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Jarkko Routtu

Genetic and Phenotypic Divergence in *Drosophila virilis* and *D. montana*

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Jari Haimi
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ABSTRACT

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Diss.

Genetic and phenotypic differentiation of *Drosophila virilis* and *D. montana* populations has proceeded along evolutionarily different pathways. *D. virilis*, "a human commensal", does not show clear diversity or population structure in mtDNA haplotypes, which refers to population growth. Microsatellites group the laboratory strains of this species into four clusters according to their geographical origin, indicating recent population differentiation. In contrast, *D. montana*, a "wild" species, has populations in Europe and North-America that are genetically diverged. In this species the mtDNA haplotypes are mixed among the two North-American populations, while microsatellites separate all study populations from each other. The courtship songs of the *D. virilis* laboratory strains showed significant inter-strain and geographic variation in several song traits. The genetic distances and the song divergence of the strains did not show significant association, which suggests that the songs have not diverged solely as a side-effect of genetic divergence. In *D. montana* the songs of the laboratory strains from different continents showed the highest divergence in song frequency, while the songs of the wild populations varied most prominently in the remaining pulse characters. *D. montana* populations also demonstrated divergence in male wing and genital morphology. The phenotypic divergence among populations did not coincide with the extent of their genetic divergence, suggesting that the first-mentioned traits are not evolving neutrally. Finally, we constructed a species recognition method for North European *D. virilis* group species to ease the identification of wild-collected flies and to detect possible misclassifications of laboratory strains.

Keywords: Genital morphology; male courtship song; natural selection; phylogeography; sexual selection; speciation; wing morphology.

Jarkko Routtu, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address Jarkko Routtu
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
jarkko.routtu@bytl.jyu.fi

Supervisors Prof. Anneli Hoikkala
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Dr. Maaria Kankare
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Reviewers Dr. Helena Korpelainen
Department of Applied Biology
P.O. Box 27
FI-00014 University of Helsinki
Finland

Prof. Perttu Seppä
Department of Biological and Environmental Sciences
P.O. Box 65
FI-00014 University of Helsinki
Finland

Opponent Prof. Leo Beukeboom
Evolutionary Genetics Group
Centre for Ecological and Evolutionary Studies
University of Groningen
P.O. Box 14
NL-9750 AA Haren
The Netherlands

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following articles which are referred to in the text by roman numbers. I have participated in sequencing and analysing mtDNA for articles I and III and recording the fly songs for article II. In addition I took part in writing these articles. I have contributed significantly to planning, data collection, analysis and writing articles IV and V, where I am the first author.

I Mirol, P.M., Routtu, J., Hoikkala, A. and Butlin, R.K. (2007). Signals of demographic expansion in *Drosophila virilis.* Submitted manuscript.

II Huttunen, S., Aspi, J., Schlötterer, C., Routtu, J. and Hoikkala, A. (2007). Variation in male courtship song traits in *Drosophila virilis*: the effects of selection and drift on song divergence at intraspecific level. Submitted manuscript.

III Mirol, P., Schäfer, M.A., Orsini, L., Routtu, J., Schlötterer, C., Hoikkala, A. and Butlin, R.K. 2007. Phylogeographic patterns in *Drosophila montana.* Mol. Ecol. 16 (5): 1085-1097.

IV Routtu, J., Mazzi, D., van der Linde, K., Mirol, P., Butlin, R.K. and Hoikkala, A. 2007. The extent of variation in male song, wing and genital characters among allopatric *Drosophila montana* populations. J. Evol. Biol. 20 (4): 1591-1601.

V Routtu, J., Hoikkala, A. and Kankare, M. (2007). Microsatellite-based species identification method for *Drosophila virilis* group species. Submitted manuscript.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
CN	the number of cycles in a sound pulse
DA	discriminant analysis
DNA	deoxyribonucleic acid
EFD	an elliptical Fourier descriptor
FRE	the carrier frequency of the song
F_{ST}	genetic differentiation of neutral markers
GPA	generalized procrustes analysis
IPI	the interpulse interval
LM	a landmark
mtDNA	mitochondrial DNA
OL	an outline (pseudo)landmark
PC	a principle component
PCA	principle component analysis
PL	the length of a sound pulse
PN	the number of sound pulses in a pulse train
PTL	the length of a pulse train
Q_{ST}	genetic differentiation of quantitative traits
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism

1 INTRODUCTION

The role of allopatry in speciation events has been demonstrated early on in evolutionary theory (Darwin 1859), and Mayr (1963) has especially emphasized its significance in speciation. Mayr (1954) showed, for example, that the mainland populations of New Guinean kingfishers do not exhibit subpopulation level differentiation, while the neighbouring geographically isolated islands harbour phenotypically differentiated subspecies. Maynard-Smith (1966) demonstrated that under special circumstances speciation may in theory occur in a sympatric situation, and Bush (1969) presented the first empirical evidence of the *Rhagoletis* fruit fly's sympatric speciation on different host species. While the majority of empirical data supports allopatric speciation, there is now abundant evidence that both types of speciation events occur (Coyne & Orr 2004). Areas with complex topography that hinder migration are often hotspots for speciation. The Hawaiian islands and Amazonian rainforest are examples of areas where mountain ridges, the ocean and meandering rivers create obstacles for migrating individuals (Salo et al. 1986, Wagner & Funk 1995). Boreal areas, which lack the ample energy resources of wet tropical areas, may experience lower speciation rates even in the presence of complex geography. These areas have, on the other hand, been influenced by expanding and retreating glaciers of the Pleistocene (Hewitt 1999).

