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Effective population size in *Drosophila subobscura*: ecological and molecular approaches

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The effective population size (*Ne)* represents the number of individuals that can contribute genes equally to the next generation and is usually smaller then the actual size of a population. The aims of this study were: (i) to assess the *Ne* for two *Drosophila subobscura* populations sampled from geographically close, but ecologically and topologically distinct habitats, (ii) to compare the results obtained from two independent approaches for estimating *Ne* [an ecological (capture-mark-release-recapture) and a molecular (microsatellite data, linkage disequilibrium and heterozygote excess)], and (iii) to obtain a long-term *Ne* estimation using a variety of mutational models for *D. subobscura* populations. The ecological method showed that the beech wood population (B) had a significantly larger *Ne* compared to the oak wood population (O). Observed sex ratio was in favor of females in both populations studied. The microsatellite analysis of populations showed that short-term effective population size in beech wood was larger when compared to population from oak wood, which is in concordance with results obtained by the ecological method. Long term *Ne* of both natural populations is infinite according to a variety of mutational models. Our results confirmed concordance between ecological and molecular methods in *Ne* estimation, but also suggested that ecological approach showed less robustness. Molecular approach provides a promising opportunity for more effective monitoring of *Ne* in *Drosophila subobscura*. However, the biology, demography and history of the populations may affect different estimators differently so we suggest that ecological and molecular approach should be combined in *Ne* estimation.

Key words: capture-mark-release-recapture, microhabitats, microsatellites, sex ratio.

INTRODUCTION

The effective population size (*Ne*) is a key concept in population genetics (Charlesworth, 2009). The rate of inbreeding and genetic drift is determined by *N*e, which is defined as the number of individuals in an ideal population (i.e. with equal sex ratio and variance in family size approximated by a Poisson distribution) that would cause the same rate of inbreeding or genetic drift as that of the actual population in question (Frankham *et al.*, 2002). The effective population size is usually far lower then the *census* numbers of breeding individuals in a species (Frankham, 1995). There is evidence that temporal fluctuations of *N*e can occur in real populations, caused by and unequal sex ratio, high variance in family size and overlapping generations.

The *Ne* is an extremely useful concept for describing expected levels of genetic diversity and for evaluating the effect of different factors of the efficiency of selection (Petit & Barbadilla, 2009; Andolfatto *et al.*, 2011). However it is important to note that it has

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some limitations as a tool for understanding patterns of evolution and variation. For instance, certain aspects of genetic variability, such as frequency of individual nucleotide variants across different sites, cannot simply be described in terms of *Ne* (Charlesworth, 2009). Furthermore, reduction in variability caused by population bottleneck, a selective sweep or background selection might as well be associated with different variant frequency distributions and so can not be described by simple reduction in *Ne* (Braverman *et al*., 1995; Simonsen *et al.*, 1995; Gordo *et al*., 2002; Kaiser & Charlesworth, 2009).

Monitoring the *Ne*, which is parallel to monitoring genetic variability, is of significant importance to population genetic studies and is applicable in conservation strategies. Field based research in combination with laboratory research can provide quick and valuable sources of data in such analyses (Frankham, 1999).

Direct counting of the exact number of individuals of organisms is difficult if not impossible. However, sampling of populations to estimate their size has become an extremely important component of studies in population dynamics and structure in ecology and evolution. Capture-mark-release-recapture technique is often used to estimate population size for species that are mobile (i.e. insects) and thus cannot be counted directly (Krebs, 1989). *Ne* can be safely estimated from the proportion of marked and unmarked individuals under the following assumptions (Southwood, 1978): (i) the marked animals are not affected (neither in behavior nor in life expectancy); (ii) the marked animals are completely mixed in the population; (iii) the probability of capturing a marked animal is the same as that of capturing any member of the population and (iv) sampling must be at discrete time intervals and the actual time involved in taking the samples must be small in relation to the total time.

