'Junk' DNA and long-term phenotypic evolution in *Silene* section *Elisanthe* (Caryophyllaceae)

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Nuclear DNA content variation over orders of magnitude across species has been attributed to 'junk' repetitive DNA with limited adaptive significance. By contrast, our previous work on *Silene latifolia* showed that DNA content is negatively correlated with flower size, a character of clear adaptive relevance. The present paper explores this relationship in a broader phylogenetic context to investigate the long-term evolutionary impacts of DNA content variation. The relationship between nuclear DNA content and phenotype variation was determined for four closely related species of *Silene* section *Elisanthe* (Caryophyllaceae). In addition to a consistent sexual dimorphism in DNA content across all of the species, we found DNA content variation among populations within, as well as among, species. We also found a general trend towards a negative correlation between DNA content and flower and leaf size over all four species, within males and females as well as overall. These results indicate that repetitive DNA may play a role in long-term phenotypic evolution.

Keywords: genome size; flow cytometry; quantitative traits; *Silene*; non-coding DNA

1. INTRODUCTION

In the 1970s, nuclear DNA content was found to vary among species by several orders of magnitude, giving rise to the C-value paradox (Gregory 2001). Such variation was written off as 'junk' DNA of marginal phenotypic significance (Doolittle & Sapienza 1980; Orgel & Crick 1980), and it emerged that much DNA content variation was attributable to non-coding repetitive DNA (Flavell *et al.* 1983; Kubis *et al.* 1998). However, recent insights suggest that certain repetitive DNA elements may have considerable adaptive significance (Bennetzen 1998; Kidwell & Lisch 2000). For example, repetitive elements can have a regulatory impact on gene expression (Zuckerlandl 1997; Kumar & Bennetzen 1999; Kashkush *et al.* 2003). Thus, accumulation or loss of repetitive sequences could provide a mechanism for evolutionary change.

These new insights into properties of repetitive DNA open opportunities for addressing long-standing evol-

utionary controversies. Two specific premises are that phenotypic evolution is based on allelic substitution over many genetic loci (Fisher 1918; Bulmer 1980) (for example, the infinitesimal model for quantitative genetics) and that long-term evolution is the cumulative result of such allelic substitution (Bateson 1894; Orr & Coyne 1992). If changes in certain fractions of DNA content underlie the short-term response to selection, and there is growing evidence that this is sometimes the case (Rayburn *et al.* 1994; Meagher & Costich 1996; Bennett *et al.* 1998; Kalendar *et al.* 2000), then such changes in DNA content could contribute to long-term evolution.

Our work on *Silene latifolia* has shown that nuclear DNA content is negatively correlated with flower size and that changes in nuclear DNA content underlie the short-term response to selection on flower size (Meagher & Costich 1994, 1996). We explore the variation in DNA content across related *Silene* species and its correlation with flower and leaf size.

2. MATERIAL AND METHODS

This study focuses on four species in *Silene* section *Elisanthe*: *S. latifolia*, *S. dioica*, *S. diclinis* and *S. maritima*. These species are all dioecious (Talavera 1990) and native to Europe. The first two species are widespread in Europe and have been introduced elsewhere, whereas the last two species are Iberian endemics restricted to Valencia and northern Portugal/adjacent parts of Spain, respectively (Talavera 1990). In May 1992, populations of each species were located in Spain and Portugal (figure 1; electronic Appendix A, table 3), and seeds were collected and transported to Rutgers University (USA), where they were raised in the greenhouse to flowering maturity. Floral (calyx diameter, petal claw length (= calyx length) and petal limb length (= half the corolla diameter)) and leaf (length and width) measurements were taken. Calyx diameter is of particular interest as it is sexually dimorphic (Meagher 1992, 1999) and has a sex-specific ecological role: pre-emption of ovule predators in females (Pettersson 1991; Wright 2000) versus no known function in males.