Given enough time, genetic and phenotypic differences between populations accumulate and at some stage lead to speciation. Biological speciation is the development of a reproductive isolation barrier between differentiated populations before (prezygotic) or after (postzygotic) fertilization of an egg cell, fuelled by an increase of developmental disturbances in hybrids. In an allopatric situation, pre- and postzygotic barriers accumulate at a near equal pace, while in sympatry, prezygotic barriers become the dominant form of isolation (Coyne & Orr 1997). Development of isolating barriers in sympatric or parapatric situations is a direct consequence of continuous hybrid formation, which creates a strong selective response to reduced fitness of interbreeding individuals. Any behavioural, morphological or physiological changes in characters that prevent mating with non-conspecifics are favoured (Ritchie et al.

1989, Butlin & Ritchie 1991). The process called reinforcement enhances and stabilises species-specific mating signals especially in areas where populations that have diverged come in to secondary contact (Butlin et al. 1991).

The role of sexual selection is essential in speciation of promiscuous species. Fisher's run-a-way process and selection for "good genes" are examples of mechanisms that may enhance changes associated with sexual signals (Fisher 1930, Zahavi & Zahavi 1997). The theories postulate that the trait(s) for which a female has a preference for will increase in frequency in the given population. However, a character that has initially arisen through sexual selection may get fixed in a population and lose its information value to the female in selection for "good genes", which may lead to changes in the target of female preference (Klappert et al. 2007). These selective processes are characterized by an arms race of both sexes, as the benefit of a male often does not coincide with that of a female (Parker 1979). It can be said that the line between sexual selection and conflict is subtle (Arnqvist & Rowe 2005). In this framework, it is not surprising that speciation rates of promiscuous species are elevated (Arnqvist et al. 2000).

Integration of genetic methods to speciation and evolutionary ecology studies has made the previously unattainable estimation of neutral population differentiation, size, history and migration straight from DNA sequences possible. This has opened up new possibilities in speciation studies, where the estimation of genetic differentiation and gene flow between populations and species is of primary concern. In fact, the estimation of neutral genetic differentiation forms the base line for inferring the levels of selection that different characters undergo (Merilä & Crnokrak 2001).

1.1 Genetic divergence

A wide variety of molecular markers is used to estimate genetic differentiation of populations. These include RFLP, RAPD, AFLP, microsatellites and mtDNA sequences. The two latter markers have the highest impact in ecological and evolutionary studies of animal species. The level of neutral genetic differentiation of populations is best estimated with neutral or nearly neutral markers like mtDNA sequences and microsatellites. Recently, new methods, like the DNA microarray technology, have become available for ecological and evolutionary studies (Gibson 2002). Although still very costly, microarrays reveal the exact genes involved in a particular phenotypic change by measuring their expression patterns.

Long term population demographic history and species phylogenies are best inferred by mtDNA haplotypes. There are a few qualities that make mtDNA sequencing in animals an especially attractive marker for evolutionary research. DNA sequences of mtDNA have higher replication and mutation rates than nuclear DNA. All alleles are haploid, which is a great benefit for DNA sequencing, though sometimes nuclear copies of mtDNA may cause problems (Gellissen et al. 1983). In most animal species, cytoplasm and mtDNA are

maternally inherited, which prevents a conflict of two parental cytoplasts in offspring (Hurst & Hamilton 1992). Selection acting on mtDNA is thought to be restricted, even though recent studies have cast some doubt on the neutrality of mtDNA variation (Nigro 1994, Hurst and Jiggins 2005, Dowling et al. 2007).

Present-day knowledge of nuclear DNA evolution predicts that so called "junk" or non-coding DNA is mostly functionally neutral and thus no selection pressures act upon it (but see Cheng et al. 2005, Rigoutsos et al. 2006). Most microsatellites are located in non-coding DNA, while microsatellites found close to the promoter area or within the coding region of the genes can cause drastic changes in the phenotype (Yu et al. 1992). Microsatellites consist of 2-6 bp motifs that are arranged as repetitive structures scattered randomly all over a nuclear genome. They are codominant markers and are therefore a preferred marker type in studies of recent history in population demography and in the analysis of population genetic structures. The mutation rate of microsatellites is elevated compared to the rest of the nuclear DNA due to a slipped-strand mispairing which acts as the chief mutational force (Tautz 1989). The mechanism ensures the continuous inflow of polymorphic alleles to a population, but the drawback of the system is the downward direction of mutations in long repeats (Harr & Schlötterer 2000). The result of this is that probability of homoplasy (i.e. convergent evolution of the size of the alleles) increases when the evolutionary distance between the alleles grows longer.

A combination of different molecular marker types offers an excellent way to estimate the demographic history and phylogeography of populations. Microsatellites have limitations, which can at least partly be bypassed with mtDNA and *vice versa*. The two markers also give information on population history on a different time scale. For example, in the *D. virilis* group, the existing estimates of population phylogeographic history based on molecular markers fit well into the overall picture of genetic changes induced by inter- and post-glacial periods of the Pleistocene (Spicer & Bell 2002, Hewitt 2004).

