual may accumulate different mutations, most of the sequence divergence of those alleles has been acquired over many generations in many different individuals. In contrast, organisms that reproduce strictly asexually do not share alleles among lineages. The two (or more) alleles in an asexual lineage begin to acquire different mutations from the moment that sexual reproduction is lost. If asexual reproduction continues for a long time, the majority of the differences in sequence between alleles is acquired after sexual reproduction was lost, and is unique to each lineage and each individual. I refer to the difference in sequence between two or more alleles in an asexual cell as intracellular allele sequence divergence, hereafter ASD.

ASD can be detected by any of the molecular methods normally used to distinguish between alleles. Sequencing provides the most detail, while restriction analysis detects ASD in a sample of sites. Enzyme electrophoresis can be used to detect heterozygosity of protein-coding genes, but is basically qualitative rather than quantitative because it fails to detect all synonymous substitutions and some nonsynonymous substitutions. Strictly speaking, ASD refers to sequence differences among the alleles in a single cell. However, the alleles are normally isolated from a clone of cells or of multicellular organisms. So long as the clone is young, the alleles in the clone will be accurate copies of those in the single cell that gave rise to the clone.

NEUTRAL ASD

In a sexually reproducing diploid species, population genetic theory predicts, and observation confirms, that the neutral sequence divergence between two alleles of a gene in a single individual will be low. In a random mating population, the expected number of substitutions between two different alleles is $k = 4N_e u$ differences per site where N_e is the effective population size and u is the mutation rate per site (base pair or amino acid) per generation. This is because the average time to the last common ancestor of two alleles, their coalescent, is $2N_e$ generations; since mutations accumulate along both lineages after the coalescent, the total divergence time is $4N_e$. Note that we are considering only selectively neutral base substitutions or amino acid substitutions, so u is the rate of neutral mutations. Because $4N_e u$ is much less than 0.1 in most sexual species that have been studied (AVISE 1994; MORIYAMA and POWELL 1996), multiple substitutions at a site can be ignored and the number of observed substitutions, d, is approximately the same as the expected number: $d = 4N_e u$.

Now consider a sexual species in which a mutant diploid individual gives rise to an asexual lineage. In the asexual lineage, the expected number of substitutions between alleles within an individual will be increased to

$$k = 4N_e u + 2tu = 2u(2N_e + t) \qquad (1)$$

where t is the number of generations since the origin of the asexual lineage.

These relationships are illustrated in Figure 1, in which the total number of substitutions separating alleles 3 and 4 in the asexual species A1, or alleles 5 and 6 in asexual species A2, is $2(t_1 + t_2)u + 4N_e u$, compared with $4N_e u$ for alleles 1 and 2 in the sexual species *S.* The difference is potentially very large; for example, if $4N_e u = 10^{-2}$ (estimated by the nucleotide diversity π; AVISE 1994; MORIYAMA and POWELL 1996), $u = 5 \times 10^{-9}$ mutations per base pair per generation (LI *et al.* 1985; WOLFE *et al.* 1989), there is one generation per year, and $t_1 + t_2 = 10$ million years, then the expected number of substitutions between alleles is 0.01 in the sexual species and 0.11 in the asexual species. In fact, $4N_e u$ will be insignificant relative to $2(t_1 + t_2)u$ when sexual reproduction was lost >10 mya, as is true for a number of cases (JUDSON and NORMARK 1996). Although the alleles may be very different with respect to base substitutions, especially in the third codon position, they will continue to be recognizable as being the same gene and coding for proteins with the same function so long as selection favors individuals with two functional copies of the gene. This is likely to be true for many, if not all, genes in a diploid because inactivation of one copy of a gene often causes detrimental gene dosage effects. Note that these comments also apply to dikaryotic organisms which have two different nuclei, usually with complete genomes, in each cell (*e.g.,* diplomonads such as Giardia and some fungi).

Of course ASD will not increase indefinitely: the rate of increase will slow as t increases because multiple substitutions at the same site are not always detectable. Consequently the observed neutral sequence divergence will asymptotically approach $^3/_4$. The true number of substitutions can be calculated from the observed number by an appropriate method of correcting for multiple hits (LI *et al.* 1985).

Phylogenetic trees with two or more asexual species: A second remarkable consequence of asexual reproduction is that the two alleles in an individual may differ from each other more than each does from an allele in a related species or clone in the same asexual clade. The argument can be made quantitative as follows. In Figure 1, consider the related asexual species A1 and A2. (For our purposes, asexual species are defined as clones or clades that are distinguishable by morphological or other criteria.) The most recent common ancestor of these two species was t_1 generations ago. The asexual lineage arose $t_1 + t_2$ generations ago as a single asexual mutant. Gene tree I, which includes all four