Flow cytometric assays of nuclear DNA content were performed as outlined previously (Costich *et al.* 1991). Two assays of nuclear DNA content emphasize different features of the nuclear DNA content (Costich *et al.* 1991). Red fluorescence of propidium iodide (PI) measures overall DNA content (PI-DNA), whereas red fluorescence of PI in the presence of chromomycin (CA3) provides an AT-biased measure (PI+CA3-DNA) that emphasizes repetitive DNA since repetitive motifs in plants are typically AT-biased (Wang *et al.* 1994). For both assays, estimates of DNA content were obtained by dividing the mean fluorescence of *Silene* nuclei by the mean for an internal chicken red blood cell (CRBC) standard and multiplying by 2.33, the DNA content (in pg) of CRBC (Arumuganathan & Earle 1991). We also tested the possibility that apparent DNA content differences are caused by interference from secondary compounds (Price *et al.* 2000) by assessing the range of variation in *Silene* nuclear fluorescence compared with that of CRBC. If there were a general inhibitor of PI uptake and fluorescence, then variance in CRBC fluorescence should track variance in *Silene* nuclear fluorescence. The variance ratio of CRBC/*Silene* ranged from 9.13 to 36.93 across our different sample groups (species, population, sex) (electronic Appendix A, table 4), showing that intraspecific variation in estimates of *Silene* nuclear DNA content were not an artefact.

3. RESULTS

Partial hierarchical analysis of variance (ANOVA) (SAS Institute Inc. 2001) of species, populations nested within species, and plant sex (table 1) was used to identify patterns within our data (figure 2; electronic Appendix A, table 5). PI-DNA showed significant differences between species and between the sexes but no detectable heterogeneity within species. AT-biased PI+CA3-DNA showed similar

Figure 1. Map of Spain and Portugal showing collection localities (circles, *S. latifolia*; squares, *S. dioica*; diamonds, *S. diclinis*; triangles, *S. marizii*). Population localities are specified in electronic Appendix A, table 3.

differences between species and sexes, but it also showed significant heterogeneity among populations within species, indicating a greater potential for short-term evolutionary change. A higher male DNA content under both assays confirms earlier findings of sexual dimorphism in DNA content in *S. latifolia* (Costich *et al.* 1991; Dolezel & Gohde 1995) and shows that the enlarged Y chromosome found in *S. latifolia* (Westergaard 1958; Ciupercescu *et al.* 1990) occurs throughout the section. The LSMEANS option in SAS was used to test for pairwise species differences, and all species pairs except for *S. diclinis* versus *S. latifolia* were significantly different (results not shown). Calyx diameter showed significant variation at all levels: among species, among populations within species and between the sexes. Moreover, the extent of sexual dimorphism differed among species, as shown by the significant sex–species interaction; sex differences are greater for species with larger flowers. Females consistently had larger calyces. The other floral characters (petal claw and limb length) also showed variation among, as well as within, species, but there was limited evidence of sexual dimorphism. Leaf dimensions primarily differed between species; significant variation among populations within species was limited to leaf length. A trend towards sexual dimorphism in leaf length was not statistically significant, but there was a significant population–sex interaction for leaf width, indicating heterogeneity in the extent of sexual dimorphism for this character.

Overall correlations between PI-DNA and floral and leaf characters were all negative (table 2; electronic Appendix A, figure 3), significantly so for calyx diameter and petal claw length. Within species, the trend is towards negative correlation between phenotype and PI-DNA, with three out of the four species showing significantly negative correlations between PI-DNA and calyx diameter. This relationship is consistent with our previous reports for *S. latifolia*, with males having smaller calyces but

greater nuclear DNA content (Meagher & Costich 1994, 1996). Within sex–species combinations, there is a trend towards negative correlation, with the strongest negative correlations between PI-DNA and calyx diameter and between PI-DNA and petal claw length for females of *S. marizii*. Correlations between phenotypic characters and PI+CA3-DNA were weaker and more variable (electronic Appendix A, table 6 and figure 3). This is in contrast to our earlier finding of a stronger correlation with PI+CA3-DNA (Meagher & Costich 1996), but the sample space for the current analysis is different in that it encompasses a greater range of genetic differentiation among populations and species.

