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Review

The biological improbability of a clone

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Summary

Emperical evidence for *intra*clonal genetic variation is described here for clonal systems using a variety of molecular techniques and implicating a diversity of mechanisms. However, clonal systems are still generally perceived as having strict genetic fidelity. As concepts of genetic variability move from primary sequence data to include epigenetic and structural influences on genetic expression, the ability to detect changes in the genome at short intervals allows precedence to be given to inherent biological variation that is often analytically ignored. Therefore, the advent of powerful molecular techniques, like genome mapping, mean that our concepts of genetic fidelity within eukaryotic clones and the whole philosophy of the 'clone' needs to be re-evaluated and re-defined to replace old unproven dogma in this aspect of science.

Introduction

A dichotomy exists in the perception of what a clone is. To some it is merely a *member of an asexual lineage within a population*. To others, it is associated with strict *genetic fidelity between members of the asexual lineage* (Abercrombie *et al*., 1990). It is often argued that the whole issue is one of semantics. However, since a clone is a scientific phenomenon, it is important that definitions are precise. The proposition in this article is that a clone is a biological improbability in terms of exact genetic fidelity between other members of that clone. Such lineages are typically produced vertically, i.e. between generations as in aphids, nematodes etc., or horizontally, i.e. within a generation. Examples of the latter are found in certain polyembryonic insects such as the Hymenopteran parasitic wasp, *Copidosoma floridanum* which produces ~ 2000 embryos (Grbic *et al*., 1998), and mammals such as armadillos which produce up to 12 'identical' siblings (Nowak, 1991), derived in both cases respectively from a single egg.

Prior to the advent of molecular markers in the 1960s, in the first instance protein markers, and more recently a plethora of DNA markers, testing of clonality in terms of genetic fidelity was solely based on morphological characters. As the use of molecular markers became widespread they were extensively used for population genetic studies and began to reveal the complexity of populations, i.e. genetic variability. Sometimes these approaches showed unexpected genetic variation in species (e.g. Hubby & Lewontin, 1966; Lewontin & Hubby, 1966), including those that had hitherto been assumed to be homogenous, e.g. asexual systems (Hebert *et al*., 1972). Some of this heterogeneity was of course believed to have adaptive significance.

Molecular Markers

Before discussing *inter*- and *intra*clonal variation, some brief mention of the molecular markers used in the study of these phenomena is pertinent here. The nature and application of the molecular markers used in variability studies, including DNA sequencing, has been extensively reviewed, notably for insects (e.g. by Hoy, 1994, Crampton *et al*., 1996; Loxdale *et al*., 1996; Loxdale & Lushai, 1998, 1999). Recent discussion of mtDNA, RAPDs, microsatellites, AFLPs and SSCP markers in insects and other organisms is given by Zhang & Hewitt (1997), Harry *et al*. (1998),

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Goldstein & Schlötterer, 1999, Vos *et al*. (1995) and Sunnucks *et al*. (2000), respectively. Tu (2001) discusses the potential use of transposons in population genetic studies and Black *et al*. (2001) review the important topic of population genomics, all aspects that are pertinent to this article.

Some species, e.g. aphids, are also known to show a significantly greater number of genotypes as a function of the genetic resolving power of the molecular marker used. In ascending order, this degree of resolution may be generally taken as beginning with whole chromosomes, then protein markers through to DNA markers, namely karyotyping, allozymes (mainly), random amplified polymorphic DNA (RAPDs), restriction fragment length polymorphisms (RFLPs), minisatellites, microsatellites, amplified fragment length polymorphisms (AFLPs) and DNA sequencing. For example, in the peach-potato aphid, *Myzus persicae*, the number of clones (genotypes) detected has increased by an order of magnitude using ribosomal DNA (rDNA) intergenic spacer (IGS) markers (Fenton *et al*., 1998*a*) compared with that found using allozymes 10 years before (Brookes & Loxdale, 1987).