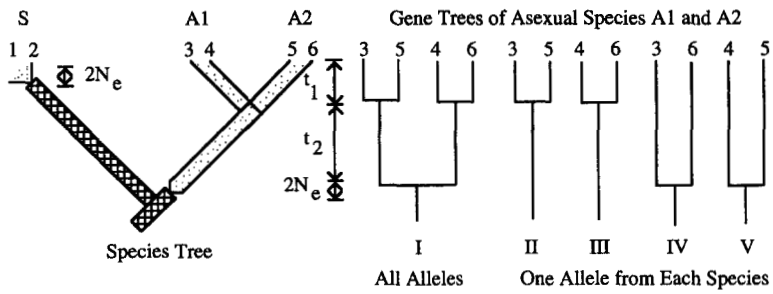

FIGURE 1.—The species tree and gene tree for two asexual species and a sexual relative. Sexual reproduction was lost when an asexual diploid mutant arose in the lineage leading to A1 and A2 at $t_1 + t_2$ generations ago and replaced its sexual relatives. Asexual species A1 and A2 (defined by morphology) diverged t_1 generations ago. Cross-hatching in the sexual lineages represents the sharing of genes by different individuals during sexual reproduction. The shading at the end of the sexual lineage indicates that the two alleles are found in a single individual, while the shading in the asexual lineage indicates that the two alleles are found in the same individual or clonal lineage of individuals. The time to coalescence of alleles 1 and 2 in the sexual species is $2N_e$ generations; the time to coalescence of the alleles in an asexual species (3 and 4, or 5 and 6) is $t_1 + t_2 + 2N_e$ generations because the two alleles in the first asexual diploid had their coalescent $2N_e$ generations earlier in an ancestral sexual individual.

alleles in the two asexual species, shows that the most closely related genes are alleles 3 in species A1 and 5 in species A2, or alleles 4 in A1 and 6 in A2.

Quantitatively, the expected substitutions between alleles in an individual are:

$$k_{34} = k_{56} = 2(t_1 + t_2)u + 4N_e u \qquad (2)$$

The substitutions between genes in different species are

$$k_{35} = k_{46} = 2t_1 u \qquad (3)$$

and

$$k_{36} = k_{45} = 2(t_1 + t_2)u + 4N_e u \qquad (4)$$

Phylogenetic trees of genes are normally based on the sequence of a single allele from each species or individual in the tree. As a result of ASD, phylogenetic trees of asexual organisms are allele-dependent, in the sense that different choices of alleles produces different trees. Some combinations of alleles will produce the correct species tree (trees II and III in Figure 1), while other combinations will result in trees in which the species divergence time and time of loss of sexual reproduction are confounded (trees IV and V in figure 1). If there are three or more asexual species, sequencing a single allele from each species can produce the incorrect topology (branching order) as well as incorrect divergence times. For three species, there are eight possible combinations of sequences of a single allele from each species. As illustrated in Figure 2, two combinations give the correct tree (II), two give the correct tree topology but incorrect distances (III), and four give the wrong topology (IV). As the number of species increases, the likelihood of sequencing the combination of genes that will give the correct tree decreases.

ASD in haploids and polyploids: Some asexual lineages may have begun as haploids, with diploidization occurring later. As shown in Figure 3A, ASD would begin to accumulate with the first diploid. Before diploidization, speciation events will be reflected correctly in the gene tree; after diploidization, the tree will be allele-dependent.

Many asexual organisms are polyploids (SUOMALAINEN *et al.* 1987). In some cases, especially in plants, these may have arisen from polyploid sexual species. In a very old asexual lineage, most ASD will have accumulated during asexual reproduction and the base of the gene tree will approximate a polytomy (Figure 3B). If an asexual lineage splits into two or more species and only one allele is sequenced from each of several species, most such trees will be not reflect the specis tree. If the organism is P-ploid and there are S species, there are P^S different combinations of alleles that may be sequenced; the probability of getting the correct tree is $P/P^S = P^{1-S}$.

Some asexual lineages probably began as haploids or diploids and subsequently became polyploid (Figure 3C). In this case the complete gene tree has no polytomy. It is similar to the case in which the ancestor was

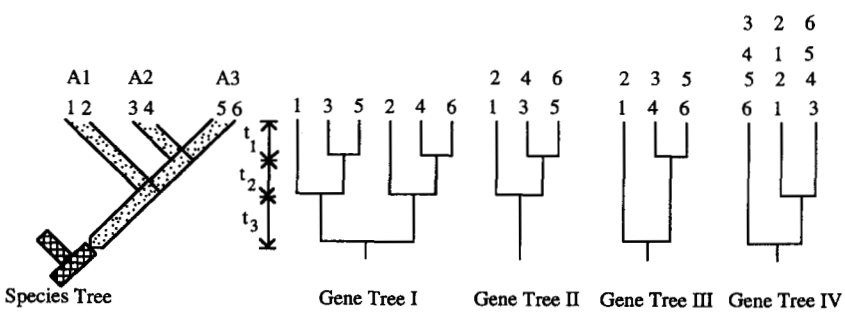

FIGURE 2.—Gene and species trees for three asexual species derived from a single asexual ancestor. Sex was lost $t_1 + t_2 + t_3$ years ago; species diverged at t_1 and $t_1 + t_2$ years ago.