1.2 Phenotypic divergence

Behavioural mating signals change fast in early stages of speciation when populations that have genetically diverged come into secondary contact with each other (Dobzhansky 1951). Also, promiscuity combined with sexual selection can lead to rapid changes in behaviour and morphology, like in the cichlids of Lake Victoria (Seehausen & van Alphen 1999). During the courtship, the males and females emit various kinds of species-specific stimuli (e.g. courtship songs) to each other. The songs may play an important role in sexual selection and/or in species recognition, and they may simultaneously be affected by directional, diversifying and balancing selection. The signals important in species recognition may not, however, vary too much if they are to retain species-specificity (Lambert & Henderson 1986). Lande (1982) has shown that the evolution of directional female mating preferences for male secondary

sexual characters can greatly amplify large-scale geographic variation in male characters. This coevolutionary process can be enhanced by variation in the strength of direct or indirect selection on female preferences through the species distribution area (see Houde 1993). For example in fish, a female sensory bias for yellow prey items may cause an indirect selection of female preference for yellow male colouration (Garcia & Ramirez 2005). At the species level, diversifying selection may speed up the evolution of species-specific courtship songs and might increase the effectiveness of prezygotic sexual isolation between sympatric species (Etges et al. 2006).

Depending on the nature of a behavioural signal, the signal and morphology related to it may co-evolve and thus cause concordant patterns. *Drosophila* flies use their wings for flying and the production of species-specific male courtship songs. Even though these are not exclusive functions, some characters of the wing may be affected mainly by sexual selection and other characters by natural selection. Variation in wing shape can also lead to functionally identical outcomes with various internal structural rearrangements due to drift or neutral diversification. For example, the convergence of clinal variation in wing size of *D. subobscura* on different continents has been achieved through analogous changes in the relative lengths of different parts of the wing (Huey et al. 2000). Additionally, wing traits have been found to evolve quite rapidly in response to geographic clines, e.g. in *Drosophila subobscura* (Huey et al. 2000, Gilchrist et al. 2000), and they have also been found to respond well to artificial selection (Houle et al. 2003, Kennington et al. 2003). These rapid changes are not surprising because of the abundant genetic variation related to wing shape (Mezey & Houle 2005, Weber et al. 2005).

The size and shape of male genitalia are rapidly evolving species-specific characters and they are often used for species identification, e.g. in *Drosophila* species (Grimaldi 1990). Genitalia have been suggested to evolve via lock-key mechanics (Dufour 1844), pleiotropy (Mayr 1963) or cryptic female choice (Eberhard 1996). The striking morphological diversity of genitalia may also have arisen through sperm competition (Parker 1970) or sexual conflict (Hosken & Stockley 2004, Arnqvist & Rowe 2005). The highest diversity in genitalia would be expected to evolve in promiscuous species which have weak sexual selection on other traits or whose genitalic behaviour maintains a significant role in mating (Peretti et al. 2006). Genital morphology is likely to be influenced by many of the above-mentioned mechanisms at the same time with varying intensities.

1.3 Why to study *Drosophila virilis* group species?

The annotated nucleotide sequence of the genetic model organism *Drosophila melanogaster* (Adams et al. 2000) is complete and also for several other *Drosophila* species, e.g. for *D. virilis*, total genomic sequences are available. The new genetic tools developed for *D. melanogaster*, as well as the availability of genetic

information, also enables comparative studies in genetically less well-known *Drosophila* species with ecologically and behaviourally interesting characters.

The *D. virilis* group is comprised of 12 species or subspecies that originated somewhere in the ancient deciduous forests of China or in the arid regions of Iran or Afghanistan (Throckmorton 1982). *D. virilis*, the name species of the group, is a domestic species distributed mainly south of latitude 35°N. *D. montana*, on the contrary, is found in natural boreal forest habitats close to water. It is distributed mainly north of latitude 40°N, with southern populations at higher elevations (Fig. 1). *D. virilis* possesses a primitive karyotype of the *D. virilis* group and in contrast to other species of the group, it shows no inversion polymorphism (Throckmorton 1982). However, *D. montana* has a large amount of inversion polymorphisms within and between populations (e.g. Moorhead, 1954, Morales-Hojas et al. 2007). This contrast between our study species is interesting in terms of the possible role of chromosomal inversions in speciation (Butlin 2005).

The males of all species of the *D. virilis* group produce acoustic signals, courtship songs, by vibrating their wings during the courtship rituals. These songs play an important role both in species recognition (Liimatainen & Hoikkala 1998) and in sexual selection (Aspi & Hoikkala 1995). Song characteristics vary much more among the *montana* phylad species than among the *virilis* phylad species (Hoikkala & Lumme 1987). Also, the importance of the courtship song varies between the species: *D. virilis* females accept the courting male even without hearing his song (e.g. Saarikettu et al. 2006), while *D. montana* females do not accept the courtship of a "mute" (wingless) male (Hoikkala 1988, Liimatainen et al. 1992).

FIGURE 1 Proposed distribution areas of *D. montana* (light grey) and *D. virilis* (dark grey) in the wild and their overlapping distribution areas in East Asia (black). In addition, *D. virilis* is found in breweries and market places around the northern hemisphere. Areas with only few observations are marked with a question mark (chart modified from Throckmorton (1982)). Collection sites of fresh *D. montana* strains (article IV) are marked as black squares in Canada (Vancouver), USA (Colorado) and Finland (Oulanka).