In the genomic era advanced molecular techniques hold a great promise for the application of genetic markers to the estimation of *Ne* in different natural populations (Xu & Fu, 2004). The use of microsatellites, a class of highly polymorphic genetic markers is valuable in assessing genetic variation at the population level (*e.g.* Bruford & Wayne, 1993). Microsatellite loci are distributed throughout the genome and are generally neutral unless linked to loci under strong selection. The effective population size can be obtained from microsatellite data using short-term *Ne* estimation methods based on linkage disequilibrium (Hill, 1981) and heterozygote excess (Pudovkin *et al*., 1996). Also, assuming Wright-Fisher mutational model it is possible to obtain measures of expected heterozygosity (Ohta & Kimura, 1973), which together with mutation rate can successfully be applied to obtain estimation of the long-term *Ne* using different mutational models.

Drosophila subobscura represents an important model species in evolutionary biology research. It has a broad Palearctic distribution and also occurs on some Atlantic islands and in the late 1970s colonized South and North America where it spread rapidly and successfully to its new environments. As a consequence, this species has developed clines for some chromosomal arrangements and for wing size similar to those found in Old World populations (Prevosti *et al*., 1988; Huey *et al*., 2000; Gilchrist *et al*., 2001).

Numerous studies have focused on population genetic structure screening of different natural populations of *D. subobscura*, based on inversion polymorphism (Prevosti, 1974; Krimbas, 1993; Andjelković *et al*., 2007; Kenig *et al*., 2010), allozymes (Marinković *et al*., 1978; Pinto *et al*., 1997; Castro *et al*., 1999) and mtDNA (Afonso *et al*., 1990; Latorre *et al*., 1992; García-Martínez *et al*., 1998; Castro *et al*., 1999; Jelić *et al*., 2012).

Furthermore, *D. subobscura* displays rich microsatellite polymorphism in all acrocentric chromosomes of the set (Pascual *et al*., 2001). The characterization of these markers in *Drosophila subobscura* confirms their high variability (Pascual *et al*., 2000; Santos *et al.*, 2010) and therefore makes them good candidates for a study of effective population size. Knowledge of *Ne* of *D. subobscura* populations originating from ecologically different habitats is important for understanding their current and future evolutionary potential for long term monitoring of microclimatic change in a habitat.

The aims of this study were: (i) to assess the effective population size for two *D. subobscura* populations sampled from geographically close, but ecologically and topologically distinct habitats, (ii) to compare *Ne* estimates obtained with an ecologically based (capture-mark-release-recapture) and a molecular based approach using microsatellite data (linkage disequilibrium and heterozygote excess), and (iii) to obtain a long-term *Ne* estimation of both population assuming that they represent a part of wider metapopulation of *D. subobscura.*

MATERIALS AND METHODS

Population samples

For the present study, *D. subobscura* flies were sampled from two localities (beech wood-B and oak wood-O) simultaneously at the end of June 2008. The beech wood-B (*Abieto fagetum*, 43°33′28.43″N and 20°45′ 10.96″E) and the oak wood-O (*Fraxineto quercetum*, 43°32′57.38″N and 20°40′02.32″E) are both situated in Mountain Goč (Central Serbia). These two wood habitats are closely located (at different expositions, approximately 6 km far from each other), but have quite distinct microclimate conditions. Beech wood features higher humidity with dense vegetation coverage, whereas the Oak has sparser trees and is slightly warmer. These two *D. subobscura* populations (B and O) represent good model populations, since both display rich inversion polymorphism in all acrocentric chromosomes of the set. Some literature data confirmed that inversion polymorphism is, to a certain degree, associated with the ecological factors at the spatial and temporal level (Živanović *et al*., 1995; Andjelković *et al*., 2003; Savković *et al*., 2004; Stamenkovic-Radak *et al*., 2008). Furthermore, both populations showed significant fluctuation in effective population size across certain period of time (Stamenkovic-Radak *et al*., 2008).