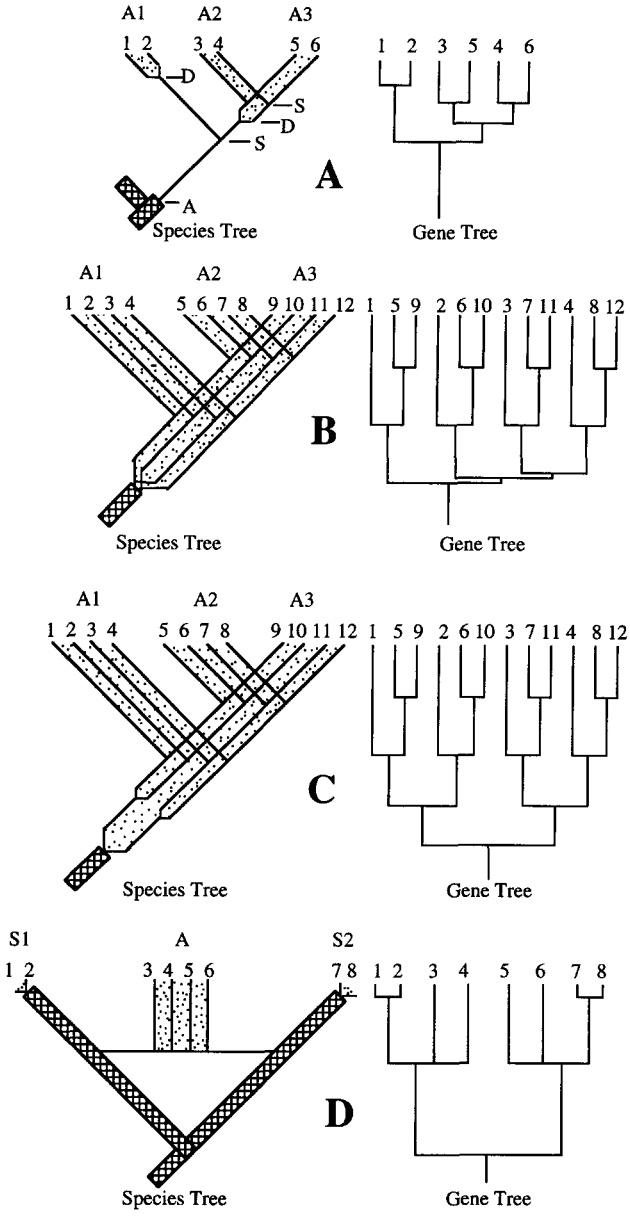

FIGURE 3.—Gene trees, species trees, and ploidy in asexual lineages. (A) Sex is lost in a haploid at point A, with subsequent diploidization (D) and speciation (S). (B) A tetraploid sexual species gives rise to a tetraploid asexual lineage which splits twice to form three different species. Gene trees based on three selections of alleles (1, 5, and 9; 2, 6, and 10; 3, 7, and 11; 4, 8, and 12) will reproduce the species tree; all other combination of alleles, *e.g.* 5, 2, and 11, will not. (C) A diploid sexual species gives rise to a diploid asexual lineage which subsequently duplicates both genomes to produce a polyploid. The polyploid asexual lineage splits twice to form three species. (D) Sexual diploid species S1 and S2 produce a tetraploid asexual hybrid A by hybridization, represented by a horizontal line.

polyploid, but with an additional branch at the origin of the asexual lineage. A practical consequence of ASD is that no two alleles in an individual or clone will be identical. Consequently the ploidy of an asexual microorganism, which is often unknown, could be determined by partially sequencing different clones of a gene to identify different alleles, increasing the sample size until no new alleles are found in a statistically significant number of clones.

Two general principles emerge from the preceding phylogenetic trees. First, the gene tree for a diploid asexual species that arose from a diploid sexual ancestor contains a dichotomy $2N_e$ generations before sexual reproduction is lost; polyploids have multiple dichotomies. Additional dichotomies are found at each point where genes are duplicated as a result of either speciation or an increase in ploidy, regardless of the ploidy of the sexual ancestor. Second, if a single allele is chosen at random from each species for sequencing, the majority of combinations of alleles will produce incorrect trees.

Many asexual lineages are produced by hybridization between diploid sexual species (SUOMALAINEN *et al.* 1987; SOLTIS and SOLTIS 1993; DUFRESNE and HEBERT 1994). Hybridization between two diploid species to produce a tetraploid results in a different phylogenetic problem, as illustrated in Figure 3D. The tree obtained by sequencing all alleles from all three species reflects the fact that one set of alleles in the asexual polyploid is more closely related to the genes in one sexual species, while the other set is more closely related to a different asexual species. The initial dichotomy reflects a speciation event in the ancestral sexual clade, and subsequent polytomies represent the loss of sexual reproduction. There is some evidence that a diploid asexual species can be fertilized by a sexual species to produce an asexual triploid (SUOMALAINEN *et al.* 1987); in this case there may be either one or two different coalescents for the three alleles in the triploid, depending on the relationship of the sexual species that fertilized the asexual species to the sexual ancestor(s) of that species.

Selection and ASD: Selection at linked sites does not affect neutral ASD even though all genes in an asexual species are effectively linked to each other. Selection for advantageous or detrimental mutations does reduce the population diversity due to hitchhiking, more strongly in asexual than in sexual organisms. However, it will not affect the accumulation of ASD due to strictly neutral substitutions. To see this for the case of directional selection, consider a selected site with three different genotypes, S/S, S/s, and s/s. These three genotypes have the same expected neutral heterozygosity at other sites, *i.e.*, the same neutral ASD, so selection for one genotype will not change the expected neutral ASD for a randomly chosen individual of the species. Overdominance (selection for heterozygotes) keeps two alleles in a population, preventing either one from being fixed; as a result it increases heterozygosity at the overdominant site and and at closely linked sites in sexual species. This phenomenon has no real equivalent in asexual species because the two alleles of a gene in a single individual or lineage are linked to each other as

if they were on the same chromosome. Selection for the heterozygote S/s will cause the S/s clone to replace the corresponding homozygotes S/S and s/s; it is analogous to directional selection, not balancing selection, in sexual species.