1.4 Objectives of the project

The main objective of this thesis was to study phenotypic divergence of *D. virilis* and *D. montana* populations in a phylogeographic framework. The extent of variations in male courtship song, wing and genital traits were compared to the genetic divergence of laboratory strains and wild populations of the species to find out whether the studied traits had evolved neutrally or whether their evolution had been enhanced by selection.

The first objective of the project was to analyse neutral genetic differentiation among the populations of *D. virilis* and *D. montana*. The data on variation in mtDNA haplotypes and microsatellite loci of these species were used primarily to estimate their phylogeography and demographic history (articles I, II & III).

The second objective of the project was to analyse the phenotypic divergence of male courtship song traits in *D. virilis* populations and male song traits as well as the size and shape of wings and genitalia in *D. montana* populations (II &IV). The data on neutral genetic divergence of conspecific

populations (I & III) was used as a reference for comparing the levels of genotypic and phenotypic divergence between populations.

The last part of the project concentrated on finding a fast and accurate molecular identification method for the North European *D. virilis* group species, as these species are very difficult to distinguish morphologically (V). So far, the species identification has been done on the basis of male genitalia, female spermathecae, RAPD fingerprinting, protein gel electrophoresis, and by mating wild caught females to laboratory males or by analysing the male courtship songs. All of these methods are very time consuming, not accurate enough and/or require special expertise. Recently there has been also interest in DNA barcoding of all species which based on sequencing mtDNA *COI* gene. However, this method does not have potential for population genetic or paternity studies like microsatellites.

2 MATERIALS AND METHODS

2.1 Study species

D. virilis strains used in this study originated from the whole distribution area of the species, from Europe to North America. The 52 isofemale strains have been collected during a time period covering almost 90 years. The most recent strains are from Chinese breweries in 2002 and from a Japanese lumberyard in 2003 (I & II). *D. montana* laboratory strains used in article III have been collected from various parts of the species distribution area during a time period of 60 years. Fresh isofemale strains of this species were established from mated females collected from three locations (Oulanka, Colorado and Vancouver) in 2003 (IV). Detailed lists of the species and strains used in this study are attached at the end of each article and manuscript.

2.2 Data analysis

2.2.1 Molecular data

Two mitochondrial genes, *cytochrome oxidase I* and *cytochrome oxidase II*, were sequenced for the subsequent analyses of *D. virilis* and *D. montana*. ARLEQUIN 2.0 (Schneider et al. 2000) was used to calculate pairwise distances between the haplotypes and the mismatch distributions. It was also used to perform Tajima's D (1989) and Fu's F (Fu & Li 1993) tests of the standard neutral model for a demographically stable population. The program FLUCTUATE (Kuhner et al. 1998) was used to make simultaneous estimates of present day θ and population growth rate g. The parameters used for the simulations were obtained by running a hierarchy of likelihood-ratio tests in Modeltest 3.0 (Posada & Crandall 1998) to choose the model of evolution with the best fit to the data. Phylogenetic trees were obtained using maximum likelihood with the molecular clock enforced in PAUP 4.0b10 (Swofford 1996), while skyline plots

were constructed using GENIE v. 3.0 (Pybus et al. 2000). GENIE was also used to calculate the fit to different models of population growth using the corrected Akaike Information Criterion. Networks of haplotypes were constructed based on statistical parsimony using the program TCS 1.06 (Clement et al. 2000).

We used for *D. virilis* (paper II) 48 microsatellites distributed along the chromosomes (see the linkage map of Huttunen et al. 2004). A linkage disequilibrium test for pairs of these loci in ARLEQUIN 2.0 (Schneider et al. 2000) showed no linkage disequilibrium in European and American groups and only small fraction of loci had significant linkage disequilibrium in Asian and Japanese groups after Bonferroni correction. Also the 16 microsatellites of *D. montana* were chosen partly on the basis of their location on different chromosomes (Schäfer et al. in preparation, III). In both species, microsatellites were used to estimate recent neutral genetic differentiation of populations. Genetic differentiation between populations was calculated using F statistics according to Weir & Cockerham (1984). Basic measures of microsatellite variability were calculated using the Microsatellite Analyser Program (MSA; Dieringer & Schlötterer 2003). The amount of genetic variation resulting from differentiation between continents relative the genetic variation from geographical separation within continents was estimated with AMOVA in ARLEQUIN 2.0. Population substructure was studied with BAPS 2.0 (Corander et al. 2003) and STRUCTURE (Pritchard et al. 2000) programs depending on which suited better for the data in question. Using individuals of old laboratory strains in phylogeographic analysis caused some troubles in data analysis. Accordingly, we have used "group" in stead of "population" when clustering the strains from different geographic areas.

2.2.2 Phenotypic data

Male courtship songs were recorded on a tape recorder at a temperature of 20±1°C and analysed with the Signal 4.0 sound analysis system (Engineering Design, Belmont, MA, USA). The analysed song traits were the number of pulses in a pulse train (PN), the length of a pulse train (PTL), the length of a sound pulse (PL), the interpulse interval (IPI, the length of the time from the beginning of one pulse to the beginning of the next one), the number of cycles in a sound pulse (CN) and the carrier frequency of the song (FRE).