Effective population size – ecological approach

Sampling was performed at both locations (B and O), in the same manner and with the same equipment. Flies were attracted by fermented fruit traps. Ten traps were set 10 m apart in a rectangular formation in the beech wood, and in a square formation in the oak wood in relation to the release point which is in the center of formation. The use of two different formations was necessary due to topography and steepness of terrain. The area covered by traps was thus 400 m^2 in each habitat and the total area of the study site was 7000 m^2. The total studied area was calculated according to Begon (1976) and Loukas & Krimbas (1979), where 30 m were added in each direction for each trap in order to take into account the range of dispersion and trap attraction of *D. subobscura* flies (Taylor *et al*., 1984). The potential effect of weather conditions at the site (e.g. temperature, humidity, wind) is acknowledged, because they may affect the assessment of *Ne* by the ecological method (Krimbas, 1993; Serra *et al.*, 1987). The flies were captured with a net in the late afternoon peak of activity (between 18:00 and 20:30), counted without etherization, dusted with UV fluorescent dust (yellow) and released at a single release point, shortly before dusk. Late in the afternoon, on the day after release, flies were recollected with the same sampling approach, brought to the lab and identified for the presence of fluorescent dust using UV light and a stereo microscope.

The species and sex of all recaptured flies were identified and flies were counted. The proportions obtained in recaptured samples were used to approximate numbers of flies of each species and sex released on the first day. Among the captured flies, *D. subobscura* was the dominant species, present at 85-95% in each sample. The species ratio and sex ratio were the same in two consecutive days.

The adapted and modified ecological method for estimating effective population size (Begon, 1977) was used. Population size for each sex (*Nm* and *Nf*) was determined ($N = rn/m$, where r is the total number of marked and released flies, n is the total number of recaptured flies, and m is the number of recaptured marked flies of each sex). Assuming that the flies have 100% survival between the time of release and the time of recapture (24 hrs), the effective population size was calculated using the following formula: $Ne = (4Nm \times Nf)/(Nm + Nf)$. *Ne* was estimated for one generation per year considering that our species is active from March to October.

Effective population size – molecular approach

For the microsatellite screening of populations, we used only the flies collected in the wild (38 and 51 individuals derived from B and O, respectively). The flies were kept frozen at –20°C prior to DNA extraction. In order to estimate the effective population size of the two *D. subobscura* populations, we conducted the fragment analysis for eleven microsatellite loci following Pascual *et al.* (2000). Each chromosome was represented with either two microsatellite loci (A: dsub05 and dsub19; U: dsub03 and dsub15; E: dsub13 and dsub20; J: dsub18 and dsub27), or three microsatellite loci for the longest chromosome (O: dsub01, dsub2 and dsub04) (Santos *et al*., 2010). Genomic DNA was extracted using the protocol of Martinez *et al*. (1992), slightly altered (the alkaline treatment was skipped). Prior to PCR reaction pureness and concentration of DNA isolates was determined on Eppendorf biophotometer. PCR was conducted in four multiplex reactions (dsub27, dsub20 and dsub04; dsub01, dsub18 and dsub05; dsub02, dsub13 and

dsub19; and dsub03 and dsub15). Primer pairs used to amplify microsatellites were as in Pascual *et al.* (2000). Four different fluorescent dyes (FAM, NED, PET and VIC) were used to end-label one primer of each primer pair. PCR conditions were set following Pascual *et al.* (2001). A single soak at 95˚C for 5 min was followed by 30 cycles of 1 min at 95˚C, 30 s at 57˚C and 30 s at 72˚C. The exception was that a final elongation step of 30 min at 60˚C was included and 2 μl (50 μg μl^{-1}) of DNA were added in the total volume of 20 μl of PCR mix. GeneScan500-LIZ size standard was used as an internal size marker. Fragment analysis was conducted on an ABI Prism 3130 automated sequencer. Only distinct and reproducible, well-marked amplified peaks were included in the genetic analysis. The obtained data were analyzed with the GeneMapper software (Applied Biosystems, Foster City, CA, USA).