Inter and *intra*clonal variation

A brief overview of *interclonal* variation (clonal lines of different genetic origin) observed across taxa is detailed here. In nature, there are numerous clonal or largely asexual animals and plants (Gill *et al*., 1995 and references therein). Recently studied examples include: cottonwoods, *Populus salicaceae* (Kearsley & Whitham 1998); dandelions, *Taraxacum officinale* (King & Schaal, 1990); grasses, e.g. clonal prairie grass, *Spartina pectinata* (Davelos *et al*., 1996) and Red mangrove trees, *Rhizophora mangle* (Klekowski, 1998). Parallels in the animal kingdom include marine bryozoa (Harvell, 1998); soft coral, *Alcyonium rudyi* (McFadden, 1997); freshwater snails, *Potamopyrgus antipodarum* (Fox *et al*., 1996); fish, *Poecilia formosa* and *Rivulus marmoratus* (Turner *et al*., 1990; 1992) and aphids, e.g. grain aphid, *Sitobion avenae* (Sunnucks *et al*., 1997; Simon *et al*., 1999) and the rose-grain aphid, *Metopolophium dirhodum* (Nicol *et al*., 1997). Irrespective of taxa, all these organisms show abundant clonal diversity (genetically typed variants) in the ecosystems that they inhabit.

In the same way that molecular markers have been shown to be useful for interclonal studies, these markers also detect *intraclonal* variation, genetic variation within an asexual lineage, even in the case of apomicts where no recombination is assumed. Since these markers likewise show increased resolution, so they will prove useful in elucidating and then defining the nature of a 'genotype'. The focus of this article is exactly this point, but with reference to genetic fidelity in a clonal lineage.

Rapid intraclonal changes have also been detected in the 'dynamic genome': phages, prokaryotes, and the mitochondrial and nuclear genomes of eukaryotes (Table 1a, b). As seen in the table, this variation is generated by a multitude of molecular mechanisms, including conventional DNA point mutations and errors of replication, slippage-mediated changes in hypervariable non-coding regions such as mini- and microsatellites, and insertion and deletion of segments of DNA, including inversion polymorphisms, some of which are governed by transposons, e.g. mariner elements (Hartl *et al*., 1997). It is now known that various mutase enzyme mechanisms are involved in such changes, certainly affecting prokaryotes and perhaps so-called 'hot spots' in the eukaryotic genome (Radman, 1999). Commonly observed in cells and plant studies, somatic mutations also contribute to changes in phenotype (e.g. leaf colour in higher plants Klekowski, 1998). In addition to these local changes of the genome, there is also the category of gross chromosomal changes, e.g. karyotype number, translocation, etc. (Blackman *et al*., 2000). Additionally still to these 'general' mechanisms, chimerism has also been observed in some colonial animals, e.g. ascidian zooids (Sommerfeldt & Bishop, 1999), whilst horizontal gene transfer of operational genes appears to occur occasionally in both pro- and eukaryotes (Jain *et al*., 1999).

Besides viruses and prokaryotes, which are well known as displaying rapid evolutionary changes, much recent intraclonal work on eukaryotes has concerned ciliates, crustaceans (e.g. especially *Daphnia*), bdelloid rotifers and aphids. Certainly, apomictic parthenogenetic aphids are one of the best-documented taxa, although even here, work is as yet limited. Aphids are amongst several groups of organisms that display a range of life cycle strategies within the same species, that is they have 'multiple-generation complex lifecycles' (Moran, 1994). Some species appear to be totally obligate asexuals, with about 20 asexual generations *per annum*, whilst others have an alternating asexual-sexual strategy with *c*. 14 parthenogenetic generations during the spring–summer field season, followed by a sexual phase. In the parthenogenetically produced offspring, each clone is *assumed* to be a genetic replica of the original parthenogenetic founder (Blackman, 1979, 1981, 2000; Dixon, 1998; Hodgson, 2001). However, recent investigations have thrown doubt on this definition by indicating the presence of intraclonal variation. De Barro *et al*. (1994), applied a synthetic oligonucleotide DNA probe $(GATA)_4$, and found DNA- fingerprint differences (the appearance of a new band variant) in the 12th generation of a laboratory clone of *S. avenae*. Using RAPD markers, Lushai *et al*. (1998) demon-