4. DISCUSSION

At the end of the nineteenth century, Bateson (1894) noted continuity of variation between the sexes, among populations and among species, implying that similar evolutionary mechanisms act at all levels. The infinitesimal model of quantitative genetics (Fisher 1918; Bulmer 1980) provided a specific theoretical framework in which such evolutionary change is attributed to accumulation of allelic substitutions at a large number of loci. This model for evolutionary change has prevailed up to the present time (Charlesworth *et al.* 1982; Orr & Coyne 1992). However, the basic inconsistency between continuous variation and particulate inheritance has been an ongoing controversy (Orr & Coyne 1992). The present finding, that variation in DNA content, a feature likely to show continuous variation, may underlie both short-term and long-term phenotypic change, offers the potential for a new model of phenotypic evolution.

Much recent research on repetitive DNA has focused on genomic plasticity (Kubis *et al.* 1998; Schmidt & Heslop-Harrison 1998; Fu & Dooner 2002). However, gradual phenotypic change in response to selection requires a genomic balance between sufficient stability to maintain germline continuity and sufficient instability to enable phenotypic adaptation. Intraspecific DNA content variation, reported for a wide range of species (Gregory 2001), must fall within limits that still permit genomic stability. Certain categories of repetitive DNA, such as tandem repetitive elements, may show these features. Our findings suggest that such elements, providing a continuously varying genetic basis for phenotypic variation, underlie gradual phenotypic evolution.

Phenotypic changes associated with DNA content variation are probably caused by regulatory impacts on patterns of gene expression. For example, localized variation in repetitive DNA may render particular genes or suites of genes more or less accessible to regulatory DNA–protein interactions (Zuckerandl 1997). Alternatively, particular DNA elements, such as retrotransposons, may have more specific impacts on gene regulation. Retrotransposons code for proteins that facilitate their own expression and replication (Kumar & Bennetzen 1999); accumulation of such retrotransposons within the genome will thus raise the overall occurrence of regulatory proteins within the nucleus; these may in turn serve as trans-acting factors in gene expression across whole suites of genes (Kashkush *et al.* 2003). Moreover, promoter sequences found in the long-terminal repeat (LTR) regions of LTR-retrotransposons

Table 1. Partial hierarchical analysis of variance (ANOVA) results testing for differences among species, populations and sexes. MS, mean square.

(a) nuclear DNA content				PI-DNA			PI+CA3-DNA			
source	d.f.	MS	F-ratio	p	MS	F-ratio	p	MS	F-ratio	p
species	3	6.77	148.68	< 0.0001	5.87	31.92	0.0004	5.87	31.92	0.0004
population (species)	6	0.08	1.65	0.1348	0.19	3.88	0.0012	0.19	3.88	0.0012
sex	1	3.16	69.48	< 0.0001	1.39	28.52	< 0.0001	1.39	28.52	< 0.0001
species × sex	3	0.03	0.64	0.5904	0.10	2.13	0.0982	0.10	2.13	0.0982
population × sex (species)	6	0.04	0.77	0.5591	0.05	0.93	0.4779	0.05	0.93	0.4779
error	157	0.05			0.05			0.05		

(b) floral dimensions				calyx diameter			petal claw length			petal limb length			
source	d.f.	MS	F-ratio	p	MS	F-ratio	p	MS	F-ratio	p	MS	F-ratio	p
species	3	2008.87	18.53	0.0019	2652.26	23.20	0.0010	665.83	41.70	< 0.0001	19.61	9.91	0.0067
population (species)	6	136.79	6.00	0.0223	144.03	7.57	0.0133	19.61	9.91	0.0067	0.67	0.33	0.5742
sex	1	581.13	37.72	0.0006	3.48	0.26	0.6249	0.67	0.33	0.5742	14.48	7.21	0.0075
species × sex	3	115.26	6.25	0.0259	230.26	14.68	0.0027	14.48	7.21	0.0075	1.98	0.93	0.4708
population × sex (species)	6	22.79	10.24	0.0001	19.03	6.00	< 0.0001	1.98	0.93	0.4708	2.12		
error	494	2.23			3.17			2.12					

