SHORT- AND LONG-TERM CHANGES IN CHROMOSOMAL INVERSION POLYMORPHISM AND GLOBAL WARMING: *DROSOPHILA SUBOBSCURA* FROM THE BALKANS

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ABSTRACT

The chromosomal inversion polymorphism of *Drosophila subobscura* is adaptive to environmental changes. The Serbian population of Petnica was chosen to analyze short- and long-term changes in this polymorphism. Short-term changes were studied in the samples collected in May, June and August of 1995. The inversion polymorphism varied over these months, although various interpretations are possible. To analyze long-term changes, samples obtained in May 1995 and May 2010 were compared. The frequency of the "cold" adapted inversions (A_{st}, J_{st}, U_{st}, E_{st} and O_{st}) decreased and that of the "warm" adapted inversions (A_2, J_1, $U_{\underline{1+2}}$ or $O_{\underline{3+4}}$) increased, from 1995 to 2010. These changes are consistent with the general increase in temperature recorded in Petnica for the same period. Finally, the possible response of chromosomal polymorphism to global warming was analyzed at the regional level (Balkan peninsula). This polymorphism depends on the ecological conditions of the populations, and the changes observed appear to be consistent with global warming expectations. Natural selection seems to be the main mechanism responsible for the evolution of this chromosomal polymorphism.

Key words: *D. subobscura* − chromosomal inversions − karyotypes − long-term changes − short-term changes − natural selection − global warming

INTRODUCTION

The impact of increasing atmospheric temperature on the thermal performance of organisms is a direct biological consequence of climate change which can be evaluated on a global scale (Deutsch et al., 2008). As a consequence of global warming, heritable changes in populations of animals as diverse as birds, squirrels and mosquitoes have been produced (Bradshaw and Holzapfel, 2006; Parmesan, 2006). The magnitude of this phenomenon has been monitored: temperature is now 0.6ºC higher than in the past three decades and 0.8ºC higher than in the past century (Hansen et al., 2006; Heerwaarden and Hoffmann, 2007). These observations are in accordance with the fact that many animals and plants are able to adapt very fast to variations in environmental conditions (Endler, 1986). In order to study the changes in genetic composition due to environmental conditions, chromosomal inversion polymorphism in *Drosophila* genus is considered an excellent model system (reviewed by Sperlich and Pfriem, 1986; Krimbas and Powell, 1992; Powell, 1997). Widespread generalist species such as *Drosophila melanogaster* and *Drosophila subobscura*, which present short generation times and rapidly develop phenotypic and genotypic clines, appear to be outstanding candidates as sensitive indicators of changes in genetic composition in response to global warming (Heerwaarden and Hoffmann, 2007; Balanyà et al., 2009). Genes located within *Drosophila* chromosomal inversions are considered to be coadapted complexes (Dobzhansky, 1970), and these complexes are associated with a variety of traits including those involved in climate adaptation (Hoffmann and Rieseberg, 2008). This conclusion was based on recent results: long-term fate of chromosomal inversion polymorphism of *D. melanogaster* in Australia (Anderson et al., 2005; Umina et al., 2005), *D. robusta* in

North America (Levitan and Etges, 2005) and *D. subobscura* in Europe and America (Rodríguez-Trelles and Rodríguez, 1998; Solé et al., 2002; Balanyà et al., 2004, 2006, 2009). The latter species is especially useful as a model organism for climate adaptation studies. *D. subobscura* is characterized by its very rich chromosomal inversion polymorphism, with 67 different inversions distributed in 93 chromosomal arrangements (Krimbas, 1992, 1993). For most of them, the frequencies vary clinally with latitude (Krimbas and Loukas, 1980; Prevosti et al., 1988; Menozzi and Krimbas, 1992; Krimbas, 1993). Of the five acrocentric chromosomes that constitute the species karyotype, O chromosome (the longest which is homologous to the 3R chromosome arm of *D. melanogaster* and the second chromosome of *D. pseudoobscura*) is the most polymorphic, with about 40 natural chromosomal arrangements (Krimbas, 1992, 1993). *D. subobscura* is a wild species that is found in woods, at the edge of woods and in urban areas (Krimbas, 1993) so, it may be influenced by human activity. It is generally accepted that anthropogenic influences have led to global warming at the global scale (Seneviratne et al., 2012), and most species (including *D. subobscura*) seem to have the potential to survive future human-induced climate change (Dynesius and Jansson, 2000). In a previous study, we examined a swamp population of *D. subobscura* unaffected by human activity (Apatin, Serbia), and thus, no human buffer effect of climate was present (Zivanovic and Mestres, 2011a). In this population, results of long-term climatic change (15 years between the samples) clearly showed that chromosomal inversions adapted to warmer conditions and that the population became more “southern” with regard to inversion polymorphism composition.