The position of the wing veins was extracted from the image of a wing. The wing landmark data were aligned using the Generalized Procrustes Analysis (GPA). Centroid size was retained as a scaling variable so that size-dependent changes in shape could be explored. Two types of data were extracted from the wings: 15 (pseudo)landmarks describing the outlines of the wing (OL) and 12 landmarks (LM) describing the junctions between wing veins or between the veins and the outline of the wing (each having x and y coordinates).

Digital images of the genitalia were analysed with the SHAPE 1.2 program (Iwata & Ukai 2002), which is a based in Principal Component Analysis (PCA) performed on elliptical Fourier descriptors (EFD) of an enclosed contour (Kuhl

& Giardina 1982). The resulting normalised Principal Component (PC) scores can be used as measured trait values that include only the allometric variation.

The structure of the phenotypic data was nested; individuals within isofemale strains and strains within populations. Subsequent analyses of variance were done using nested ANOVAs as described in Sokal and Rohlf (1997). The direction of population differentiation was estimated with linear discriminant analysis (DA). The calculated strain means for different traits were used in DA to avoid pseudoreplication.

2.3 *D. virilis* group species identification

The species identification method was based on 14 microsatellite loci. A genetic distance tree was constructed with GenAlEx (Peakall & Smouse 2006) and visualised with MEGA (Kumar et al. 2004). Clustering of species and populations in groups was done using a Bayesian maximum likelihood based program, STRUCTURE (Pritchard et al. 2000). Identification of North European *D. virilis* group species was based on one locus.

3 RESULTS AND DISCUSSION

Global populations of *D. virilis* and *D. montana* showed moderate to high genetic and phenotypic differentiation. The estimated divergence time of *D. montana* populations on different continents appeared to be vastly longer than that of *D. virilis* populations. High genetic divergence of *D. montana* populations on different continents has not, however, led to high phenotypic divergence compared to that of populations on the same continent.

3.1 Phylogeographic patterns

3.1.1 Mitochondrial haplotypes

Mitochondrial haplotypes of *D. virilis* strains from different parts of the species distribution area did not show any geographic structure (I). A low number of substitutions shared among locations suggests a rapid population expansion over a relatively short time-scale (~50,000 years with a mutation rate of 10^{-8} per year). This time-scale is consistent with an expansion of the human population and may either reflect a shift of the flies into domestic environments or postglacial colonization. The central haplotype of the network can be found around the species distribution area and thus it gives no information on the geographic origin of the species. In their study on sequence variation for six X-linked genes in 21 *D. virilis* strains from different continents, Vieira and Charlesworth (1999) found no fixed differences between the Asian strains and strains originating from Europe, North- and South-America. The fact that all the variants found outside Asia were also present in Asia, but not vice versa, refers to a large population centered in Asia with smaller migrant populations elsewhere (Vieira & Charlesworth, 1999). According to Throckmorton (1982), *D. virilis* group species originate from an ancestral form in Asia, with the most primitive species of the *virilis-repleta* section observed in South-East Asia.

Unlike *D. virilis*, *D. montana* possessed some geographic structure. mtDNA analysis indicates the presence of at least two distinct populations, one in

Eurasia and the other one representing the expansion of the species to the New World (III). The mtDNA haplotypes found in North America formed a clade nesting within the more diverse set of haplotypes present in Finland, supporting the idea that the species has spread from Eurasia to North-America (Throckmorton 1982). Genetic distances between the two major mtDNA clades range from 0.9% to 1.8%, which, assuming a mitochondrial divergence rate of 2 % per million years, implies a separation of 450,000 to 900,000 years. Päällysaho et al. (2005) obtained congruent divergence times between Finnish and American *D. montana* populations based on the silent substitutions on three *X*-linked genes (*fused*, *elav* and *su(s)*).

3.1.2 Microsatellites

Microsatellite loci, contrary to the mtDNA haplotype data, gave support to the population structure in *D. virilis* (II). The assignment test using *a priori* information about the geographical origin of the strains gave high posterior probabilities for the correct assignment of American, Asian, European and Japanese strains. Also, genetic variation measured with F_{ST} statistics suggested a significant population differentiation following the isolation-by-distance model. Variation in microsatellite loci can have a more recent origin than variation in mtDNA haplotypes. The lack of population differentiation in mtDNA indicates a shared ancestral polymorphism at the mitochondrial level, while variation in microsatellite loci indicates that differentiation among populations is still ongoing.

Microsatellite analysis distinguished Finnish and North American *D. montana* populations like the mtDNA analysis, but it also suggested genetic differentiation between North American populations (IV). Although mtDNA haplotypes from Canada and USA were not phylogenetically distinct, the populations were significantly differentiated as judged by mtDNA genetic variation. Microsatellite variation also showed evidence for different historical demographic patterns of populations. This might reflect partial or complete isolation into distinct northern (Beringian) and southern (Rocky Mountains) refugia (Hewitt 2004) during the last glaciation, or conversely to different colonization times of populations. Both of the Finnish and the Canadian samples suggest a very rapid population expansion around 35,000 years ago, somewhat before the end of the last glaciation period. The USA sample suggests an earlier gradual expansion consistent with the more southern location of the Rocky Mountains refuge.

