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Population subdivision and the Hudson–Kreitman–Aguade test: testing for deviations from the neutral model in organelle genomes

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Summary

The Hudson–Kreitman–Aguade (HKA) test is based on the prediction from the neutral theory that levels of polymorphism within a species and the divergence between two closely related species should be correlated. Population subdivision has been shown to alter both the amounts of polymorphism segregating within species and the rate of divergence between species, meaning that genomic regions with different population structures also differ in their divergence to polymorphism ratios. Population subdivision may hence hamper the utility of the HKA test for detecting deviations from the standard neutral model, especially for organelle genomes that often have different patterns of population structure compared with nuclear genes. In this paper, I show that population subdivision inflates the number of instances where the HKA test detects deviations from the neutral model. Using coalescent simulations I show that this bias is most apparent when population subdivision is strong and differs substantially between the loci included. However, if divergence time is large and population structure substantial even changes in the levels of polymorphism and divergence associated with differences in the effective population size between two loci is enough to substantially alter the number of significant outcomes of the HKA test. A dataset on cytoplasmic diversity in *Silene vulgaris* and *S. latifolia* (Ingvarsson & Taylor, 2002) is also reanalysed. The previous study had shown a marked excess of intraspecific polymorphism in both species. However, when effects of population subdivision were removed, *ad hoc*, levels of intraspecific polymorphism were no longer significantly different from neutral expectations, suggesting that population subdivision contributed to the observed excess of intraspecific polymorphism seen in both species of *Silene*.

1. Introduction

Population subdivision is a common phenomenon in many organisms and the effects of population subdivision on the structuring of genetic variation have received much attention in the population genetics literature. Recent work in population genetics has extended the standard coalescent model to incorporate the effects of population subdivision (Nordborg, 1997; Wakeley, 1998, 1999, 2000, 2003). Wakeley (1999) showed that when the number of populations is large, the genealogical history of a sample of DNA sequences can be divided into two distinct phases: a scattering phase and a collecting phase. The scattering phase is a stochastic sample size adjustment where an initial sample of n lineages is reduced to n' ancestral lineages ($1 \leqslant n' \leqslant n$). The scattering phase proceeds until all the remaining n' lineages have been dispersed by migration into different demes. After the scattering phase ends the collecting phase takes over and proceeds until the most recent common ancestor of the remaining ancestral lineages is reached. Wakeley (1998, 1999) showed that the collecting phase reverts to a standard Kingman coalescent process if time is rescaled in units of

$$2N_e = 2Nd\left(1 + \frac{1}{2M}\right), \tag{1}$$

where N is the local population size, d is the number of populations in the metapopulation and $M = 2Nm$ is

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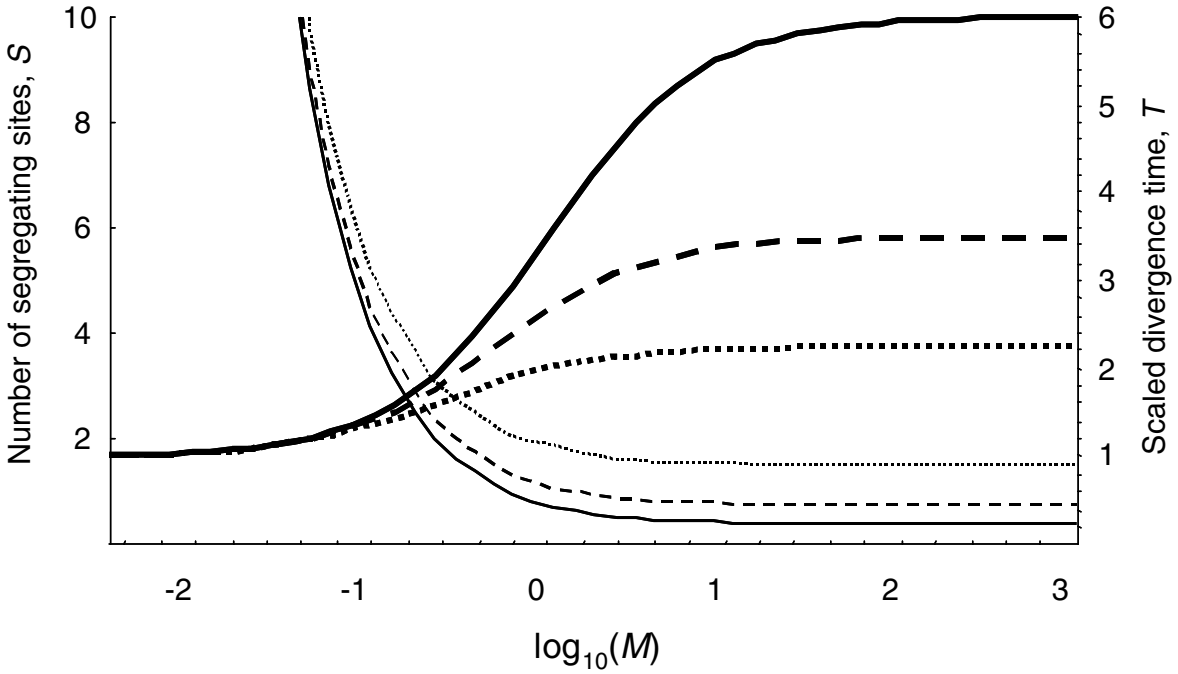

