



Divergent evolution of life span associated with mitochondrial DNA evolution

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Received May 25, 2016

Accepted October 20, 2016

Mitochondria play a key role in ageing. The pursuit of genes that regulate variation in life span and ageing have shown that several nuclear-encoded mitochondrial genes are important. However, the role of mitochondrial encoded genes (mtDNA) is more controversial and our appreciation of the role of mtDNA for the evolution of life span is limited. We use replicated lines of seed beetles that have been artificially selected for long or short life for >190 generations, now showing dramatic phenotypic differences, to test for a possible role of mtDNA in the divergent evolution of ageing and life span. We show that these divergent selection regimes led to the evolution of significantly different mtDNA haplotype frequencies. Selection for a long life and late reproduction generated positive selection for one specific haplotype, which was fixed in most such lines. In contrast, selection for reproduction early in life led to both positive selection as well as negative frequency-dependent selection on two different haplotypes, which were both present in all such lines. Our findings suggest that the evolution of life span was in part mediated by mtDNA, providing support for the emerging general tenet that adaptive evolution of life-history syndromes may involve mtDNA.

KEY WORDS: *Acanthoscelides obtectus*, artificial selection, Bruchinae, coadaptation, mitochondria, mtDNA, negative frequency dependent selection, senescence.

Although mitochondrial metabolic processes are central in ageing (Trifunovic et al. 2004; Guarente 2008; Kenyon 2010; Houtkooper et al. 2013; Monaghan et al. 2015; Riera and Dillin 2015), the role of the haploid mitochondrial genome itself for the evolution of ageing and life span has often been dismissed because of its small size, low effective population size, the lack of recombination, and the presumed effectiveness of mechanisms ridding the germline from detrimental mtDNA mutations (Levin and Mishmar 2015). More generally, mtDNA variation within species has by tradition been considered neutral (Ballard and Rand 2005) and mtDNA was assumed not to play a prominent role in the evolution of ageing in the past (see Levin and Mishmar 2015 for a historic ac-

count). However, a growing body of experimental studies has now unveiled cases where within-species mtDNA variation is not functionally equivalent, but is linked to life span (James and Ballard 2003; Maklakov et al. 2006; Rand et al. 2006; Clancy 2008; Camus et al. 2012; Zhu et al. 2014; Immonen et al. 2016), metabolic (Arnqvist et al. 2010; Meiklejohn et al. 2013), and other life-history (Dowling et al. 2008) phenotypes. Many of these mtDNA effects are, to a large extent, expressed through intergenomic epistatic interactions with nuclear-encoded mitochondrial genes (Dobler et al. 2014; Levin et al. 2014; Wolff et al. 2014).

The combination of experimental evolution or artificial selection experiments with genotyping or genome sequencing represents one of the most powerful methods available to uncover the

This article is dedicated to the memory of Professor Nikola Tucić.

genetic basis of adaptation (Kawecki et al. 2012): associations between phenotypic evolution brought about experimentally and changes in allele frequencies provides a powerful way to uncover evolutionary genotype–phenotype links (Schlötterer et al. 2015). We tested whether the evolution of ageing and life span is associated with the evolution of mtDNA in an artificial selection experiment involving a nonmodel organism: the seed beetle *Acanthoscelides obtectus* (Coleoptera, Bruchinae). Replicated selection lines were founded already in 1986 from an outbred population harboring three distinct mtDNA haplotypes. Selection lines were then subjected to artificial selection for reproduction early or late in life (i.e., E and L lines) for 193 and 282 generations, respectively. These beetles have shown a dramatic evolutionary response to this divergent selection regime: detailed survival analyses have shown that beetles from the E lines now age about twice as rapidly and live for about half as long as do beetles from the L lines, and these differences apply to both sexes and reproductive states (Stojković et al. 2010; Stojković and Savković 2011; Đorđević et al. 2015, 2016). For example, the average life span of virgin females when housed alone are 10.3 days in E lines (rate of senescence = 0.65), 25.6 days in L lines (rate of senescence = 0.23) and 14.6 days in the unselected base population (Stojković et al. 2010; Stojković and Savković 2011). Using mitonuclear introgressive backcrossing experiments, we have elsewhere demonstrated links between mtDNA and life-history traits, including life span and mitochondrial bioenergetics across these selection lines (Đorđević et al. 2015, 2016) as well as across populations of other seed beetle species (Arnqvist et al. 2010; Kazancıoğlu and Arnqvist 2014; Løvlie et al. 2014; Immonen et al. 2016). Here, we thus ask whether mtDNA evolution is associated with life span evolution by genotyping samples of beetles from our selection lines, both with regards to mtDNA haplotype and a series of nuclear microsatellite loci, at two separate points in time.