In addition to neutral mutations, detrimental mutations can accumulate in an asexual lineage by chance fixation (MULLER's ratchet) or because they are recessive. This accumulation will be limited by natural selection, *i.e.* by the extinction of lineages with many detrimental alleles (LYNCH *et al.* 1993; KONDRASHOV 1994). But before this happens, significant numbers of recessive detrimental mutations might accumulate in some lineages. The resulting nonneutral ASD may be reduced when the recessive mutations are brought to expression and exposed to selection because they are made homozygous by convergence events or by the occurrence of mutations in both alleles of a gene. If a gene is not subject to dosage limitations, *i.e.,* if the organism can function with only one copy (or fewer than P copies in a P-ploid organism), then null mutations will inactivate the copies that are not needed. Even if this is slightly detrimental, a lineage with inactive copies could still be fixed in a species by chance. Inactive alleles may not be detectable by allozyme analysis, which could cause the apparent heterozygosity detected by allozymes to increase and then decrease as the age of an asexual lineage increases (SUOMALAINEN *et al.* 1987).

Automictic reproduction: The preceding treatment is for apomictic parthenogenesis and other forms of asexual reproduction involving only equational (mitotic) divisions. Another form of asexual reproduction is automixis, which involves a reductional division (*e.g.,* the first meiotic division), after which diploidy is restored in various ways. There are a number of different mechanisms, but they fall into three different classes with respect to their genetic consequences (ASHER 1970).

1. Heterozygosity is lost completely at every division (*i.e.,* ASD is reduced to 0). This is the case with organisms reproducing by automictic parthenogenesis, in which a normal meiosis produces a haploid nucleus, followed by fusion of the products of the subsequent mitosis to restore diploidy. An example is autogamy in Paramecium.
2. Heterozygosity is lost unless there is a cross-over between the gene and centromere, so the organism may be heterozygous only distal to the centromere or if crossing-over is very frequent. This happens when diploidy is restored by fusion of the products of the second meiotic division, provided that the fusing nuclei came from the same product of the first meiotic division; or it can happen when the second meiotic division is suppressed.
3. Heterozygosity is retained unless there is a cross-over between the gene and centromere; heterozygosity will be greater proximal to the centromere. This happens when diploidy is restored by fusion of the products of the second meiotic division, provided that the fusing nuclei came from different products of the first meiotic division (central fusion).

Overall, ASD in automicts is likely to be greatly reduced compared to that in apomicts, unless it is exclusively by central fusion or a similar mechanism *and* crossing-over is suppressed. Automixis will not be considered further in this paper.

Experimental evidence for ASD: The bdelloid rotifers are highly successful freshwater invertebrates that reproduce by apomictic parthenogenesis; no males have been found. This group is believed to be an ancient asexual lineage derived from the monogonont rotifers, which are diploid animals that alternate between apomictic parthenogenesis and sexual reproduction (WALLACE and SNELL 1991). MATTHEW MESELSON and DAVID WELCH (personal communication) sequenced both alleles of several protein-coding genes from two species of bdelloids, finding >30% allele sequence divergence in third codon positions and allele-dependent trees as shown in Figure 1. These data provide strong confirmation for long-term obligate asexual reproduction in bdelloids. Candida is a diverse group of organisms defined as yeasts for which no sexual reproduction has been detected. Clonal reproduction in *C. albicans* has been verified by population genetic analyses of allozyme data (PUJOL *et al.* 1993). OHKUMA *et al.* (1995) identified 13 different copies of the *CYP52* gene in *C. maltosa.* Some of these were identified as alleles on the basis of chromosome location; nucleotide divergence between alleles was 2–5% in the third codon position, which is substantially higher than in sexual species where it is usually <1%. Similar investigations have been initiated on another asexual eukaryote, the amoeba *Acanthamoeba castellanii* (R. RUMPF and C. W. BIRKY, JR., unpublished results). In strain Neff of *A. castellanii,* which is believed to be polyploid (BYERS *et al.* 1990), preliminary evidence suggests that the gene coding for transcription factor IID exists in at least twelve alleles with up to 2.5% sequence divergence (synonymous and nonsynonymous). Electrophoretic studies suggest that *Acanthamoeba* is heterozygous at many, but not all, loci coding for enzymes (DE JONCKHEERE 1983; COSTAS and GRIFFITHS 1984; BYERS *et al.* 1990); similar results were obtained for asexual oribatid mites (PALMER and NORTON 1992).

ALLELE SEQUENCE CONVERGENCE

Returning to apomixis, we saw that ASD will increase during asexual reproduction by apomixis and that phylogenetic trees produced by sequencing a single allele from two or more asexual species will often fail to represent the species tree. But there are several plausible mechanisms by which that sequence divergence can be reduced or eliminated entirely. If these events are suffi-

ciently frequent, the asexual organism may appear to be essentially haploid. At the other extreme, if they are infrequent they can be identified with appropriate sequence data and will allow extensive allele sequence divergence, but will usually make it impossible to produce correct phylogenetic trees from sequence data.