Fig. 1. Number of segregating sites (thin lines) and divergence time (thick lines) as a function of strength of population subdivision. Values are shown for three different effective population sizes, Λ (cf. eqn 5). $\Lambda = 2$ (dotted line), $\Lambda = 1$ (dashed line) and $\Lambda = 0{\cdot}5$ (continuous line). Parameter values used were $N = 1000$, $d = 1000$, $\mu = 10^{-7}$ and $\tau = 10^{7}$.

number of migrants exchanged between populations each generation. Polymorphism at the species level is thus proportional to $\theta = 4N_e\mu = 4Nd\mu(1 + 1/2M)$ and it is easy to see from this expression that strong population subdivision (i.e. low M) will increase the levels of intraspecific polymorphism maintained in a species (Fig. 1). This observation is important, because it implies that as long as all the appropriate rescalings are done, it should be possible to apply the whole statistical machinery that has been developed for analysing polymorphisms in species living in unstructured populations to those living in subdivided populations as long as all samples are taken from different populations (i.e. there is no scattering phase; cf. Wakeley, 2003).

Another important consequence of population subdivision is that the divergence time between closely related species appears to be more recent, and this effect becomes more pronounced for low migration rates (Wakeley, 2000, 2003). Thus, in species that are subdivided into local populations interconnected by low levels of migration, isolation or speciation events appear to be more recent than they in fact are, because divergence between populations or species is reduced relative to the expectation based on segregating variation within the population or species (Fig. 1). This is intuitively easy to understand since the fixation time of a segregating variant in a subdivided population is proportional to the effective metapopulation size and this can be substantially increased if the migration rate among populations is low (Crow & Kimura, 1970; Whitlock & Barton, 1997; Whitlock, 2003).

An added complexity occurs when different genomic regions have different levels of population structure, either because of differences in effective population size or because different genomes experience different migration rates. For example, in angiosperms, cpDNA and mtDNA are maternally inherited and since seed and pollen dispersal often occur over quite different spatial scales, levels of population structure can be quite different between maternally inherited genomes (cpDNA and mtDNA) that are dispersed only through seeds and nuclear genes that are dispersed through both pollen and seeds (McCauley, 1994; Hu & Ennos, 1999; Hamilton & Miller, 2002). Also, many animals have sex-biased dispersal where one sex (often males) is the dispersing sex while the other sex is philopatric (Shields, 1987). Such sex-biased migration can also create different population structures in mtDNA and nuclear genes and this has been shown to affect the phylogenetic utility of mtDNA and nuclear genes (Hoelzer, 1997). Since population structure not only changes the amount of variation that is segregating within a population or species but also changes the rate by which populations or closely related species diverge from a common ancestor, genomic regions that have different population structures will have different divergence to polymorphism ratios.