Materials and Methods

SELECTION LINES

A base population of *A. obtectus* was established in 1983 by mass mating equal numbers of adults from three local subpopulations *A. obtectus* captured near Belgrade (Tucić et al. 1996). The base population was then maintained at large size ($N \approx 5000$ individuals) in the laboratory for three years (27 generations). Replicated selection lines were then established from the base population, using four replicates for each of two contrasting selection regimes: one in which phenotypic selection for long life was removed and one in which we selected for long life. Throughout the course of our long-term selection experiments, reproduction was permitted either in only very young beetles (E regime) or in individuals at age 10 days and above (L regime). In the E regime, where fitness

was accrued during days 1–2 of adult life, life span beyond two days thus had no effect on fitness. In the L regime, where fitness was accrued after day 10 of adult life, individuals with a life span shorter than 10 day had zero fitness. For a full description of the selection lines, we refer to Tucić et al. 1996 (see also Supporting Information).

GENOTYPING

Four hundred eighty randomly selected individuals were sampled from the selection lines to be used for molecular analysis. Each selection line (four replicate lines of each regime) was sampled at two chronological time points (generation 223/282 in E lines, generation 158/193 in L lines) and a sample of 30 beetles was collected from each line and time ($2 \times 2 \times 4 \times 30$ beetles). In addition, we sampled 60 beetles from the founding base population at generation E223/L158. Beetles were stored individually in 96% ethanol at 4°C until DNA extraction. Total DNA was extracted from each beetle using the sodium dodecyl sulfate (SDS) extraction method detailed in Rees et al. (2001). All individuals were then genotyped for three nuclear microsatellite loci (F01, F09, and E07), using PCR, and a single mitochondrial COI amplicon, using RFLP (see Supporting Information for details).

STATISTICAL EVALUATION

Our inferential strategy proceeded in two steps. Differences in mtDNA and microsatellite haplotype frequencies between the two time points within lines and between selection regimes across lines were first analyzed in a log-linear model of the four-way contingency table containing haplotype, time, selection treatment, and replicate line per selection treatment (i.e., $3 \times 2 \times 2 \times 4$). The inferential model contained all four terms, as well as the two focal interactions: haplotype \times time and haplotype \times selection. Given that the haplotype frequency data provided strong evidence for selection on mtDNA haplotypes (see below), we further characterized selection by parameterizing formal models of selection, which included elements of both positive (additive) selection and negative frequency-dependent selection. This was achieved in a series of Monte Carlo (MC) simulations of our selection lines, closely mimicking the demography during selection, using the Python-based simulation package simuPOP (version 1.1.1; Peng et al. 2012; Supporting Information). This was performed separately for the E and L lines under the general fitness model:

$$W = 1 + s - (tf),$$

where f denotes haplotype frequency, s represents additive, and t represents negative frequency-dependent components of the haplotype-specific relative fitness W . Due to the complexity of these MC simulations, we refer to Supporting Information for a more detailed account.