Our aim was to analyze the *D. subobscura* chromosomal inversion polymorphism and climatic data from another Balkan population, because this region presents a different chromosomal inversion composition and climatic pattern (Menozzi and Krimbas, 1992; Zivanovic, 2007; Araúz et al., 2009; Zivanovic and Mestres, 2010a, 2010b, 2011a) than Western Europe, the most studied Palearctic region with regard to these topics (Solé et al., 2002; Balanyà et al., 2004, 2006, 2009). Moreover, this region has the additional interest that it was a continental refuge for both animals and plants during the last glaciation period (Taberlet et al., 1998; Hewitt, 1999, 2000; Heckel et al., 2005). We chose the Petnica population (at continental part of Balkan peninsula, Serbia), which was studied in depth for chromosomal inversion polymorphism fifteen years ago (1995) in May (unpublished), June (Zivanovic et al., 2002) and August (unpublished). Our first objective was to present and reanalyze these data to carry out a short-term monthly study. In *D. subobscura*, it is well documented that the O chromosome shows different degrees of seasonality for different arrangements: O_{st}, $O_{\underline{3+4}}$, $O_{\underline{3+4+7}}$ and $O_{\underline{3+4+8}}$ (for a recent review see Zivanovic and Mestres, 2010b). These arrangements were also significantly associated with the seasonal variation of the climate – temperature, rainfall, humidity and insolation (Rodríguez-Trelles et al., 1996). We attempted to ascertain whether the inversion chromosomal polymorphism reacts to climate changes in a short period of time, as monthly variation in the same season. Furthermore, we examined long-term changes in chromosomal polymorphism by comparing samples collected from exactly the same site in May 1995 and in May 2010, taking special care to revise calendar and climatology data. Finally, chromosomal inversion polymorphisms in Petnica samples were also compared with those of other *D. subobscura* populations in Serbia and Montenegro studied over the last twenty years (Zivanovic et al., 1995, 2002; Zivanovic, 2007; Zivanovic and Mestres, 2010a, 2010b, 2011a) to ascertain general patterns of these polymorphisms in this Balkan region and their potential relationship with environmental conditions including climatic change.

MATERIAL AND METHODS

POPULATION SAMPLES AND CHROMOSOMAL PREPARATION

Drosophila subobscura flies were collected in a dense horn-beam wood (*Carpinus betulus)* in Petnica (44º14´N/19º55´E, approximately 100km SW of Belgrade). Meteorological data for Petnica were recorded using the values registered from near the town of Valjevo by the Republic Hydrometeorological Service (Serbia). Samples were collected between 14-17 May 2010 from the same place that had been sampled fifteen years earlier (1995): between 24-26 May, on 28 June and on 5 August. To compare May 2010 and May 1995 samples, they were collected 2.5 days earlier per decade, because spring/summer has advanced an average of 2.5 days per decade in Europe (Menzel et al. 2006). Unfortunately, trapping on precise days limited the sample size obtained. We have used both wild males and sons of wild females (only one son per wild female) for the chromosomal inversion polymorphism analysis of Petnica (May 2010). However, for the analysis of previous samples only wild males (May 1995, June 1995) and male offspring of wild females (August 1995) were used. In the latter case, only one son per wild female was used. Males were individually crossed with virgin females of the Kussnacht strain, which is homokaryotypic for standard chromosomal arrangements in all five chromosomes. The polytene chromosomes were stained and squashed in aceto-orcein solution. At least eight larvae from the progeny of each cross were examined. For the cytological analysis of chromosomal arrangements, the Kunze-Mühl and Müller (1958) chromosome map was used. The designation of chromosomal arrangements followed that of Kunze-Mühl and Sperlich (1955).

The chromosomal polymorphisms of Petnica were compared with those found in other Balkan populations: Jastrebac (June 1990, June 1993 and June1994) (Zivanovic et al., 1995), Apatin (June 1994, June 2008+2009) (Zivanovic et al., 2002; Zivanovic and Mestres, 2011a), Kamariste (June 1996) (Zivanovic et al., 2002), Zanjic (June 1997) (Zivanovic et al., 2002), Djerdap (June 2001, August 2001 and June 2002) (Zivanovic, 2007) and Avala (September 2003, June 2004, September 2004 and September 2005) (Zivanovic and Mestres, 2010a, 2010b). The geographical location of these populations is presented in Figure 1. All these populations are situated in a region comprised between 45º40'N and 42º24'N of latitude and 18º37'E and 21º20'E of longitude. Climatic information was obtained from the Republic Hydrometeorological Service (Serbia).