The comparison of levels of polymorphism within species with levels of divergence between species forms

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the basis of several statistical tests of neutrality, such as the Hudson–Kreitman–Aguade test (HKA test; Hudson *et al*., 1987) or the McDonald–Kreitman test (MK test; McDonald & Kreitman, 1991). These tests are based on the prediction from the neutral theory that levels of polymorphism within a species and the degree of divergence between two closely related species should be correlated. However, since population subdivision may change the polymorphism to divergence ratio it is likely that this could bias statistical tests relying on this ratio. Population subdivision may thus hamper the utility of statistical tests of neutrality, such as the HKA test, for detecting deviations from the standard neutral model. In this paper I quantify the degree to which population structure affects the HKA test and in particular I focus on attempts to detect natural selection acting on organelle genomes (mtDNA and cpDNA). Organelle genomes often have different patterns of population structure compared with nuclear genes for the reasons described above, and the effects of population subdivision are therefore expected to be particularly pronounced for such genomes. Statistical tests of neutrality, based on comparing diversity and divergence, also require unlinked loci to be used as 'controls' for detecting differences in, for instance, effective population sizes between two species. When studying selection on organelle genomes, nuclear genes are required as 'controls' due to the non-recombining nature of cpDNA and mtDNA, and in such cases it is impossible to avoid the confounding effects of population structure.

2. Polymorphism and divergence in subdivided populations

The HKA test is a goodness-of-fit test that compares estimates of parameters that describe within-species diversity and between-species divergence with their observed values using data from two or more loci (Hudson *et al*., 1987). Under a standard neutral model the expected number of segregating sites S in a sample of n individuals is

$$E[S] = C(n)\theta, \tag{2}$$

where $\theta = 4N\mu$ is the scaled mutation rate and $C(n) = \sum_{i=1}^{n-1} 1/i$. Similarly, the expected divergence between two species is given by

$$E[D] = \theta(T + (1+f)/2), \tag{3}$$

where T is the time since the split of the two species, τ, scaled in units of $2N$ such that $T = \tau/2N$ and f is the relative population size of the species used for comparison. As noted above, in cases where all samples are all taken from different populations, (i.e. there is no scattering phase) these expressions also hold for a subdivided population if the local population size, N, in eqns (2) and (3) is replaced by the effective metapopulation size, eqn (1). Thus, as population subdivision increases, a greater proportion of the genetic variation will be segregating within a species. The divergence time between the two species, τ, is rescaled according to

$$T = \frac{\tau}{2N_e}. \tag{4}$$

The effective population size of a genome region also has direct effects on genetic differentiation. The expected level of genetic differentiation under an island model of population structure is given by

$$F_{ST} \approx \frac{1}{2\Lambda Nm + 1}, \tag{5}$$

where ΛN is the number of copies of the genomic region contained in the population. Thus for nuclear genes in diploid individuals $\Lambda = 2$ while for organelle genes in hermaphrodites $\Lambda = 1$ and for organelle genes in species with separate sexes (and an equal sex ratio) $\Lambda = 0{\cdot}5$.

Under neutrality, the polymorphism to divergence ratio ($E[S]/E[D]$) is expected to be constant across different genome regions within the bounds set by stochastic variation arising from sampling and genealogical processes. However, when comparing genome regions with different effective population sizes this is not necessarily true, as regions with greater population structure will have an apparent increase in polymorphism levels and an apparent reduction in divergence times. Polymorphism to divergence ratios can thus differ substantially between different genome regions when population subdivision is present (Fig. 2).