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strated a mutational change in the germ line of a clonal lineage after 14 generations. The study revealed 14 mutations in total over the course of 32 generations (number of individuals tested = 344), 13 being somatic (cell line) mutations. Note that in both these studies, clonal hygiene was monitored against contamination by other aphid strains using RAPDs. Most recently, Forneck *et al.* (2001*a*, *b*) using AFLP markers in an attempt to show genetic differences within and among lineages on different hosts (natal and novel grape root stocks) over several generations, have revealed interclonal as well as intraclonal variation in the gall forming grape aphid phylloxera, *Daktulosphaira vitifoliae*. Thus for example, in one study (Forneck *et al.*, 2001*b*), genetic variability was low among 40 individuals from the fifth (G_5) generation of four independent parthenogenetic lineages reared on different hosts, including the original natal host, whilst one lineage tested showed intraclonal differences. In this insect group, in which chromosomes appear to be quickly evolving at the molecular organisational level (Sunnucks *et al.*, 1996; Wilson *et al.*, 1999; Blackman *et al.*, 2000), presumably the use of additional molecular markers of greater resolution would, as suggested earlier, reveal DNA mutations at generations earlier than the 5–14 quoted above.

It is also possible that aphids and other cyclical parthenogens show rapid changes in the genome over a short relative time frame due to transposable elements (however, in some exceptional cases this trend is significantly different, *cf.* Arkhipova & Meselson 2000). Such elements are known to occur in the *Drosophila* genome with transposition rates in the range 10^{-5} to 10^{-2} (Nuzhdin *et al.*, 1996). More recently, other families of miniature inverted repeat transposable elements (e.g. MITEs) have been found to be widespread (40–10^4 copies) in the genomes of mosquitoes of several genera (Tu, 2001). It has also been recently shown that inversion break points in *Drosophila buzzatii* are related to transposable element 'hotspots' along the genome (Cáceres *et al.*, 2000). It appears that natural selection directly affects inversion length polymorphism, the 'most extensive evidence [being] in favour of the notion that the adaptive value of inversions stems from their effect on recombination' (Cáceres *et al.*, 1999). Lastly, it is now well established that various cyclical eukaryotic protozoans have several mechanisms of large-scale nuclear genomic re-arrangements, possibly resulting from the action of transposons (e.g. Yao, 1996), and which are likely to have adaptive consequences.

Besides these direct structural changes to the genome leading to intraclonal variation, sometimes rapidly produced variation arises due to epigenetic influences (Wolfe & Matzke, 1999), which affect the expression of DNA in clonal lineages, although as far as is known, the DNA sequence *per se* is not altered. This could perhaps be described as a form of 'near-Lamarckism', since environmental 'feed-back' governs gene expression. An excellent example of this is found in Aphids. For the past 30 years or so, extensive application of pesticides in both greenhouses and the field has selected for resistance to these compounds. In Britain, the peach-potato aphid, *Myzus persicae*, one of four resistant species of aphid in the UK, now displays a range of resistance genotypes, designated 'S' (susceptible) to R_3. In the highly resistant genotypes (R_2 and above), the conferment of resistance is related to an autosomal (A1, 3) translocation, whilst many genotypes are also cross resistant to different classes of chemicals e.g. primarily carbamates and organophosphates. The basic mechanism of resistance is related to amplification of two closely-related carboxylesterases, E4 and FE4. The copy number of the genes coding for these enzymes increases with increasing resistance to pesticides (Field *et al.*, 1996, Field *et al.* 1999), although other resistance mechanisms such as '*kdr*' (knockdown resistance) and MACE (modified acetylcholinesterase) are also known (Devonshire *et al.*, 1998; Foster *et al.*, 2000).

Of interest is the fact that in the absence of high pesticide selective pressure, the highly resistant forms spontaneously revert to lower levels of resistance, although the copy number of E4/FE4 genes is unchanged (Field *et al.*, 1999). The loss of resistance (but not its recovery on re-application of pesticide pressure) is known to be controlled by methylation of the DNA (Field *et al.*, 1989; Field, 2000; Hick *et al.*, 1996). These highly resistant forms, however, appear to carry a fitness cost, probably associated with a pleiotropic association of E4/FE4 expression with other resistance mechanisms, especially knockdown resistance to pyrethroids. This concerns a mutation of the sodium channel gating of the nervous system (Devonshire *et al.*, 1998; Foster *et al.*, 2000). Since the highly resistant forms are less responsive to alarm pheromones, less likely to leave senescent leaves and may be less aerially mobile (Foster *et al.*, 1996), i.e. less likely to produce winged forms, they tend to decline in frequency in the field over the winter months in the UK (Foster *et al.*, 1996). Hence, a clone is seen to change with time, showing yet again, that genetically, here in a phenotypic sense, they are unstable and adapt and evolve in accordance with environmental selection.