Mitotic recombination: If a crossover occurs between homologous chromosomes in G2 of the cell cycle, all loci distal to the crossover will be homozygous after the next mitotic division, with a probability of $^1/_2$. Frequent mitotic crossing-over in a single asexual lineage will result in a gradient of decreasing ASD distal from the centromere. If mitotic gene conversion occurs between homologous chromosomes in G1 of the cell cycle, the region included in the conversion tract will become homozygous, unless the conversion is reciprocal. If conversion occurs in G2, half of the progeny will be homozygous whether the conversion is reciprocal or nonreciprocal. Gene conversion in a single asexual lineage will result in patches with different levels of ASD. Patches will be less frequent in regions of reduced recombination, *e.g.,* near the centromere, but there will be no overall distal gradient. If conversion is infrequent, the patches will usually be smaller than a gene because gene conversion tracts are smaller than most genes (HILLIKER *et al.* 1994) and could be recognized in gene sequences.

The many copies of ribosomal RNA genes and some other tandemly repeated sequences are highly homogeneous within a single chromosome in sexual organisms, although they may be highly variable in different species and individuals (LI and GRAUR 1991). This concerted evolution of the repeats is due to unequal crossing-over and gene conversion, which appear to occur at high frequency during mitotic as well as meiotic divisions. The ribosomal RNA genes are hot-spots for mitotic recombination (THOMAS and ROTHSTEIN 1991), so concerted evolution of these genes may be possible even when other genes show high levels of ASD. In asexual organisms, crossing-over and gene conversion could maintain the homogeneity of ribosomal RNA genes on the same or different chromosomes. Consequently, phylogenetic trees based on ribosomal RNA sequences are probably not subject to errors due to ASD and are no less reliable in asexual than in sexual species. This may not be true for organisms that are dikaryotic; if both nuclei contain ribosomal RNA genes, the genes in different nuclei can accumulate ASD unless the partitioning of nuclei at cytokinesis is reductional (R. D. ADAM, A. C. BARUCH, and C. W. BIRKY, JR., unpublished results). Mitochondria and chloroplast genes are present in many copies in each cell. Nevertheless, as a result of gene conversion, random replication, and random segregation, all the copies of an organelle gene in an individual are usually identical (BIRKY 1994). Phylogenetic trees based on organelle genes are thus not confounded by ASD and, like those based on ribosomal RNA genes, can be used to help interpret gene trees that are affected by ASD.

Rare sex: The effect of occasional sexual reproduction will be essentially the same as that of mitotic recombination. Presumably it will involve a reduction division like meiosis, followed by fusion of reduced nuclei to restore diploidy (or polyploidy). It will take place between closely-related individuals with similar alleles, or will be selfing, or both. It will only be detectable if it produces an individual that is homozygous for the gene being studied, or if it produces a mosaic gene due to recombination. Either way, the two alleles (or a segment of the alleles) in the lineages initiated by the participating cells will have a new coalescent at the time of sexual reproduction.

Ploidy cycle: A ploidy cycle consists of a decrease in ploidy followed by an increase; a variety of mechanisms have been described (KONDRASHOV 1994). Ploidy might decrease and increase by one-step processes involving successive halving and doubling of chromosome number. ASD would be reduced at a similar rate at all loci. Alternatively, ploidy could be decreased and increased again *via* aneuploid intermediates resulting from nondisjunction. In this case ASD would be lost at different times in different chromosomes. The loss of a chromosome is usually lethal in diploid animals and plants, but not in polyploids, and not in diploid fungi.

Consequences of mitotic recombination and ploidy cycles for phylogenetic trees: Mitotic gene conversion, crossing-over, and ploidy cycles affect parts of genes, chromosome segments, or whole chromosomes, respectively. But they have the same basic consequences for the regions they affect: the sequence on one chromosome replaces the homologous sequence on other chromosomes. This has the effect of producing a new coalescent at the time of the event and erasing all traces of previous ASD from the lineage. Convergence events on basal branches (event *a* in the species tree of Figure 4) do not affect the topology of the gene tree but reduce the time to the coalescent of the two sub-trees. Convergence events on internal (*b*) or terminal branches (*c*–*f*) can change the topology of the gene tree and the likelihood of recovering the correct species tree from sequences of one allele from each species. If there are multiple events on the same branch, *e.g. d* and *e,* the first event determines tree topology but the last event determines the coalescent of the affected genes. One combination of terminal events (*c, e, f*) produces a gene tree with the same topology as the species tree, so that sequencing any combination of one allele from each species will give the correct species tree. Some convergence events (or combinations of events) increase the number of combinations of allele sequences that give the correct species tree. After the internal event *b,* for example, four combinations of alleles give the correct species tree (1, 3, 4; 1, 3, 6; 1, 5, 4; 1, 5, 6), compared with two in the absence of convergence. The remaining four combinations of se-

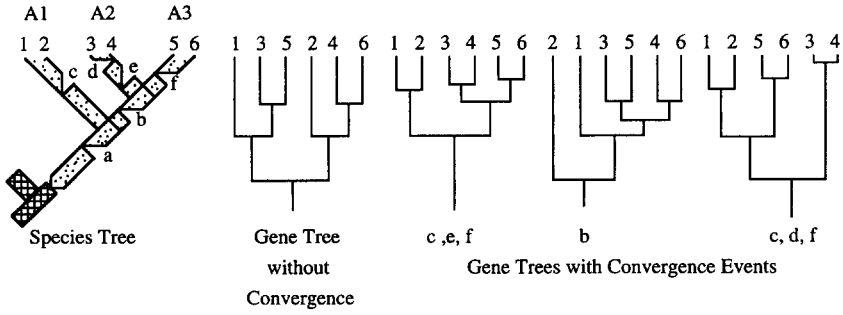

FIGURE 4.—Effects of gene conversion, crossing-over, or ploidy cycles on allele sequence divergence. The species tree is the same as that of Figure 2, but with allelic convergence events (gene conversion, crossing-over, or a ploidy cycle) occurring at six different points, *a–f*, on the tree. For example, event *c* replaces allele 2 with a copy of allele 1. Each event is assumed to affect the entire gene being studied.