Effective population size (*N*e) was estimated from microsatellite data using short-term *Ne* estimation methods, namely linkage disequilibrium (Hill, 1981) and heterozygote excess (Pudovkin *et al*., 1996). The effective number of breeders using linkage disequilibrium data ($Nb_{(D)}$) was estimated using multiple pairs of unlinked loci. The standard linkage disequilibrium method (Hill, 1981) was recently shown to be biased when sample size is less than the true effective size (England *et al*., 2006). LDNE software (Waples & Do, 2008) contains an empirical correction that effectively eliminates the bias. The software can be downloaded from http://fish.washington.edu/xfer/LDNE/.

The effective number of breeders using Heterozygote Excess method ($Nb_{(H)}$) can be estimated examining the excess of heterozygotes in the sample compared to the proportion predicted under Hardy-Weinberg equilibrium (Pudovkin *et al*., 1996; Luikart & Cornuet, 1999). For the statistical data analysis we used NEESTIMATOR software (Peel *et al*., 2004; Ovenden *et al*., 2007, http://www.dpi.qld.gov.au/28_6908.htm).

The long-term *Ne* estimation (historical effective population size) on molecular data set was performed using expected heterozygosity assuming three different mutational models: (i) the infinite-alleles model [IAM: $H = 4Ne\mu/(1 + 4Ne\mu)$; Kimura & Crow, 1964], (ii) the stepwise mutation model [SMM: $H = 1 - (1 / (1 + 8Ne\mu)^{1/2})$; Ohta & Kimura, 1973] and, (iii) the single-step stepwise mutation model [SSMM: $var(nA) = 4Ne\mu$; Slatkin, 1995] was performed using data of variance of repeat number. The IAM assumes that each mutation creates a novel allele; the SMM assumes that new alleles arise by gain or loss of an integer number of repeat units; the SSMM consider that each mutation can increase or decrease the allele size by a single repeat, and it has been widely used as an approximation of the process underlying the genetic diversity at microsatellite loci (Goldstein *et al*., 1995; Slatkin, 1995; Zhivotovsky & Feldman, 1995).

The dinucleotide repeat mutation rate of *Drosophila melanogaster* (9.3×10^{-6} per locus per generation) empirically determined by Schug *et al.* (1998) was used, since it produces estimates of *N*e concordant with those obtained from sequences of single nuclear genes (Pascual *et al*., 2000). By rearranging the equations, solving for *N*e at each locus, and averaging across all 11 polymorphic loci, we obtained estimates for both sampled populations of *D. subobscura*. The expected heterozygosity for each of 11 loci for both populations was calculated in Arlequin 3.5. Software (http://cmpg.unibe.ch/software/arlequin35/Arl35Downloads.html, Excoffier *et al*., 2005). The variance in repeat number for each of loci was calculated using the freely download MICROSAT Software (http://genetics.stanford.edu/hpgl/projects/microsat/). The values of both the parameters (H and var(nA)) were evaluated for significance using non-parametric Mann-Whitney U test with the free download software PAST (http://folk.uio.no/ohammer/past/, Hammer *et al*., 2001).

RESULTS

The results of the ecological approach to estimate population size (*N*), effective population size (*Ne*) and their ratio (*Ne*/*N*) in two populations for both sexes are presented in Table 1. The results show that females outnumbered males in both B and O populations. The effective population size in beech popula-

TABLE 1. Ecological approach: Population size (*N*), effective population size (*Ne*) and their ratio in B (beech) and O (oak) population of *D. subobscura. Nf*: number of females; *Nm*: number of males

	N_f	N_m	*N*	*Ne*	*Ne*/*N*
Beech (B)	3419.18	1976.8	5395.98	5010.42	0.93
Oak (O)	106.55	35.87	142.42	107.34	0.75

TABLE 2. Details and variability of the microsatellite loci in Beech (B) and Oak (O) *D. subobscura* populations