3. Coalescent simulations

Since the diversity to divergence ratio changes with the strength of population subdivision it is likely that this will affect the performance of the HKA test. When samples are taken from a subdivided population, and this subdivision is ignored, it is possible that population structure could increase the number of instances where the HKA test detects significant deviations from a neutral model (i.e. the Type 1 error rate is inflated). To test this idea I performed coalescent simulations to simulate data from two independent loci sampled from two species that shared a common ancestor τ generations ago. Species i ($i = 1, 2$) was assumed to be subdivided into d_i local populations, each of size N_i with migration among local populations occurring at a rate m_i so that $M_i = N_i m_i$ individuals are exchanged among populations each generation. No migration was assumed to take place between the two species after the time of isolation, τ. Mutations occur at a rate μ and are assumed to follow the infinite sites model. All simulations assumed no recombination

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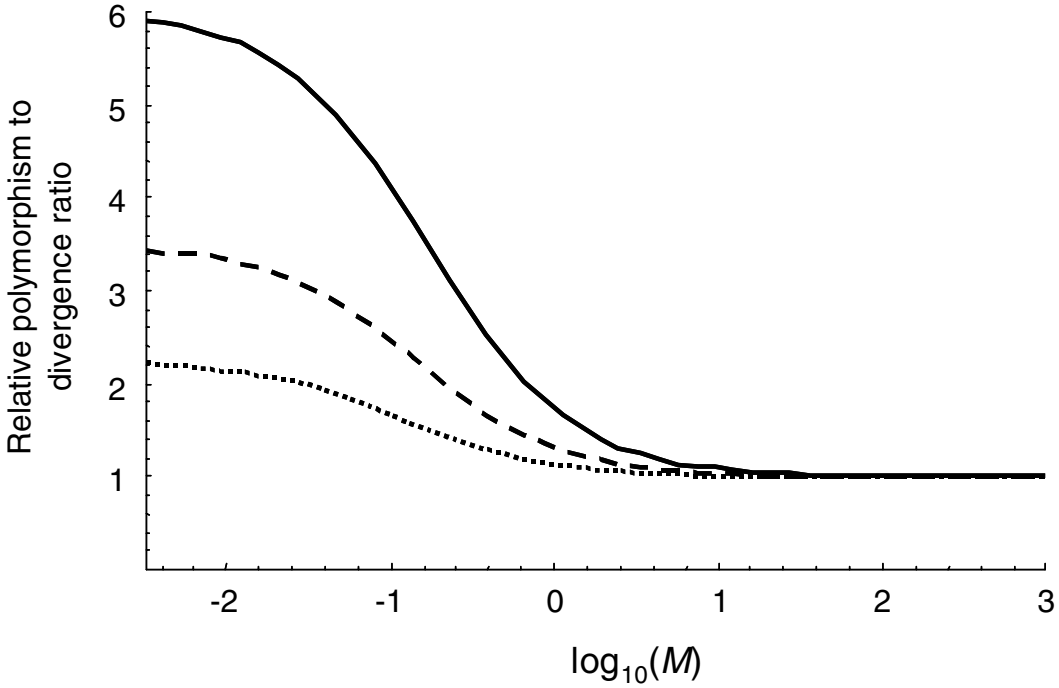

Fig. 2. The ratio of within-species polymorphism to between-species divergence, relative to the situation without population subdivision, as a function of the migration rate among local demes. Results are shown for three different effective population sizes, Λ (cf. eqn 5). $\Lambda = 2$ (dotted line), $\Lambda = 1$ (dashed line) and $\Lambda = 0{\cdot}5$ (continuous line). Parameter values are as in Fig. 1.

within loci, an assumption that makes the HKA test conservative (Hudson *et al.*, 1987). All simulations were performed using a sample size of $n = 25$ individuals from each species, all sampled from different populations (i.e. the simulations did not include any scattering phase), since in this situation the coalescent of the sample reverts to a standard Kingman coalescent but on a timescale determined by the effective metapopulation size. Sampling multiple individuals from one or more populations is much more likely to yield data that are incompatible with a neutral model, especially if subdivision is strong (Wakeley, 1998). After each simulation the number of segregating sites, S, was scored in each species and the pairwise divergence between the two species, D, was calculated for each of the two loci. These results were fed into J. Hey's program, HKA (available at http://lifesci.rutgers.edu/~heylab/DistributedProgramsandData.htm#HKA), to perform HKA tests on the simulated data. Statistical significance of the HKA tests was calculated by comparing the observed results with the result from 500 coalescent simulations using parameters estimated from the simulated data set (Hudson *et al.*, 1987). For each parameter combination in Tables 1 and 2, 1500 independent replicate coalescent simulations were performed and the proportion of replicates where the HKA test was significant was scored to estimate the Type 1 error rate empirically (p_{sim} in Tables 1 and 2). As the HKA test performs quite well under strict neutrality (Hudson *et al.*, 1987) the expectation is that approximately 5% of the replications should show a significant HKA test statistic.