(c) leaf dimensions				leaf length			leaf width			
source	d.f.	MS	F-ratio	p	MS	F-ratio	p	MS	F-ratio	p
species	3	19271.00	7.34	0.0183	4290.43	26.62	0.0006	4290.43	26.62	0.0006
population (species)	6	3137.98	14.78	0.0023	191.64	2.73	0.1236	191.64	2.73	0.1236
sex	1	199.87	0.87	0.3638	2.63	0.05	0.8291	2.63	0.05	0.8291
species × sex	3	298.49	1.35	0.3148	4.45	0.07	0.9727	4.45	0.07	0.9727
population × sex (species)	6	212.28	0.82	0.5582	70.23	3.46	0.0023	70.23	3.46	0.0023
error	516	260.31			20.32			20.32

Figure 2. Population means for (a) PI-DNA, (b) PI+CA3-DNA, (c) calyx diameter, (d) petal claw length (= calyx length), (e) petal limb length, (f) leaf length and (g) leaf width (circles, *S. latifolia*; squares, *S. dioica*; diamonds, *S. diclinis*; triangles, *S. marizii*). Diagonal lines in each panel indicate equal male and female values.

can have a generalized influence on downstream gene expression (Kumar & Bennetzen 1999). Evidence of such effects has been associated with *BARE-1*, an LTR-retrotransposon of the Ty1-copia group, which has been shown to correlate with localized ecological adaptation in barley (Kalendar *et al.* 2000). Evolutionarily related LTR-retrotransposons of this type include Ty1-copia-like elements in the genus *Silene* (Matsunaga *et al.* 2002).

On another level, our finding that within-species correlations carry over to the interspecific level suggests that repetitive elements may play a role in longer-term evolutionary response. It appears that, in addition to allelic substitution, aspects of genome composition and organization not factored into the present theoretical framework of quantitative genetics are also important in evolutionary response.

Table 2. Correlations by species and sex between PI-DNA and phenotype. (Numbers in parentheses are sample sizes of females and males, respectively).

trait	<i>Silene</i> species	<i>diclinis</i> (26,30)		<i>dioica</i> (11,15)		<i>latifolia</i> (22,29)		<i>marizii</i> (7,20)		overall by sex		overall	
		<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
calyx diameter	females	-0.15	0.4592	-0.52	0.1005	+0.01	0.9667	-0.73	0.0606	0.1262	0.0028	-0.23	0.0028
	males	+0.02	0.9302	-0.25	0.3742	-0.14	0.4727	+0.19	0.4151	0.6934			
	species overall	-0.42	0.0012	-0.69	0.0001	-0.47	0.0004	-0.18	0.3814				
petal claw	females	-0.02	0.9303	-0.59	0.0570	-0.24	0.2634	-0.73	0.0606	0.0547	0.0050	-0.22	0.0050
	males	-0.27	0.1548	-0.21	0.4420	-0.09	0.6377	+0.08	0.7263	0.0424			
	species overall	+0.09	0.5075	+0.01	0.9746	-0.43	0.0015	+0.01	0.9739				
petal limb	females	+0.02	0.9302	-0.13	0.7007	+0.08	0.7314	-0.64	0.1221	0.0699	0.2284	-0.10	0.2284
	males	-0.24	0.1920	-0.00	0.9941	+0.02	0.9318	+0.55	0.0114	0.5355			
	species overall	-0.10	0.4735	-0.00	0.9954	-0.32	0.0207	+0.39	0.0425				
leaf length	females	-0.18	0.3738	-0.01	0.9526	+0.16	0.4527	-0.30	0.4727	0.0198	0.0786	-0.13	0.0786
	males	+0.31	0.0963	-0.13	0.5902	-0.02	0.8997	+0.30	0.1942	0.9173			
	species overall	+0.11	0.4098	-0.18	0.2536	-0.06	0.6498	+0.19	0.3403				
leaf width	females	-0.26	0.1974	0.03	0.8851	+0.28	0.1974	-0.22	0.5940	0.0275	0.0060	-0.21	0.0060
	males	+0.04	0.8186	-0.03	0.8944	+0.15	0.4480	+0.30	0.1939	0.0646			
	species overall	+0.03	0.8001	-0.02	0.8881	+0.10	0.4828	+0.13	0.4980				

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