				Beech				Oak		
Locus	allelic size range	chr.	alleles	H_O	H_E	p (χ^2 test)	alleles	H_O	H_E	p (χ^2 test)
dsub05	130-183	A	15	0.8571	0.9245	0.0049	13	0.7586	0.9020	0.0228
dsub04	176-214	O	17	0.7895	0.9014	0.0139	16	0.7708	0.8283	0.0040
dsub18	193-231	J	17	0.8684	0.8775	0.0001	13	0.7872	0.8364	0.0029
dsub27	213-266	J	20	0.8947	0.9126	0.0004	17	0.6735	0.8668	0.0431
dsub20	237-295	E	17	0.5790	0.8407	0.0815	15	0.3256	0.7841	0.2682
dsub01	257-289	O	15	0.7632	0.8811	0.0158	13	0.8913	0.8858	0.0000
dsub03	131-162	U	13	0.8684	0.8211	0.0027	11	0.9546	0.8286	0.0191
dsub13	99-150	E	15	0.9474	0.8351	0.0151	13	0.6222	0.6347	0.0002
dsub19	174-218	A	11	0.7143	0.8873	0.0337	9	0.7500	0.8734	0.0174
dsub02	203-265	O	18	0.7632	0.9197	0.0266	12	0.8837	0.8583	0.0008
dsub15	225-269	U	16	0.7632	0.8891	0.0178	12	0.8182	0.8383	0.0005
				$\Sigma = 8.8082$	$\Sigma = 9.6900$	$\Sigma = 0.2125$, p = n.s.		$\Sigma = 8.2357$	$\Sigma = 9.1367$	$\Sigma = 0.379$, p = n.s.

H_O: observed heterozygosity, H_E: expected heterozygosity, p: probability level for Hardy-Weinberg equilibrium (df = 10)

TABLE 3. Effective number of breeders in *D. subobscura* populations estimated with disequilibrium ($Nb_{(D)}$) and heterozygote excess ($Nb_{(H)}$) methods. S: sample size; L: number of loci; r^2: mean squared frequencies of at pairs of loci; 95% CI: confidence intervals for *Ne* estimates

			Linkage disequilibrium method			Heterozygote excess	
Population	S	L	r^2	$Nb_{(D)}$	95% CI	$Nb_{(H)}$	95% CI
Beech (B)	38	11	0.03617	154	110.3-245.8	infinite	not available
Oak (O)	51	11	0.03138	77	62.7-97.8	21.1	not available

TABLE 4. Molecular approach: estimation of effective population size (*Ne*) using microsatellite data. IAM: infinite-alleles model; SMM: stepwise mutation model; SSMM: single step stepwise mutation model

	IAM		SMM		SSMM	
locus	*Ne* (B)	*Ne* (O)	*Ne* (B)	*Ne* (O)	*Ne* (B)	*Ne* (O)
dsub05	329215	247422	2344605	1386649	825341	246022
dsub04	245752	129671	1369364	451641	485499	185770
dsub18	192633	137452	882616	489962	361487	143254
dsub27	280795	174963	1748140	745930	940200	306057
dsub20	141867	97646	516352	274990	806020	304108
dsub01	199110	208530	935772	1020471	269905	243712
dsub03	123337	129982	406586	445292	338488	110840
dsub13	136127	46708	482584	87315	572904	605729
dsub19	211728	185421	1044895	826613	246778	135413
dsub02	307676	162800	2086694	658602	878376	185025
dsub15	215558	139363	1088269	501535	426387	382011
Mean	*216709*	*150905*	*1173261*	*626273*	*559217*	*258904*

tion is higher (46×) compared to *Ne* in oak. The ratio *Ne*/*N* for B population showed high value (0.93) which suggests that effective population size is close to *census* size. However, in O population the ratio *Ne*/*N* showed lower value then 0.8 which might have been caused by a strongly unbalanced sex ratio in this particular year.

The microsatellite variability data represented by expected heterozygosity and variance in repeat number for locus/loci are presented in Table 2. The mean expected heterozygosity showed high values in both populations (0.830 and 0.881 for O and B, respectively). Population of *D. subobscura* beech wood had greater expected H value than oak population from the same mountain (Mann-Whitney U test: 0.881 *vs* 0.830, $Z = -2.101$, $p = 0.034$). The beech population showed higher variance in repeat number then oak populations (Mann-Whitney U test: 7.77 *vs* 3.72, $Z = -2.896$, $p = 0.002$).