Initially I compared a situation where only the effective population size of the two loci differed and the underlying level of population structure was the same (i.e. Nm was the same and only Λ differed between loci). The results from these coalescent simulations are summarized in Table 1. With only a few exceptions, the HKA test is robust against changes in the polymorphism to divergence ratio that arise from differences in effective population sizes. Only where population subdivision is very strong and divergence time is long are there instances where the HKA test appears to reject the neutral model more often than expected by chance.

I also performed simulations where the two loci had both different effective population sizes and different levels of population structure, and these are summarized in Table 2. In this case there are far more instances where the HKA test is biased and the neutral expectation is rejected far more often than expected by chance. Once again, this effect is most severe when population subdivision is great ($M < 1$) and differs substantially between the loci ($M_1/M_2 > 4$), and when species divergence is not very recent.

4. An example: cpDNA polymorphisms in *Silene*

Two recent studies have shown remarkably high levels of nucleotide diversity in the cytoplasmic genomes in

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Table 1. *The effect of population subdivision on the HKA test when effective population sizes differ between the loci compared*

M	$2Nd\mu_1$	T_1	p_{sim}
$\Lambda_1=2$ and $\Lambda_2=1$			
∞	24	3·75	0·044
10	24	3·75	0·040
1	24	3·75	0·049
0·25	24	3·75	0·047
∞	24	25	0·049
10	24	25	0·051
1	24	25	**0·093**
0·25	24	25	**0·207**
∞	80	0·94	0·030
10	80	0·94	0·035
1	80	0·94	0·051
0·25	80	0·94	0·061
$\Lambda_1=2$ and $\Lambda_2=0·5$			
∞	24	3·75	0·046
10	24	3·75	0·047
1	24	3·75	**0·127**
0·25	24	3·75	**0·130**
∞	24	25	0·052
10	24	25	0·074
1	24	25	**0·299**
0·25	24	25	**0·716**
∞	80	0·94	0·044
10	80	0·94	0·041
1	80	0·94	0·061
0·25	80	0·94	0·058

The Type 1 error rate of the HKA test, i.e. the proportion (out of 1500 replicates) of coalescent simulations where a given parameter combinations showed a significant deviation from neutral expectations is given in the column denoted p_{sim}. Values of p_{sim} greater than 0·075, representing a 50% increase in the Type 1 error rate, are highlighted in bold.

Table 2. *The effect of population subdivision on the HKA test when both effective population size and population subdivision differ between the loci compared*

M_1	M_2	R	$2Nd\mu_1$	$2Nd\mu_2$	T_1	T_2	p_{sim}
$\Lambda_1=2$ and $\Lambda_2=1$							
10	5	2	24	12	3·75	7·5	0·060
1	0·5	2	24	12	3·75	7·5	**0·082**
10	5	2	24	12	25	50	0·067
1	0·5	2	24	12	25	50	**0·279**
10	5	2	80	40	0·94	1·88	0·040
1	0·5	2	80	40	0·94	1·88	0·058
4	1	4	24	12	3·75	7·5	**0·079**
1	0·25	4	24	12	3·75	7·5	**0·231**
4	1	4	24	12	25	50	**0·197**
1	0·25	4	24	12	25	50	**0·681**
4	1	4	80	40	0·94	1·88	0·045
1	0·25	4	80	40	0·94	1·88	**0·067**
10	1	10	24	12	3·75	7·5	**0·077**
1	0·1	10	24	12	3·75	7·5	**0·246**
10	1	10	24	12	25	50	**0·215**
1	0·1	10	24	12	25	50	**0·983**
10	1	10	80	40	0·94	1·88	0·057
1	0·1	10	80	40	0·94	1·88	**0·113**
$\Lambda_1=2$ and $\Lambda_2=0·5$							
10	5	2	24	6	3·75	15	0·073
1	0·5	2	24	6	3·75	15	**0·227**
10	5	2	24	6	25	100	**0·093**
1	0·5	2	24	6	25	100	**0·693**
10	5	2	80	20	0·94	3·75	0·051
1	0·5	2	80	20	0·94	3·75	0·065
4	1	4	24	6	3·75	15	**0·202**
1	0·25	4	24	6	3·75	15	**0·369**
4	1	4	24	6	25	100	**0·439**
1	0·25	4	24	6	25	100	**0·969**
4	1	4	80	20	0·94	3·75	0·067
1	0·25	4	80	20	0·94	3·75	**0·087**
10	1	10	24	6	3·75	15	**0·222**
1	0·1	10	24	6	3·75	15	**0·449**
10	1	10	24	6	25	100	**0·484**
1	0·1	10	24	6	25	100	**1·000**
10	1	10	80	20	0·94	3·75	**0·089**
1	0·1	10	80	20	0·94	3·75	**0·139**