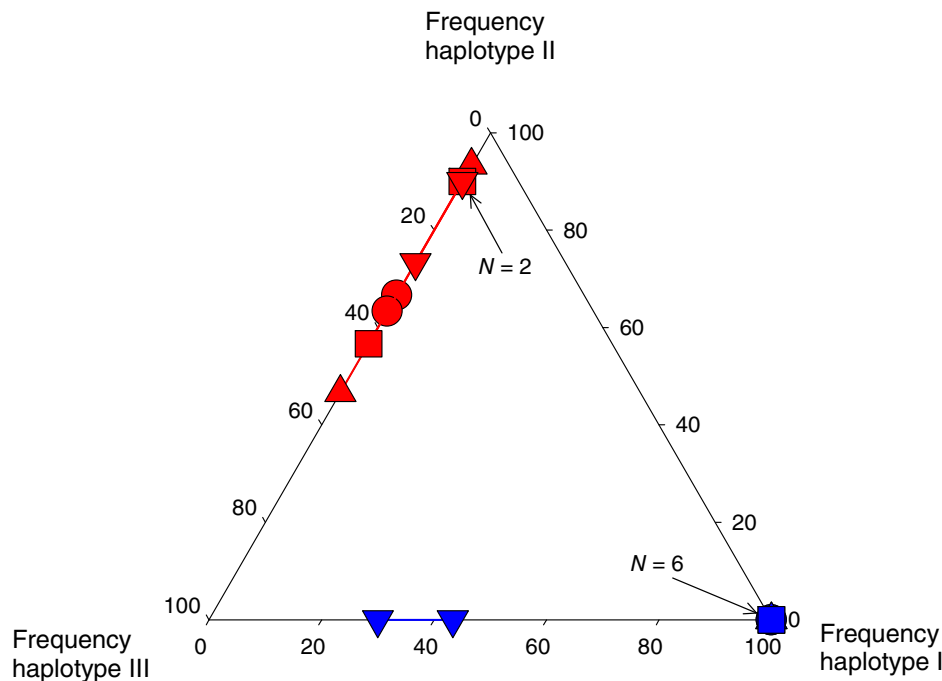


Figure 1. Ternary plot of mtDNA haplotype frequencies at two time points in the life span selection lines. Lines selected for short life ($N = 4$; red symbols) evolved very different mtDNA haplotype frequencies compared with lines selected for long life ($N = 4$; blue symbols; $G_2^2 = 472.44$, $P < 0.001$). Arrows indicate overlapping observations, the number of which is given. The two observations for each individual selection line share a unique symbol and are connected with solid lines.

Results and Discussion

We retrieved three distinct mtDNA haplotypes, named here I, II, and III, all of which were present in the base population (at the frequencies I:0.75, II:0.20, III:0.05) as well as in our selection lines. The distribution of mtDNA haplotypes across lines and selection regimes suggested that selection on life span generated differences in mtDNA haplotype frequencies (Fig. 1 and Table 1). For example, haplotype I was lost in all four E lines, whereas haplotype II was instead lost in all four L lines. A log-linear model showed that mtDNA haplotype frequencies changed over time ($G_2^2 = 9.58$, $P = 0.008$) and it confirmed that selection regime had a very strong and significant effect indeed on the observed mtDNA haplotype frequencies ($G_2^2 = 472.44$, $P < 0.001$). This general result was validated by analyses of molecular variance (AMOVAs): while 5.6% of mtDNA haplotype diversity was due to repeated sampling in time within lines ($P_{\text{rand}} < 0.001$), 63.7% was due to differences between selection regimes ($P_{\text{rand}} < 0.001$). Although the haplotype frequencies at the start of our selection experiment (i.e., in 1986) are unknown, the above analyses clearly show that the probability of seeing such divergent distribution of haplotypes 30 years later as a result of random processes is exceedingly low. Hence, we conclude that phenotypic selection on life span apparently translated into selection for specific mtDNA haplotypes.

Analogous log-linear models of the three microsatellite markers used (Supporting Information) showed that microsatellite allele frequencies did not change significantly over time for any of the loci (F01: $G_2^2 = 3.59$, $P = 0.166$; F09: $G_3^2 = 3.85$, $P = 0.278$; E07: $G_3^2 = 4.03$, $P = 0.258$), but differed highly significantly between the two selection regimes (F01: $G_2^2 = 21.71$, $P < 0.001$; F09: $G_3^2 = 91.04$, $P < 0.001$; E07: $G_3^2 = 520.54$, $P < 0.001$). AMOVAs showed that 4.1% of the total genetic variation in microsatellite loci was due to repeated sampling in time within lines ($P_{\text{rand}} = 0.001$), while 46.5% was due to differences across selection regimes ($P_{\text{rand}} < 0.001$). These findings are consistent with selection on nuclear loci. One possibility is that changes in mtDNA generated concerted changes in nuclear genes with mitochondrial function through mitonuclear epistasis (Trier et al. 2014; Bar-Yaacov et al. 2015).

Our MC simulations provided three additional insights (see Table 1). First, they confirmed that selection on specific mtDNA haplotypes imposed by age at reproduction was sizeable. Second, there was opposing selection on mtDNA haplotypes in the two selection regimes. Although positive selection favored haplotype I in the L selection regime, selection instead favored haplotypes II and III in the early regime. Third, they revealed that negative frequency dependent selection formed an integral part of net selection on haplotypes II and III in the early selection regime

Table 1. Frequency of the three mtDNA haplotypes (HI—HIII) in the selection lines ($N = 30$ for all samples) at two time points.

		Generation ↓	H I	H II	H III
Lines selected for early-life reproduction	E1	223	0	20	10
	E2	223	0	14	16
	E3	223	0	17	13
	E4	223	0	22	8
	E1	282	0	19	11
	E2	282	0	28	2
	E3	282	0	27	3
	E4	282	0	27	3
Selection coefficient	s		0	≥ 0.05	≥ 0.025
	t		0	≥ 0.05	≥ 0.025
Lines selected for late-life reproduction	L1	158	30	0	0
	L2	158	30	0	0
	L3	158	30	0	0
	L4	158	13	0	17
	L1	193	30	0	0
	L2	193	30	0	0
	L3	193	30	0	0
	L4	193	9	0	21
Selection coefficient ¹	s		≥ 0.025	0	0
	t		0	0	0

Given are also estimates of the minimal significant (at $\alpha = 0.05$) additive (s) and negative frequency dependent (t) components of haplotype-specific relative fitness in each selection regime. These represent the weakest selection coefficients that significantly improved Monte Carlo model fit to data over a null model involving only genetic drift (Supporting Information).