The estimation of short-term effective number of breeders using linkage disequilibrium (LD) and heterozygote excess methods on microsatellite data are shown in Table 3. The results of LD analysis showed that Beech population size [$Nb_{(D)} = 154$, CI (95%) = 110.3 – 245.8] is twice higher compared to size of Oak population [$Nb_{(D)} = 77$, CI (95%) = 62.7 – 97.8]. The heterozygote excess method showed that Beech population has infinite effective number of breeders, however Oak population showed smaller effective population size ($Nb_{(H)} = 21$).

The estimation of long-term (historical) effective population size using microsatellite data due to three mutational models [infinite alleles model (IAM), stepwise mutation model (SMM) and single-step stepwise mutation model (SSMM)] are presented in Table 4.

The results based on microsatellite analysis showed the higher effective population size in Beech population compared to Oak but with different ratios for three mutation models (1.44×, 1.87× and 2.16× for IAM, SMM and SSMM, respectively). Also, the results showed extremely high values of effective population size for both sampled populations of *D. subobscura.*

DISCUSSION

The aim of the present study was to assess the effecive population size (*Ne*) using two independent approaches: ecological and molecular. *Ne* represents the average size of a population in terms of the number of individuals that can contribute genes equally to the next generation and is usually smaller than the actual population size. Theoretically, the ecologicaly based approach could be usefull to understand the effect of demographic characteristics of population (population size, sex ratio, mortality, etc.) and to assess the changes in effective population size caused by microhabitat and/or microclimatic variation, while the molecular approach provides an estimation of the expected rate of loss of genetic variation, the rate of increase in inbreeding, and the strength of selection required to counter the effect of genetic drift (Crow & Denniston, 1988).

The presented results of effective population size estimated by ecological method (capture-mark-release-recapture) showed that beech (B) population

has a significantly larger effective population size compared to oak (O) population which fit into data obtained for several years for the two populations under study (Stamenkovic-Radak *et al*., 2008). The effective population size of *D. subobscura* in beech wood can be considered as infinite (more that 4000 breeders) according to Wright (1943) and Begon *et al*. (1980). However, the population sampled in oak wood showed extremely low effective population size (less then 1000 individuals) and this was the lowest effective population size measured at that locality in the last eleven years (Stamenkovic-Radak *et al*., 2008). Extremely unequal sex-ratio and fluctuating population *census*, detected in the oak wood population, could lead to reduction in effective population size and consequently to reduction of genetic diversity and reproductive fitness of population (Woodworth *et al*., 1994).

The differences between numbers of each sex of *D. subobscura* obtained in this study are large in both populations, and have an obviously significant effect on *Ne.* The sex ratio differs between populations and is more unbalanced towards females in oak wood population. The abundance of females over males in both populations was detected also in previous seasons at both localities (Stamenkovic-Radak *et al*., 2008). That could be the result of the fact that females showed higher longevity than males (Christie *et al*., 2004). However, Christie *et al*. (2004) presented results obtained in controlled laboratory conditions and such explanation cannot be fully applied to natural conditions. Furthermore, the occurrence of biased sex-ratio can be of considerable ecological and evolutionary importance. This bias can result from X-linked meiotic drive that affects Y-bearing sperm and causes males to produce female progeny. This trait was described in several *Drosophila* species (Jaenike, 2001). Contrary to our findings, Pascual *et al.* (2004) presented that wild samples of *D. subobscura* yielded a significantly higher number of males than females during those months, when the species is more abundant. In other cases, they detected no significant deviation of 1:1 in sex proportion in wild populations.