3.2 Phenotypic differentiation

3.2.1 Male courtship songs in *D. virilis* and *D. montana*

In *D. virilis*, the male courtship songs showed significant interstrain and geographic variation (IV) in the number of pulses in a pulse train (PN) and the pulse train length (PTL), as well as in two sound pulse characters: the number of cycles in a pulse (CN) and the carrier frequency of the song (FRE). The strains from continental Asia and Europe expressed higher values than the American and Japanese strains for all of these traits. The genetic distances and the song divergence of the strains did not show significant association, which suggests that the songs have not diverged solely as a side-effect of genetic divergence. The length of time the strains had been kept in the laboratory prior to song recording decreased the values of CN and FRE. Also, the songs of laboratory strains showed a slight, but significant, decrease in CN and FRE and higher increases in pulse length (PL) and interpulse interval (IPI) when compared to the songs of the progenies of wild-caught females. Laboratory rearing had no effect on PN or PTL, and it did not eradicate interstrain and geographic variation in CN and FRE. However, parallel changes in the songs of the laboratory strains could explain the lack of geographic variation in PL and IPI. Lack of intra- and interspecific variation in IPI has also been observed in other *Drosophila* species (e.g. in *D. ananassae* and *D. pallidosa*), where the phenomenon has been suggested to be due to selection on other species-specific song characters requiring constant IPI (Yamada et al. 2002).

The male courtship songs of the laboratory strains of *D. montana* originating from a wide geographic area showed the highest divergence in the pulse characters of the song, especially in FRE (III). Also, the songs of males from wild populations (F1 progenies of wild-caught females) in Oulanka (Finland), Colorado (USA) and Vancouver (Canada) showed divergence in pulse characters PL and CN (IV). The fact that the songs of the males from wild populations showed no significant divergence in FRE was mainly due to high variation in this trait within the isofemale strains. The song frequency is quite sensitive to changes in environmental factors (Hoikkala & Suvanto 1999) and has a low heritability (Aspi & Hoikkala 1993, Suvanto et al. 1998), and thus one would expect any change under selection in this character to be slow.

In addition to PL and CN, IPI also varied significantly among *D. montana* populations (IV). This trait has been found to play an important role in species recognition in *D. montana* (Saarikettu et al. 2005) as well as in several other *Drosophila* species (e.g. Cowling & Burnet 1981). The songs of all the species of the *montana* phylad have a species-specific IPI (Hoikkala & Lumme 1987). Variation between *D. montana* populations in the male courtship song could have been enhanced by character displacement if the flies of different populations are sympatric with different species of the *D. virilis* group and if they interact/hybridize with them in the wild. In Finland, interspecific courtships are quite common between *D. montana*, *D. ezoana*, *D. littoralis* and *D.*

lummei (Aspi et al. 1993, Liimatainen & Hoikkala 1998). In Colorado, *D. montana* occurs sympatrically with *D. borealis* and *D. flavomontana* (Hoikkala & Mazzi personal observation), while in Vancouver it is probably the only representative of the *D. virilis* group (Klappert & Orsini unpublished observation). It is not possible to trace the historical patterns of sympatry between different species, but it is intriguing that a species-specific song trait (IPI) shows such a high variation between conspecific populations (see Hoikkala & Lumme 1987). It is important for the species-recognition signals to differ from those of other sympatric species to effectively prevent hybridization.

3.2.2 Wing size and shape in *D. montana*

Drosophila species are known to evolve latitudinal wing morphology clines when introduced into novel environments (Gilchrist et al. 2000, Santos et al. 2004), reflecting increasing body size in a colder climate (Huey et al. 2000). Similarly, altitude has been shown to have an effect on wing morphology by increasing wing load due to colonisation or the scarcity of food resources (Norry et al. 2001). In the present study (IV), variation between the wing measures of males from the Oulanka, Colorado and Vancouver populations was mainly due to changes on the tip as well as on the front and back edges of the wings; the differences were highest between the two latter populations. The males from the Vancouver population also diverged by the size and the internal landmarks of the wings from the other two populations. The differentiation, which is likely to have aerodynamic consequences, could be due to natural selection on the wing form to adjust to the aerodynamic optimum of a local climate. Also, a wing landmark pinpointing the endpoint of the second long vein on the outline of the wing showed co-variation with the combined effect of three pulse characters of the song.

3.2.3 Genitalia size and shape in *D. montana*

Male genitalia size and shape showed divergence between Vancouver and Oulanka as well as the Vancouver and Colorado populations in the discriminant analysis (IV). The cross-validated classification test showed an overlap between the Colorado and Oulanka populations. Nested ANOVA revealed a strong within-strain variance for all genitalia shape traits suggesting that they are sensitive to environmental factors and/or to effects of sample preparation. Size and shape of male genitalia could be affected in the wild by cryptic female choice or by variation of a male's ability to remove the previous male's sperm with the genitalia. These factors might also be important in *D. montana* as the females of this species mate repeatedly in the wild where the last male sires most of the offspring (Aspi & Lankinen 1991). Also, sexual conflict over the duration of copulation (Klappert & Mazzi unpublished results) may influence the curvature of the genitalia and the genital hook angle to increase the male's ability to prolong copulation (Arnqvist & Rowe 2005).

3.3 Genetic species identification in the *D. virilis* group

Species identification was based on 14 microsatellite loci that were amplified in all of the *D. virilis* group species. This set of markers was divergent enough to separate the species according to their species status with a few exceptions. *D. montana* clustered to three separate groups according to their geographic origin (Fig. 2). Subspecies *D. a. americana* and *D. a. texana* were grouped together, thereby supporting the view that these subspecies are just chromosomal forms of one single species of *D. americana* (Schlötterer 2000, Schäfer et al. 2006) Additionally, microsatellite data did not support species status for *D. canadiana*.