¹Estimates based on replicates L1–L3 (see Supporting Information).

(Table 1 and Supporting Information). The maintenance of these two mtDNA haplotypes in all four E lines for almost 300 generations was, in part, due to an inverse relationship between relative fitness and haplotype frequency within our replicate populations. The fact that selection on mtDNA haplotypes in the E lines, where phenotypic response to selection has been larger than in the L lines (Stojković et al. 2010; Stojković and Savković 2011), showed a negative frequency-dependent component is interesting in light of recent experimental evidence for negative frequency-dependent selection on mtDNA haplotypes in another seed beetle species (Kazancıoğlu and Arnqvist 2014) as well as in *Drosophila* (Arnqvist et al. 2016). Balancing selection through negative frequency dependency may represent an underappreciated contributor to the maintenance of genetic variation in mtDNA within populations.

Our results are in line with the general view that the mitochondrion has a central place in ageing (Guarente 2008), but they also provides direct support for a role for mtDNA in the evolution of life span and ageing. It has become increasingly clear that intraspecific genetic variation in mtDNA often affects a suite of life-history traits in seed beetles (Dowling et al. 2007; Arnqvist et al. 2010; Løvlie et al. 2014) as well as in other taxa

(James and Ballard 2003; Maklakov et al. 2006; Rand et al. 2006; Clancy 2008; Camus et al. 2012; Meiklejohn et al. 2013; Zhu et al. 2014; Kurbalija Novičić et al. 2015), including life span, growth rate, metabolic rate, behavior, viability, and even fitness. It therefore seems likely that the mitochondrion plays an important role not only for ageing, but more generally for multidimensional life-history syndromes (Ballard and Pichaud 2014; Løvlie et al. 2014). Presumably, these effects are caused by differences across mtDNA haplotypes in their effects on metabolic and bioenergetic processes (Pichaud et al. 2012). In fact, we show this to be the case in an accompanying study of these selection lines, where we use mitonuclear introgression to unravel mitochondrial genetic effects on mitochondrial bioenergetics (Đorđević et al. 2016). This implies that mtDNA genes that affect life span and ageing may have extensive pleiotropic effects. The prospects of ageing having a simple genetic architecture may thus seem rather dim, an interpretation which is underpinned by the fact that mtDNA genetic effects are typically embroiled within a complex web of interactions with both environmental factors (Arnqvist et al. 2010; Meiklejohn et al. 2013) and nuclear genes (Clancy 2008; Dobler et al. 2014; Levin et al. 2014; Wolff et al. 2014), sometimes with sex-specific effects (Jelić et al. 2015; Đorđević et al. 2016; Immonen et al. 2016). It is indeed very likely that the association between mtDNA evolution and ageing phenotypes seen in our experiment to a large extent involved important epistatic interactions with nuclear-encoded mitochondrial genes and, as a result, concerted evolution between mtDNA and the nuclear genome. This is consistent with the fact that nuclear markers also evolved in response to selection and supported by the results of introgressive cytonuclear backcrossing between these selection lines. The latter efforts unveiled a large effect of the nuclear genome, but also sizeable mitonuclear epistatic effects, on life span as well as on other life-history traits (see Đorđević et al. 2016). We note that mitonuclear epistasis may also have been responsible for the fact that selection on mtDNA in one of our populations selected for long life span (i.e., L4) apparently differed from the other three replicate populations in this selection regime (Supporting Information), assuming that the nuclear genetic background in this population differed from the others. However, other factors may also be involved and further experiments would clearly be required to unambiguously unveil the proximate causes of the distinct evolutionary path toward prolonged life span shown by the L4 population.