The short-term effective population size obtained using microsatellite data of both sampled *D. subobscura* population is in concordance with previous literature data for lethal allelism for *D. subobscura* (Begon *et al*., 1980; Loukas *et al*., 1980; Mestres *et al*., 1990; Zivanovic *et al*., 2007; Araúz *et al.*, 2009). Most of these authors (Begon *et al*., 1980; Loukas *et al*., 1980; Mestres *et al*., 1990; Araúz *et al.*, 2009) suggested that the population of *D. subobscura* is extremely large and effectively infinite. However, Zivanovic *et al.* (2007) found that the *Ne* values varied greatly from 370 to 19413 and that was attributed to the habitat conditions and some environmental factors. In our study, both short-term methods for *Ne* estimation (Linkage Disequilibrium and Heterozygote Excess methods) performed on microsatellite data showed

TABLE 5. Comparison of ecological and molecular approaches

	Ecological approach	Molecular approach
	Capture-mark-release-recapture	Genome wide research
Description:	Usefull to asseses the demographic *Ne* and to understand the effect of demographic characteristics of population (population size, sex ratio, mortality etc.) and to detect the changes in effective population size caused by microhabitat and/or microclimatic variation	Provides an estimation of the expected rate of loss of genetic variation, the rate of increase in inbreeding, and the strength of selection required to counter the effect of genetic drift by using short and long term evaluation of *Ne*
Field/laboratory work:	field work field and laboratory work	
Output information:	*N*, *Ne*, *N*/*Ne*, sex ratio, demographic parameters	*Ne*, migration rate, bottleneck evaluation
Sample size:	Large sample size	Small/moderate sample size
Cost:	cheap time consuming	expensive efficient
Disadvantages:	Fluctuation in weather condition (temperature, precipitation, wind etc.) during sampling and re-sampling	*Ne* valuation dependence of short or long term evaluation of effective population size

significantly larger population size in beech wood compared to population from oak wood which is also in concordance with results obtained by ecological method. However, it should be noted that different methods are not necessarily estimating the same *Ne* because they are subjected to different bias. The heterozygosity excess methods estimate the effective size of the parental population; the LD methods infer the short- to intermediate-term (mean) effective population size, the length of the term being dependent on the linkage between markers, while the methods considering the mutational process explicitly estimate the long-term *Ne* in the past on a time-scale of the order of *Ne* generations.

The long term *Ne* estimates from current genetic variation of beech and oak populations have been derived independently from a variety of mutational models (IAM, SMM and SSMM) and we have detected the same *Ne* ratio between populations but three different *Ne* values. Colson & Goldstein (1999) suggested that most alleles are generated by gain or loss of a repeat unit according to SMM model, while some alleles originate by more complex mutations, as observed by Van Oppen *et al.* (2000) using a different approach. For microsatellites, the widely used mutation model is the single stepwise mutation model (SSMM; Ohta & Kimura, 1973), which assumes that a mutation leads to one repeat unit increase or decrease in allele size with an equal probability. The effective population size of both populations in the present study evaluated using three models predicted significantly larger *Ne* in beech compared to oak population. Therefore, based on microsatellite data, it is quite clear that the long-term *Ne* of these natural populations of *D. subobscura* is extremely large.

Regarding molecular approach, it is important to understand the time-scales behind each method, because natural populations rarely have constant *Ne*; rather, they are dynamic entities changing significantly in size and distribution over time. Therefore, using the same data, different methods could yield considerably different estimates of *Ne*. Additionally, the biology, demography and history of the populations may affect different estimators differently. *Ne* is a parameter summarizing the effects of many other demographic parameters in determining a given genetic property of the population (Caballero, 1994). In some cases, such a highly summary parameter is desirable, simplifying both the explanation of the pattern and amount of genetic variation observed in a population and the prediction of the genetic properties (such as loss of variation, fixation probability, changes in fitness of the population) in the future. In other cases, however, it is more helpful to know the details that determine *Ne*. This is especially true in conservation biology, where appropriate management can be exercised only when detailed knowledge of the population is available. In the present scheme, we showed the advantage/disadvantage of both, ecological and molecular approach (Table 5).

Finally, our results suggest that both, ecological and molecular approaches combined provide a great opportunity for more precise monitoring of effective population size in *Drosophila subobscura*. However, in general, careful consideration of the natural history of each particular population or species must also dictate the selection of the most appropriate analytical method for *Ne* monitoring efforts.

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