quences (2, 3, 4; 2, 3, 6; 2, 5, 4; 2, 5, 6) give species trees that show the correct cladistic relationships of the species but overestimate the time to species divergence. After some other convergence events (*e.g., c, d, f*), the correct species tree topology cannot be recovered from gene sequences. Of course any combination of convergence events will reduce ASD in some or all species or clones.

Even when convergence makes it difficult or impossible to deduce the correct species tree from sequences of one allele per species, it may be possible to deduce the occurrence of asexual reproduction and convergence if (1) all alleles of a gene are sequenced and (2) the species tree is already known from morphological data or from sequences of organelle genes and ribosomal RNA genes that are not affected by ASD. Sequence data from several different loci may also be useful if they have not all been affected by the same convergence events.

The frequency of convergence events may be limited: It is difficult to calculate the expected level of ASD for two reasons. First, it must depend on the relative frequencies of mutation and convergence, and while the mutation rate can be estimated from evolutionary data, there are no data on the frequencies of mitotic crossing-over, gene conversion, or ploidy cycles in most organisms. Second, even if all these parameters were known, complex interactions between mutation, mitotic recombination, and ploidy reduction make it impossible to calculate the frequency of conversion events without additional theory which is in preparation. A complete theoretical treatment of these interactions is in progress (C. W. BIRKY, JR. and J. B. WALSH, unpublished results); briefly, they are as follows:

1. Mutation causes allele sequences to diverge, which in turn reduces the sequence homology that is essential for recombination. Thus as sequence divergence between homologous sequences increases, the frequency of mitotic crossing-over and gene conversion decrease rapidly and sequence convergence due to these events becomes increasingly unlikely, and eventually impossible (WALSH 1987).
2. Mitotic crossing-over is self-limiting because it leads to chromosome rearrangements which act as crossover suppressors (GARCIA-BELLIDO and WANDOSELL 1978; GOLIC and GOLIC 1996; C. W. BIRKY, JR., unpublished results). These include inversions, reciprocal translocations, and nonduplicative transposition.
3. Mitotic crossing-over also limits ploidy reduction or ploidy cycles when it produces cells that are heterozygous for chromosome rearrangements. If a diploid cell that is heterozygous for a deletion, interchromosomal transposition, or reciprocal translocation undergoes ploidy reduction, some of the resulting cells will be homozygous for a deletion and will be inviable if the deletion contains at least one essential gene or other sequence.

Some kinds of selection will also favor the maintenance of heterozygosity. Consider a cell that is heterozygous for a recessive detrimental mutation and a pair of neutral alleles: *D N1/d N2*. Mitotic crossing-over proximal to the site of the detrimental mutation will produce the following genotypes: $^1/_2$ *D N1/d N2*, $^1/_4$ *D N1/D N1*, and $^1/_4$ *d N2/d N2*. Selection favors the first two genotypes and consequently favors heterozygosity at the neutral site. Alternatively, suppose that there is overdominance at a locus such that the heterozygote genotype *H/h* is favored over either homozygote *H/H* or *h/h*. This kind of selection will favor the mode of segregation that maintains heterozygosity at the *h* locus and also at any linked loci where neutral alleles are segregating.

Experimental evidence for convergence: No sexual reproduction has been observed in the parasitic diplomonad *Giardia lamblia*. Moreover, a population genetic analysis of allozyme data by TIBAYRENC *et al.* (1991) showed that Giardia meets all four of their criteria for lack of recombination: overrepresented, widespread identical genotypes; absence of recombinant genotypes; linkage disequilibrium; and correlation between independent sets of genetic markers. Giardia may have been asexual for a very long time; sequences of the gene encoding the small-subunit rRNA show about 36% sequence divergence between Giardia and another asexual diplomonad, *Hexamita* (LEIPE *et al.* 1993; VAN KEULEN *et al.* 1993), while two strains of *G. lamblia* show 9% amino acid substitutions in the *tim* gene (MOWATT *et al.* 1994 and GenBank Accession no. L02116). Nevertheless, both allozyme and sequence data show that there is much less ASD than would be expected in the

absence of convergence (R. D. ADAM, A. C. BARUCH and C. W. BIRKY, JR., unpublished data).