The Type 1 error rate of the HKA test, i.e. the proportion (out of 1500 replicates) of coalescent simulations where a given parameter combinations showed a significant deviation from neutral expectations is given in the column denoted p_{sim}. Values of p_{sim} greater than 0·075, representing a 50% increase in the Type 1 error rate, are highlighted in bold.

two plant species associated with cytoplasmic male sterility (CMS) (Ingvarsson & Taylor, 2002; Städler & Delph, 2002). CMS elements are known to be associated with complex patterns of selection, and different theoretical models predict that CMS-associated selection can either reduce or enhance polymorphism levels in cytoplasmic genomes relative to a species that does not experience CMS. An interesting question is therefore to determine whether the high levels of intraspecific polymorphism seen in these two studies (Ingvarsson & Taylor, 2002; Städler & Delph, 2002) could be explained by CMS-associated natural selection that acts to maintain cytoplasmic diversity.

Ingvarsson & Taylor (2002) used DNA sequence data from four non-coding regions in the chloroplast genome to study the pattern of selection exerted by CMS in *Silene vulgaris* and its close relative, the dioecious *S. latifolia*. The individuals included in the study were collected from a set of populations covering central Europe and this sampling scheme should thus approximate a situation where the scattering phase can be ignored (Wakeley, 1998, 2000). The relevant cpDNA data from Ingvarsson & Taylor (2002) is given in Table 3, together with data from two nuclear loci serving as 'controls' for potential differences in effective population sizes between the two species (*G3pdh* is previously unpublished data). These data clearly do not fit a neutral model, as evidenced by the highly significant HKA test (Table 3). By partitioning the total χ^2 value from the HKA test into contributions from individual loci, it becomes clear that

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Table 3. *Sample sizes* (n), *segregating sites* (S) *and pair-wise divergence* (D) *for cpDNA and two nuclear loci in* Silene latifolia *and* S. vulgaris

	Ingvarsson & Taylor (2002)			Corrected using McCauley (1997)				Corrected using McCauley (1998)			
	S. vulgaris	*S. latifolia*	Deviance	F_{ST}	*S. vulgaris*	*S. latifolia*	Deviance	F_{ST}	*S. vulgaris*	*S. latifolia*	Deviance
cpDNA											
n	29	25		0·546	29	25		0·624	29	25	
S	15	29	12·45		6·81	13·17	2·19		5·64	10·9	2·04
D	31·68		5·01		69·78		0·14		84·25		0·06
Slx1											
n	8	9		0·056	8	9		0·222	8	9	
S	26	30	0·93		24·544	28·32	0·64		20·228	23·34	0·72
D	91·06		2·29		96·46		0·97		117·04		1·08
G3pdh											
n	17	11		0·056	17	11		0·222	17	11	
S	79	32	1·17		74·58	30·208	1·38		61·462	24·9	1·62
D	53·21		0·27		56·37		1·61		68·39		1·83
HKA-test											
χ^2			22·10				6·92				7·35
p			0·004				0·180				0·156
df			4				4				4

The fit of these data to a neutral model is evaluated using an HKA-test. Two independent estimates of population structure (McCauley, 1997, 1998) are also used to 'correct' the data for population subdivision. For further details see the text.