All these genomic changes or indicators of genomic expression are testament to the inherent variability of clones. Although difficult to verify, it can be assumed that all such mutations and related phenotypic expressions are under selection e.g. Müller's ratchet (Müller, 1964) and the accumulation of *deleterious* mutations must continually be purged from the environment over time (see Charlesworth &

Table 1. (a) *Studies indicating intraclonal genetic variation and* (b) *rates of genetic change* (*per gene per generation*) *for various genomes*

(a)		
Phage		
Whole genome sequence	Between 12 and 26 substitutions occurred in each of the 9 genome lines traced, i.e. 119 substitutions at 68 nucleotide sites. A deletion event involving 27 bases occurred in one lineage	Bull *et al*., 1997 (see also Yin, 1993; Hillis *et al*., 1994; Cunningham *et al*., 1997)
Escherichia coli		
IS-elements and 1 kb sequencing	1500 days of 'evolutionary' time traced to detect genetic changes around 2 K generations	Papadopoulos *et al*., 1999
Daktulosphaira vitifoliae (aphid)		
AFLPs	Mutant bands after 2–5 generations. ($n = 40$)	Forneck *et al*., 2001*a*, *b*
Megoura viciae (aphid)		
NORs	Intraclonal and intra-individual NOR heteromorphism	Mandrioli *et al*., 1999
Myzus persicae (aphid)		
rDNA	Variation within individuals, indicating two ITS haplotypes	Fenton *et al*., 1988*b*
Rhopalosiphum padi (aphid)		
RAPDs	Band variation between phenotypes of the same clone	Lushai *et al*., 1997
Sitobion avenae (aphid)		
$(GATA)_4$	5 kb. Mutant band after 12th generation.	De Barro *et al*., 1994
RAPDs	14 mutations over 32 generations, including 1 gametic mutation after the 14th generation ($n = 344$)	Lushai *et al*., 1998
	Band variation between phenotypes of the same clone (winged, wingless, and sexual forms)	Lushai *et al*., 1997
Sitobian miscanthi (aphid)		
Karyotype	Clonal karyotypic variation linked to host adaptation	Sunnucks *et al*., 1996
S. miscanthi & *S. nr. fragariae*		
Karyotype Microsatellites	Intraclonal karyotypic variation greater than microsatellite evolution	Wilson *et al*., 1999
Tramini (aphids)		
EF-1a & mtDNA	Low levels of clonal sequence divergence, indicative of possible sexual leakage in predominantly parthenogenetic lines	Normark, 1999
Daphnia pulex (water flea)		
rDNA	Both intra- and inter-chromosomal exchanges occur between rDNA arrays in the absence of meiosis.	Crease, & Lynch, 1991; Crease, 1995
Drosophila melanogaster (fruit fly)		
mtDNA	High genetic polymorphism in cytochrome-b and ATPase 6 genes within 59 lines	Rand & Kann, 1996
D. Simulans	High genetic polymorphism in cytochrome b and ATPase 6 genes within 29 lines	
Diplosoma listerianum (clonal marine ascidian)		
RAPDs	Here, chimeric colonies are composed of several genotypes	Sommerfeldt & Bishop, 1999
Gracilaria chilensis (algae)		
RAPDs	As growth occurred and biomass accumulated, intraclonal genetic changes were detected	Meneses *et al*., 1999

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Table 1 (*cont.*)