The amount of ASD in the asexual yeast Candida appears to be significantly lower than expected in the absence of convergence. *C. albicans* and seven other species form a clade that probably represents a monophyletic loss of sexual reproduction (BARNS *et al.* 1991; OHKUMA *et al.* 1993a). Two different protein coding genes show 15–16% amino acid substitutions between pairs of species in this clade (KAWAI *et al.* 1992; OHKUMA *et al.* 1993b), which suggests that the lineage has been asexual long enough to have accumulated >15% synonymous sequence divergence between alleles. However, OHKUMA *et al.* (1995) found a maximum of 5% ASD in the third codon position between alleles of a *CYP52* gene in *C. maltosa.* Moreover, the percentage of loci that are heterozygous for allozymes ranges from 10 to 37% in three studies (LEHMAN *et al.* 1989; CAUGANT and SANDVEN 1993; PUJOL *et al.* 1993), which is much lower than expected if ASD were not limited by convergence. Overall, the Candida data suggest that the amount of ASD may be highly variable among loci, perhaps indicating infrequent convergence due to mitotic recombination. This may also be true for bdelloid rotifers, in which an RNA polymerase gene shows significantly lower ASD than does a heat shock protein gene (M. WELCH and M. MESELSON, personal communication).

HOMOLOGUE STRUCTURE DIVERGENCE

It has been suggested that chromosomes might become heterozygous for rearrangements in the absence of pairing constraints enforced by meiosis (WHITE 1973). Heterozygosity for chromosome structure is called chromosome heteromorphy; I will refer to the accumulation of such changes within a cell or individual as homologue structure divergence or HSD. In sexual species, HSD is kept low, primarily by random drift and inbreeding. In addition, chromosome rearrangements that put genes in new chromosomal environments sometimes change their expression, for example by separating the gene from its normal control elements; this will often be detrimental and the clone containing the rearrangement will be eliminated by selection. In asexual species, intracellular HSD is not limited by random drift. It can be reduced by the same processes that reduce ASD, *i.e.,* natural selection, mitotic crossing-over, gene conversion (very small rearrangements only), or ploidy cycles. The rate of accumulation of structural differences both within and between individuals may also be lower for asexual than sexual species because many chromosome rearrangements are due to crossing-over between repeated sequences, which are often due to transposable elements. Asexual reproduction reduces the spread of such elements, possibly to the point where mutations destroy the repeats faster than new ones are produced (HICKEY 1982). Rearrangements due to unequal crossing-over between homologous chromosomes may be limited by sequence divergence between the chromosomes, which will eliminate some of the sequence homology required to initiate recombination.

Evidence for high levels of intracellular HSD in asexual organisms comes from cytological studies of bdelloid rotifers (HSU 1956a,b; PAGANI *et al.* 1993) and aphids (BLACKMAN 1980), and from molecular mapping of the chromosomes of Giardia (ADAM 1992; LE BLANCQ *et al.* 1992), *Candida albicans* (THRUSH-BINGHAM and GORMAN 1992; CHU *et al.* 1993), and *C. maltosa* (OHKUMA *et al.* 1995). In theory, continued HSD might lead to complete loss of recognizable pairs of homologues. In two aphid species, homologues cannot be recognized in Giemsa-stained squashes (BLACKMAN 1980). However, homologous *C. albicans* chromosomes can be recognized by detailed molecular mapping (THRUSH-BINGHAM and GORMAN 1992; CHU *et al.* 1993).

ALLELE SEQUENCE DIVERGENCE CAN BE USED TO VERIFY LONG-TERM ASEXUAL REPRODUCTION

There is strong evidence for asexual reproduction in some animals and plants, in which virgin females can be isolated and tested for parthenogenetic reproduction, detailed cytological studies can show that all eggs are produced by equational maturation divisions, or the absence of males from a population can be demonstrated by exhaustive field studies (JUDSON and NORMARK 1996). It is more difficult to obtain convincing evidence of asexual reproduction in small invertebrates, fungi, algae, protozoa, and other eukaryotic microorganisms. The failure to detect sexual reproduction is not definitive, because sexual reproduction may be (1) cryptic, having been seen but not recognized as such; (2) furtive, occurring only under circumstances not yet detected in nature or fulfilled in the laboratory; or (3) rare, occurring so infrequently as to be undetected but still sufficiently frequently to have a significant effect on population genetics and evolution. Hemoflagellates were long believed to be asexual, but genetic experiments demonstrated sexual reproduction by showing that when a host is simultaneously infected with two different genotypes of a hemoflagellate, cells with recombinant genotypes can be recovered subsequently (GLASSBERG *et al.* 1985; JENNI *et al.* 1986). Even if direct observation clearly demonstrated that a particular species is reproducing asexually now, it would not tell how long the lineage has been asexual. Biologists are increasingly using molecular evidence to identify lineages with clonal reproduction (*i.e.,* asexual or selfing) (*e.g.,* TIBAYRENC *et al.* 1991; PUJOL *et al.* 1993) and to estimate their ages (AVISE 1994). Allele sequence divergence can be used to solve both problems at once, provided that convergence events do not interfere. High ASD and allele-dependent tree structure are strong evidence for asexual reproduction when they are found in several

different genes. The age of the asexual lineage can be estimated from the amount of divergence and the substitution rate (if the latter is known); this will be a minimum estimate if convergence has occurred. Finding homologue structural divergence by cytogenetic or molecular mapping methods would be strong confirmation of long-term asexual reproduction.