Our results could, in theory, have been affected by maternally inherited proteobacteria (i.e., *Wolbachia*), which affect fitness and cosegregate with mtDNA. Three facts collectively make this highly unlikely in our case. First, endosymbiotic bacterial infections have previously been screened for in many *A. obtectus* populations but have never been detected (Kageyama et al. 2010; Li et al. 2015). Second, we conducted PCR assays for the

presence of *Wolbachia* in our selection lines, but these were all negative (Supporting Information). Third, using in vitro assays of the activity of the multisubunit complex of the electron transport chain (ETC) involved in oxidative phosphorylation (OXPHOS) in mitochondria of beetles from these selection lines, we show elsewhere (Đorđević et al. 2016) that (i) selection regimes differ markedly in ETC activity and (ii) this difference is to a large extent due to cytogenetic and cytonuclear genetic effects. Because the mitochondrial ETC complex is in part built and regulated by mtDNA genes, this validates our findings and provides a likely mechanistic basis for our results. In this sense, we suggest that the results of Đorđević et al. (2016) provide support for a causal relationship underlying the association between mtDNA evolution and life span evolution seen here.

Uncovering the genetic basis of adaptation is challenging (Kawecki et al. 2012; Schlötterer et al. 2015), and this is particularly true for ageing phenotypes (Kenyon 2010). Associational studies can suggest causal links, but validation often requires the study of, for example, null mutants or RNAi knock down of particular candidate genes. We suggest that the link between the evolution of life span and mtDNA documented here is strengthened by the particularities of mtDNA. mtDNA carries several candidate genes for life span (Guarante 2008; Levin and Mishmar 2015) and its nonrecombining and haploid nature essentially renders it a potential life-history “supergene,” both in general (Ballard and Rand 2005; Ballard and Pichaud 2014) and in *A. obtectus* (Đorđević et al. 2016). The fact that it is maternally inherited and thus segregates independently of the nuclear genome in males also rules out confounding effects of selection on linked loci. We note that *A. obtectus* is a nonmodel species and neither its mitochondrial, nor its nuclear genome has been assembled. It is thus not possible to ascribe the effects seen here of entire mtDNA haplotypes to particular genes or mtDNA nucleotides (Camus et al. 2015). This said, naturally occurring sympatric mtDNA haplotype polymorphism in *A. obtectus* is known to harbor several nonsynonymous substitutions both in cytochrome oxidase subunit I and in cytochrome b, as well as substitutions in 12S rRNA and 16S rRNA (Alvarez et al. 2005, 2006; Oliveira et al. 2013), and a list of potential candidate genes would thus need to include these.

In conclusion, we show that divergent evolution of life span and ageing has involved the evolution of mitochondrial DNA in our selection lines. The fact that the phenotypic response to selection has been dramatic in these populations, as has genetic divergence in mtDNA haplotype frequencies, suggests that the phenotypic effects of mtDNA on life-span evolution can be significant and likely involves mitonuclear epistasis. Because mtDNA is haploid, shows maternal inheritance, does generally not recombine, is profoundly pleiotropic, and is involved in intergenomic epistatic interactions with the nuclear genome, our findings add a layer of complexity to the evolution of life span and ageing.

ACKNOWLEDGMENTS

We extend our deepest gratitude to the late N. Tucić, who was a mentor to several of us and started the E and L lines 30 years ago. We thank U. Friberg, A. Maklakov, and the GENCON laboratory group for comments and discussions. This contribution was financially supported by the European Research Council (GENCON AdG-294333; to GA), the Swedish Research Council (621-2010-5266, to GA), and the Serbian Ministry of Education, Science and Technological Development (grant no. 173007). The authors declare that they have no conflict of interest.

Accession Numbers

All relevant data are within the article and its Supplemental Information files. All sequence data are available from GenBank, accession numbers KU168414–KU168416.

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Associate Editor: T. Flatt
Handling Editor: M. Noor

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Three-dimensional mesh plots of the improvement in fit to the observed data compared to a null model with no selection (i.e., genetic drift alone) among all four replicate E lines, for MC models with different values of the additive- and frequency-dependent component of relative fitness of haplotype II (top panel) and III (bottom panel; range 0–1; increments of 0.05 shown).

Figure S2. Three-dimensional mesh plots of the improvement in fit to the observed data compared to a null model with no selection (i.e., genetic drift alone) among all four replicate L lines, for MC models with different values of the additive- and frequency-dependent component of relative fitness of haplotype I (top panel) and III (bottom panel; range 0–1; increments of 0.05 shown).

Figure S3. Three-dimensional mesh plots of the improvement in fit to the observed data compared to a null model with no selection (i.e., genetic drift alone) among three replicate L lines (excluding replicate L4) for MC models with different values of the additive- and frequency-dependent component of relative fitness of haplotype I (top panel) and III (bottom panel; range 0–0.2; increments of 0.025 shown).

Table S1. Absolute frequency of microsatellite genotypes across the three microsatellite loci in the eight selection lines in generation 223/158 (below) and 237/165 (overleaf).