Table 4. *Coalescent simulations using parameter values estimated from the data in Taylor and Ingvarsson (2002), using a range of M_1/M_2 values taken from McCauley (1997)*

			cpDNA		Nuclear		
M_1/M_2	M_1	M_2	θ_1	T	θ_1	T	p_{sim}
1	∞	∞	1·5	18	10	4·5	0·052
3·4	0·588	2	4·05	6·667	11·25	4·0	0·302
9·2	0·217	2	8·4	3·21	11·25	4·0	0·609
124	0·016	2	94·5	0·286	11·25	4·0	0·755

The migration rate for nuclear genes was fixed at $M_2 = 2$, yielding an expected F_{ST} value of 0·11, which is within the range of values seen for nuclear loci in both *S. latifolia* and *S. vulgaris*. p_{sim} is the Type 1 error rate, i.e. the proportion of coalescent simulations where the HKA test detects a significant deviation from neutral expectations.

much of the total deviation can be explained by an excess of intraspecific polymorphism in both *S. vulgaris* and *S. latifolia* compared with between-species divergence (Table 3). As mentioned above, one possible interpretation of this is that natural selection associated with CMS acts to maintain cytoplasmic diversity. However, earlier studies have demonstrated the existence of substantial population structure in both species of *Silene*, with chloroplast loci showing much higher levels of population subdivision than nuclear loci (McCauley, 1997, 1998), and population structure is thus a viable alternative explanation for the excess of cytoplasmic diversity in *S. vulgaris* and *S. latifolia*. One way to test this is to 'correct' for the presence of population subdivision. Population subdivision increases intraspecific polymorphism and reduces divergence by a factor ($[1+1/(\Lambda M)]$; eqns 1 and 4). If population structure in *Silene* is assumed to be adequately described by an island model (Wright, 1930), this factor equals ($1–F_{ST}$), where F_{ST} is Wright's measure of genetic differentiation among populations (Wright, 1951). McCauley (1997, 1998) has shown that F_{ST} is around 0·6 for cpDNA data in both *S. latifolia* and *S. vulgaris*, whereas genetic differentiation at several nuclear allozyme loci ranges between 0·05 and 0·25 depending on the spatial scale of study. If these values are used to 'correct' the data from Ingvarsson & Taylor (2002) for the effects of population subdivision, the HKA test is no longer significant and the cpDNA data also contribute far less to the overall deviance (Table 3). Both nuclear loci are far less affected, suggesting that population subdivision is an important contributor to the observed excess of intraspecific polymorphism in both species of *Silene*.

McCauley (1997) estimated genetic differentiation at cpDNA restriction fragment length polymorphism (RFLP) markers and nuclear isozymes over three spatial scales in *Silene latifolia*, ranging from individuals separated by a few metres to populations separated by several kilometres, and used these data to estimate the ratio of pollen to seed migration over these scales. He showed that the ratio of pollen to seed gene flow ranged from 3·4, over the largest scale, to 124, over the smallest spatial scale. Table 4 shows the results of a set of coalescent simulations based on the data from Ingvarsson & Taylor (2002) where population subdivision is varied across a range of migration rates corresponding to the data from McCauley (1997). These simulations clearly highlight the importance to the two modes of migration of establishing differential population structure at organelle and nuclear loci in *Silene* and how these bias the HKA test.

5. Discussion

Population subdivision has been shown to alter the ratio of polymorphism to divergence in a very predictable manner (Wakeley, 2000). Population subdivision is expected to yield an excess of segregating variation within a species compared with the levels of divergence in a between-species comparison and this apparent excess becomes more pronounced as levels of population structure increase. The number of instances where the HKA test detects a deviation from the neutral model can thus be inflated if the test is applied to samples from subdivided populations, especially when levels of population structure differs between gene regions. Coalescent simulations show that this bias is most apparent when population subdivision is strong and differs substantially between the loci included. However, if divergence time is large and population structure substantial, even changes in levels of polymorphism and divergence associated with differences in the effective population size between two loci are enough to bias the HKA test substantially (Table 1). Notice that this effect of population subdivision is observed even when a single sample is collected from each population – a situation where intraspecific data are well described by the standard coalescent (albeit on a different timescale).