In some cases, it will not be possible to demonstrate allele-dependent tree structure, either because of convergence events on terminal branches of the phylogenetic tree or because the asexual lineages being studied are too closely related. High ASD in a single lineage would still be good evidence for long-term asexual reproduction if two conditions are met. First, it is necessary to rule out the possibility that the organism is sexual with high heterozygosity due to balancing selection. Fortunately, genes with very ancient coalescents due to balancing selection appear to be in the minority and balancing selection may affect only a portion of a gene (KREITMAN 1991). Consequently, balancing selection in a sexual species can probably be distinguished from sequence divergence in an asexual species by sequencing completely both alleles of several different genes in a clone. Second, the observed ASD must be much higher than is likely for a sexual species. In a random-mating population, the expected divergence between two alleles in an individual is equal to the nucleotide diversity (π). Nucleotide diversity has been determined for only a few organisms, and is unlikely to be known for a sister group of most asexual organisms. The largest values of π of which I am aware are in *Drosophila simulans,* in which the range for 12 loci is 0–0.07 and the average is 0.03 for synonymous sites (MORIYAMA and POWELL 1996). This suggests that finding ASD values of $\geq$0.10 at several loci would be good evidence for long-term asexual reproduction.

There are several potential problems with the identification of asexual lineages by sequencing alleles:

1. Finding low allele sequence divergence in a gene does not necessarily mean that the species is sexual, because the gene may have been affected by a recent convergence event. Such events can be detected if they are infrequent by sequencing many different genes (or all alleles of a smaller number of different genes in a polyploid). Gene conversion will generally affect segments of genes; crossing-over affects only genes distal to the crossover; and ploidy cycles affect all the genes on the chromosomes that underwent ploidy reduction, and only those. But if any of these events are frequent enough, they can cause allele sequence divergence in an asexual species to be reduced to the level found in sexual species and make it impractical to detect allele-dependent tree structure.
2. The deletion of one allele of a gene will eliminate ASD for that gene, while loss of an entire chromosome will do the same for a large number of genes. A mutation that causes loss of expression of a gene will eliminate heterozygosity detected by enzyme electrophoresis but the allele and ASD will still be detected by sequencing. There is some evidence for each of these events in asexual polyploids (SUOMALAINEN *et al.* 1987; SOLTIS and SOLTIS 1993; DUFRESNE and HEBERT 1994).
3. Extensive outbreeding causes the observed heterozygosity, and allele sequence divergence, to exceed $4N_e u$, mimicing asexual reproduction. This will not affect phylogenetic trees and can be distinguished from high ASD by sequencing both alleles in two or more related species.
4. The trees shown in Figures 1 and 2 could also be produced by gene duplications in sexual or asexual species. Alleles can be distinguished from duplicate genes by showing that they occupy the same positions on homologous chromosomes, using molecular mapping or comparing the sequence of flanking regions (*e.g.,* OHKUMA *et al.* 1995). This could be confounded by chromosome rearrangements that might accumulate. Duplicate genes may produce functionally different proteins, and this may be detectable from their sequence or by gene disruption experiments (OHKUMA *et al.* 1995). Alleles could be defined historically in asexual species by using phylogenetic analysis to demonstrate their origin from alleles, as opposed to gene duplications, in a sexual ancestor (B. NORMARK, personal communication). If ASD is found in a number of different genes that are present in single copies in related organisms, it is unlikely that all were duplicated. If the number of different alleles is greater than the ploidy of the organism, then some must have arisen by duplication.
5. Sexual species that arise by hybridization of distantly related species might be expected to have high ASD of all genes. These will usually be allopolyploids that could be recognized by cytogenetic studies or by the similarity of their alleles to those in the parent species. In microorganisms where this information is not available, sexual allopolyploids might be identified because sexual reproduction will keep alleles on homologous chromosomes similar.

CONCLUSIONS

Asexual eukaryotic diploids and polyploids show allele sequence divergence, sequence convergence, and karyotype divergence. These interesting phenomena are potentially important for a number of reasons:

1. If convergence events are sufficiently rare, diploid or polyploid organisms that have been reproducing asexually for a long time can be identified by high allele sequence divergence and by unique features of phylogenetic trees that are based on the sequences of all alleles of several genes. When convergence

events are frequent enough to affect some but not all genes or lineages, this technique may give ambiguous results by itself but may be a useful adjunct to population genetic and phylogenetic methods.

2. In organisms suspected of reproducing strictly asexually for a long time, phylogenetic trees should not be based on sequences, restriction analysis, or allozyme analysis of protein-coding genes or other regions unless the (asexual) genetics of the organism is well understood and it is known that allele sequence divergence is negligible due to frequent convergence events. It would be possible to deduce the correct tree topology if all alleles of the gene were sequenced and resulting gene tree structure was as shown in Figure 2, in which each subtree reflects the correct species tree.
3. The long-term survival of asexual lineages may depend on the extent to which they accumulate a load of detrimental mutations (KONDRASHOV 1994). Recessive detrimentals might accumulate in diploids or polyploids and only be brought to full expression when there are sequence convergence events. We will not fully understand why so many organisms reproduce sexually, and why some do not, until we know more about the interplay between sequence divergence due to mutation and convergence due to recombination and ploidy reduction.

There is some preliminary evidence for allele sequence divergence, sequence convergence, and karyotype divergence in a few organisms. However, the analysis of these cases has only begun, and most asexual species have not been studied at all. Until we know much more about the extent of these phenomena and their interactions, we will not understand either the genetics or the evolution of asexual organisms.

Finally, it should be apparent that polymorphism in an asexual species is not directly comparable to that in sexual species: it may be inflated by mutations that have accumulated in different alleles since the loss of sexual reproduction (by a factor that approaches tu when t is large) or the last convergence event, and reduced by periodic selection (C. W. BIRKY, JR. and J. BRUCE WALSH, unpublished results).

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