Glomus spp., *Scutellospora castanea* (asexual fungi)		
rDNA	Highly polymorphic for 18s and ITS in different nuclei within the same multinucleate fungal spore	LloydMacgilp *et al.*, 1996; Hijri *et al.*, 1999; Hosny *et al.*, 1999; Sanders *et al.*, 1995; Sanders, 1999
Horizontal gene transfer	Another mechanism responsible for operational genes in both prokaryotes and eukaryotes being moved between genomes causing genetic variability	Jain *et al.*, 1999
Somatic variation		
	Extensive examples in the plant kingdom, e.g. variegated leaves	see Gill *et al.*, 1995; Klelowski, 1998
	Mammalian micro- and minisatellite variation	Jeffreys *et al.*, 1997; Sajantila *et al.*, 1999
Epigenetic variation	e.g. methylation induced	Wolfe & Matzke, 1999
Transposable elements	Host genome variation	see Kidwell & Lisch 2000
(b)		
Coding genes, including proteins/allozymes	$\sim 10^{-9}$–10^{-5}	Dobzhansky *et al.*, 1977; Jarne & Lagoda, 1996; Kondrashov, 1998
Microsatellites	$\sim 10^{-5}$–10^{-2}	Jarne & Lagoda, 1996; Luikart & England, 1999; Goldstein & Schlötterer, 1999
Minisatellites	Up to 10^{-2}	Bruford *et al.*, 1996
Mitochondrial DNA		
insects	$\sim 10^{-4}$	Rand, 1994; Zhang & Hewitt, 1997
mammals		Parsons *et al.*, 1997
Transposons	10^{-5}–10^{-2}	Dimitri & Junakovic, 1999; Nuzhdin *et al.*, 1996

Charlesworth, 1997; Lynch & Blanchard, 1998). That is not to say that non-lethal changes do not occur and persist within the environment for short periods.

Conclusion

Certainly in nature nothing is static and all living organisms must, and are, responsive to selection pressure and evolve in order to fill novel habitats and ecological niches. Thereby this reduces interspecific competition for resources, including mates, in the case of members of the same species. Similarly, clones must also be under selective pressure and they too must undergo adaptive change, leading to a reduction of intraclonal competition for resources (this is outside the scope of the present article but we discuss it elsewhere; Loxdale *et al.*, 2002).

According to Dobzhansky (1973) 'Nothing in biology makes sense except in the light of evolution', and indeed, why should clones be any different in this respect and not evolve like everything else? [Note. *The point here is not a comparison with the evolutionary success of sexually reproducing organisms and their capacity for generation of adaptive variation.*] From what has been said, it is clear that clones do mutate by a variety of mechanisms. Sometimes this happens quickly, in other instances, over very long periods of time, such that the phenomenon of 'genome freeze' seems to be apparent, as in bdelloid rotifers, where the animals have hardly changed over aeons and seem to consist of relatively few asexual lineages (Welch & Meselson, 2000). In more general cases of strictly apomictic lineages where no meiosis or recombination is assumed to occur, other factors affect genotype including 'sexual leakage'. For example, rare lifecycle strategies sometimes allow recombination between asexual and sexual lines (*cf.* Delmotte *et al.*, 2001) as does the similar phenomenon of rare recombination, where an uncommon sexual morph influences the population at large (Normark, 1999).

Accepting that certain mechanisms of clonal evolution do occur in all asexual lineages, both vertical and horizontal (the relative time scales of operation of which are discussed in Loxdale *et al.*, 2002), the

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existence of a 'genotype' is only presently assessed following the testing of a relatively few genetic loci. We do not know what variation exists at other parts of the genome and hence, clones should more accurately be described as '*clonotypes*', a term already in use in immunology (Song *et al*., 1999). This argument is strengthened by genotype assessment where markers revealing a greater level of resolution proportionally increase the number of genotypes determined. Ultimately, sequencing parts of the clonal eukaryote genome (Normark & Moran, 2000) or its mapping (Black *et al*., 2001, Hawthorne & Via, 2001) is likely to reveal much more genetic variation, even to the point that each individual within a clonal lineage is different from another.

If this is so, then concepts of genetic fidelity within eukaryotic clones are untenable and the whole philosophy of the 'clone' must be re-defined and the topic re-visted, hopefully using the armoury of advanced molecular markers now available. We encourage people not to accept the old, unproven dogma that clones are 'genetically identical', not only in terms of sequence fidelity but also DNA expression. This point is duly exemplified by the recent developments in cloned mammals which show epigenetic changes, potentially affecting fitness and ultimately survival and reproduction (Humpherys *et al*., 2001).

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