Another important point is that the effect of subdivision on the HKA test depends on the absolute values of population structure. Thus, even in cases where the relative difference in population structure is the same, i.e. pairs of loci have the same M_1/M_2 ratio, cases with greater absolute levels of population structure are more affected (Table 2). This result is not surprising given the highly non-linear relationship between gene flow and levels of population structure (e.g. Crow & Kimura, 1970).

As noted above, the HKA test is most severely affected when population subdivision differs between the

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loci compared. This situation is most likely to occur when nuclear loci are compared with loci located in organelle genomes, i.e. in mtDNA or cpDNA. Furthermore, since both mtDNA and cpDNA are non-recombining, loci in these genomes must be contrasted with nuclear loci if the HKA test is to be applied. The results presented here make it clear that population subdivision will inevitably interfere with our ability to detect selection in organelle genomes using the HKA test. mtDNA and cpDNA have long been the molecules of choice for screening variation in many non-model animal and plant species, and given the rapidity with which sequence data are accumulating, data from nuclear genes will probably be available from many more non-model organisms in the near future. This will increase the possibilities for studying how patterns of sequence variation differ between nuclear and organelle genomes in organisms with very different life histories.

The re-analysis of the data from Ingvarsson & Taylor (2002) highlights how population subdivision can introduce large biases into the HKA test, even when all samples are taken from different populations. The cpDNA dataset had an unusually high level of intraspecific polymorphism in both species and this resulted in a highly significant HKA test and a rejection of the neutral model. However, when the effects of population subdivision were removed *ad hoc*, the HKA test was no longer significant. A direct contrast of cpDNA sequence diversity between the two species showed that cytoplasmic diversity was about 50% lower in *S. vulgaris*, the species associated with CMS. However, this direct comparison cannot demonstrate whether diversity is reduced in *S. vulgaris* or enhanced in *S. latifolia* – information that the HKA test could have provided if it had not been biased by population subdivision.

In a similar study, Städler & Delph (2002) showed that *Silene acaulis*, another species associated with CMS, also had unusually high levels of mtDNA polymorphism. However, the study by Städler & Delph did not include any estimates of nucleotide diversity of nuclear genes so it is difficult to assess exactly how high mtDNA diversity is in *S. acaulis*. Population subdivision is also pronounced in *S. acaulis*, although the data analysed by Städler & Delph (2002) showed no correlation between genetic and geographic distance of haplotypes. An answer to the question of whether mtDNA diversity observed in *S. acaulis* is unusually high and may have been the result of balancing selection imposed by CMS or is simply a by-product of strong population subdivision, must await more detailed studies of both nuclear and other cytoplasmically inherited genes in this and closely related species.

Finally, Wakeley (2003) showed that another test that compares within-species polymorphism with between-species divergence, the McDonald–Kreitman (MK) test, is fairly robust to any underlying population subdivision. The reason for this is that the MK test compares differences between synonymous and non-synonymous mutations from a single coding region and population subdivision is therefore expected to affect these types of mutation in the same way. It is thus clear that using the MK test is the preferred option if population subdivision is potentially a problem. However, the MK test requires that mutations can be classified as either synonymous or non-synonymous and is therefore only applicable to data from coding regions. The MK test can therefore not be used when testing for deviations from the neutral model in non-protein coding regions such as introns or regulatory elements located 5′ or 3′ to the coding region. The HKA test thus remains one of only a few alternative statistical tests that can be used to detect deviations from the neutral model in non-coding genomic regions. Since studies have indicated that many important adaptations are mediated by changes in gene regulation rather than through changes in coding sequence (e.g. Doebley & Luekens, 1998), population subdivision may hamper our ability to detect such evolutionary processes in organelle genomes.

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