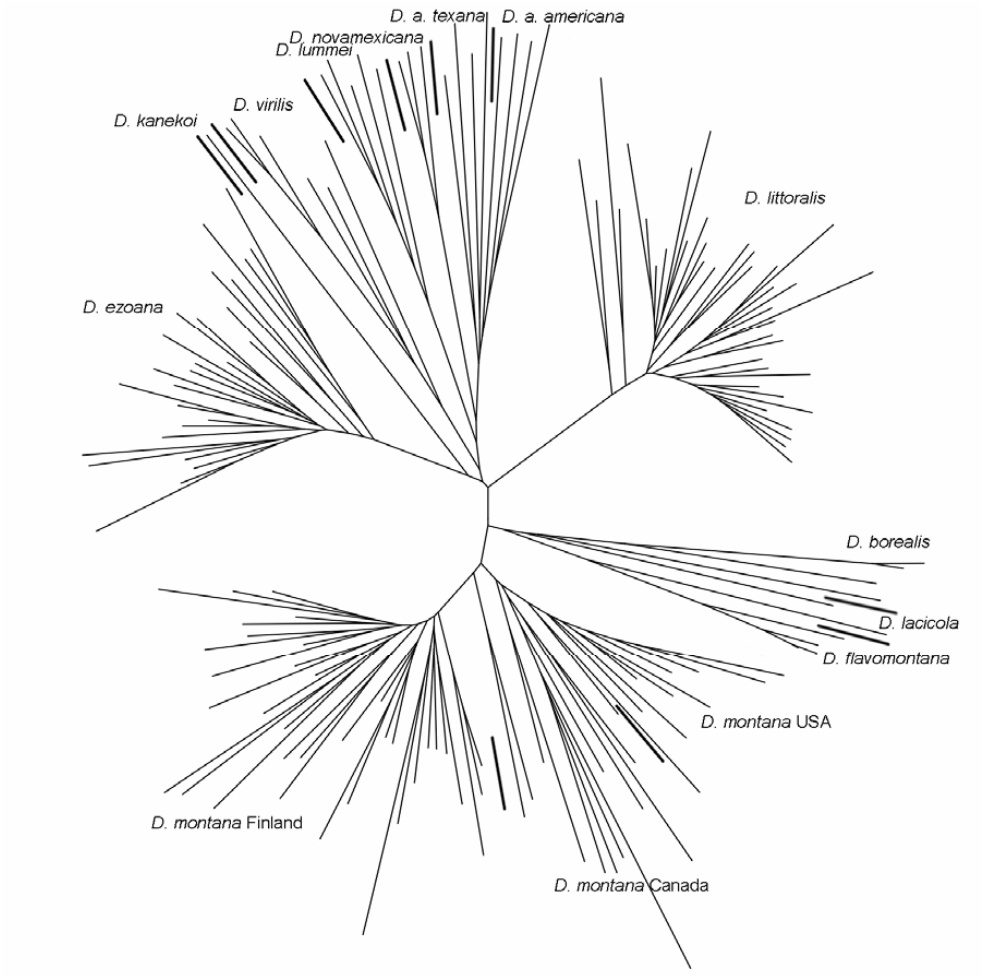

FIGURE 2 Unrooted neighbor joining tree based on 14 microsatellite loci.

4 CONCLUSIONS

D. virilis and *D. montana*, two species with different ecological adaptations, show remarkably contrasting phylogeographic patterns and phenotypic divergence. The expansion of *D. virilis* on different continents has probably been associated with evolutionarily recent human population expansion. The environmental niches of this species are rich in resources and relatively free of predators, which may have lead to low levels of genetic and phenotypic differentiation. *D. montana*, on the other hand, lives in highly heterogeneous environments, which has led to high genetic and phenotypic divergence between populations. The studied *D. montana* populations have unique combinations of phenotypic characters, which have not followed convergent evolutionary pathways.

The study on geno- and phenotypic variation in *D. virilis* (II) reveals possible pitfalls in evolutionary studies. The male courtship songs of all laboratory strains of this species have evolved in the same direction compared to wild populations, which may have led to diminished geographic variation in the lengths of sound pulses and interpulse intervals. While the laboratory strains provide good material for genetic crosses and studies on gene structure and function, the studies on the genetic and geographic variation in traits like the male courtship song should be done within the framework of a wild population.

D. montana populations have intriguing divergence patterns that are demonstrated in article IV. The males of the laboratory strains from USA (III) as well as the males from the Colorado population (IV) have a higher song frequency than the males from Canada and Finland. The Finnish *D. montana* females have previously been shown to prefer the males that produce a high frequency song as their mating partner (e.g. Aspi & Hoikkala 1995, Ritchie et al.1998). Surprisingly, the females from the Colorado population, where the males sing with a high frequency, do not show this preference or show a preference for lower song frequency (Klappert et al. 2007). Does this mean that all of the males from this population sing with a high frequency so that this signal has lost its value in mate choice? This question requires further studies

on male song / female preference covariation in the Colorado population, similar to the one made for the Finnish population (Ritchie et al. 2005). Also, mate choice experiments between the flies from different populations could give more information on the genetic basis and evolution of male songs and female song preferences. Another interesting discovery worth studying further is the coevolution of male song traits and wing shape. The ease of extracting data from the wing morphology opens new possibilities also for evo-devo studies.

Due to a small number of study populations, the genetic and phenotypic variance was not compared to each other directly (for example, using F_{ST} and Q_{ST} statistics; Merilä & Crnokrak 2001). Instead, we used a multivariate analysis and Mantel test to trace the direction of phenotypic divergence between populations as well as its correlation with genetic divergence. Differences between populations in male song, wing and genital traits were not compared with each other because of different time and scale measurement units (e.g. between song and wing traits) and also because of scale differences between wing and genital traits.

The two species, *D. montana* and *D. virilis*, together form a powerful model system in evolutionary ecology and speciation studies of wild populations. The fully sequenced *D. virilis* genome can be used for designing experiments in *D. montana*, the species more interesting in evolutionary and ecological aspects. Also, behavioural mutations of the genetic model species, *D. melanogaster*, could be traced down in the *D. virilis* genome and then eventually in *D. montana*. Studies that require massive sequencing operations of previously unknown sequences in *D. montana* (e.g. like the construction of microarrays), benefit greatly from the known genetics of the model species.

The species concept is a controversial topic. It depends on how the species is defined and what kinds of methods are used to distinguish species and conspecific populations. The results of our species identification analyses using microsatellite markers were not fully identical with the previously known species and subspecies boundaries. The genetic method may help clarify inconsistencies and mistakes in previous cases of species identification, and even more importantly, this method will enable large-scale studies on the flies of wild populations among North European *D. virilis* group species. More studies with more microsatellite loci and fly samples are necessary to reliably identify all of the species in the *D. virilis* group.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Geneettinen ja fenotyyppinen erilaistuminen *Drosophila virilis* ja *D. montana* lajien mahlakärpäsillä

Drosophila virilis ja *D. montana* lajien mahlakärpästen geneettinen ja fenotyyppinen erilaistuminen on edennyt evolutiivisesti eri reittejä. Ihmisen seuralaislajin, *D. virilis* -lajin, eri maantieteellisiltä alueilta peräisin olevat kannat eivät erotu toisistaan mtDNA haplotyyppien osalta. Lajin leviäminen ja populaatioiden koon kasvu on voinut tapahtua ihmispopulaatioiden kasvun ja uudelle mantereelle levittäytymisen myötä. Mikrosatelliitti-analyysi ryhmittelee *D. virilis* -lajin laboratoriokannat maantieteellisen alkuperän mukaan neljään ryhmään, mikä viittaa populaatioiden viimeaikoina tapahtuneeseen erilaistumiseen. Luonnossa vesistöjen äärellä elävän *D. montana* -lajin Euroopassa ja Pohjois-Amerikassa elävät populaatiot ovat eriytyneet toisistaan sekä mitokondriaalisten haplotyyppien että mikrosatelliittialleelien osalta. Tällä lajilla mtDNA haplotyypit eivät eroa kahden pohjois-amerikkalaisen populaation välillä, kun taas mikrosatelliitti-analyysi erottelee kaikki populaatiot omiksi ryhmikseen.

Fenotyyppistä eriytymistä tutkittiin *D. virilis* -lajilla koiraan kosintalaulun ja *D. montana* -lajilla koiraan kosintalaulun sekä siipien ja genitaalien koon ja muodon perusteella. *D. virilis* -lajin laboratoriokantojen laulut olivat erilaistuneet kantojen maantieteellisen alkuperän mukaan. Geneettinen erilaistuminen ja laulujen eriytyminen eivät olleet edenneet yhdenmukaisesti, mikä viittaa siihen, että laulut eivät ole erilaistuneet pelkästään neutraalin geneettisen erilaistumisen sivutuotteena. *D. montana* -lajilla laboratoriokantojen koiraiden laulut poikkesivat toisistaan selkeimmin laulun frekvenssin suhteen, kun taas luonnon populaatioiden koiraiden laulut erosivat toisistaan enemmän äänipulssien muissa ominaisuuksissa. *D. montana* –lajin populaatiot poikkesivat toisistaan myös siipien ja genitaalien koon ja muodon suhteen. Populaatioiden välinen fenotyyppinen eriytyminen ei ole kulkenut samaan tahtiin genotyyppisen eriytymisen kanssa, mikä viittaa siihen, että ominaisuuksissa syntyneet erot eivät olleet kehittyneet neutraalisti.

Viimeisessä osatutkimuksessa kehitimme pohjois-eurooppalaisille *D. virilis* -ryhmän lajeille lajintunnistusmenetelmän helpottamaan luonnosta kerättyjen yksilöiden tunnistusta ja mahdollisesti väärin tunnistettujen laboratoriokantojen identifioimista. Menetelmä perustuu kaikissa *D. virilis* -ryhmän lajeissa monistuvien polymorfisten mikrosatelliittien käyttöön. Tasaisesti koko genomiin jakautuneet markkerit selkeyttävät *D. virilis* -ryhmän lajien keskinäisiä sukulaisuussuhteita. Näitä molekyylimarkkereita voi myös käyttää mm. isyystutkimuksiin ja geenien paikallistamiseen samoin kuin luonnonpopulaatioiden laajamittaiseen tutkimiseen.

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