All strains, stocks and crosses were kept at 19°C. Flies were fed a standard cornmeal-sugar-agar-yeast medium and kept at 60% relative humidity under a 12 h/12 h light/dark cycle.

STATISTICAL ANALYSES

Departure from expectations of chromosomal arrangements and observed karyotypes frequencies was tested by χ^2 test contingency tables. In the population analyzed, the index of free recombination (IFR) was used to measure the degree of chromosomal inversion polymorphism (Carson, 1955). For analyzing the temperature changes along years we have carried out time series analyses (Box et al., 1994; Chatfield, 1996; Brockwell and Davis, 2002). Time series are data collected over time; in our case are yearly values of temperatures (maximum, minimum and mean). An important step is trend identification, that is, to estimate whether data present a long-term increase or decrease. The first step in the process of trend identification is smoothing. The most common technique is *moving average* smoothing which replaces each element of the series by either the simple average of k surrounding elements, where k is the smoothing window (see Velleman and Hoaglin, 1981; Box et al., 1994). After smoothing, data series can be adequately approximated by an appropriate function. In our case, we consider a smoothing window equal to k=3, which has the interpretation of a three year period.

In order to compare the inversion polymorphism of Petnica with those from other Balkan populations, a principal coordinate analysis was computed following Balanyà et al. (2004, 2006) and Mestres et al. (2009). In our case, we had 18 populations and studied the five chromosomes of *D. subobscura* karyotype (A, E, J, O and U). To reduce possible “noise” due to seasonal effects, we repeated the analysis using only those populations sampled in June. In this case, the principal coordinate analysis was carried out using data from 11 populations. Moreover, a principal component analysis was computed using the climatic data available for the period and location in which these populations were sampled (maximum, minimum and mean temperatures and rainfall). All these analyses were carried out using STATISTICA 9.0 software (StatSoft, Tulsa, OK). Finally, the similarities and relationships between the Balkan populations were analyzed using a cluster procedure. It is known that different clustering problems need different approaches to achieve the best results (Krzanowski 1993). Irigoien et al. (2010) showed that the GEVA-Ward clustering gives the best results for chromosomal polymorphism data, even better than those obtained by standard hierarchical clustering. For this reason, we used GEVA-Ward clustering to carry out two cluster analyses: one for the 18 and the other for the 11 populations described above.

RESULTS

The frequencies of chromosomal arrangements observed in Petnica in May 1995, June 1995, August 1995 and May 2010 are shown in Table 1. As commented in the Introduction, only data from Petnica in June 1995 have been published (Zivanovic et al., 2002). With regard to short-term changes, when comparing all three 1995 samples (May *vs*. June; May *vs*. August; June *vs*. August) significant differences were observed for some J and E chromosomal arrangements, whereas a trend was detected for some of the arrangements on the O chromosome. The J_{st} chromosomal arrangement showed a significant decrease during June, comparing to May (χ^2 = 4.55; p = 0.0329; df = 1, with Yates' correction). However, the frequency of J_{st} increased in August. Furthermore, E_{st} chromosomal arrangement showed a trend of frequency increase during August, comparing to May (χ^2 = 3.41; p = 0.0648; df = 1, with Yates' correction) and it is significant in June (χ^2 = 8.0; p = 0.047; df = 1, with Yates' correction), while $E_{\underline{1+2+9}}$ showed a trend of frequency decrease when comparing May to August (χ^2 = 3.28; p = 0.0708; df = 1, with Yates' correction) and June to August (χ^2 = 3.58; p = 0.0584; df = 1, with Yates' correction). Finally, O_{st} arrangement showed a non significant frequency decrease during August, comparing to May (χ^2 = 2.16; p = 0.1419; df = 1, with Yates' correction). Furthermore, O_{3+4} and all the other O_{3+4+x} chromosomal arrangements derived from O_{3+4} (O_{3+4+1}, O_{3+4+2}, O_{3+4+8}, O_{3+4+22}) showed a trend of frequency increase during August compared with May ($\chi^2 = 2.88$; p = 0.0896; df=1, with Yates' correction).

In the long-term analysis, a total of seventeen different chromosomal arrangements were observed between the old (May 1995) and new (May 2010) samples. Several particular chromosomal inversions found in May 1995 (O_6 and O_{22}) were not present in May 2010. Also, some chromosomal arrangements like U_{1+8+2}, $E_{1+2+9+12}$, O_{3+4+8}, not found in May 1995 were detected in May 2010 (Table 1). We found significant difference in four U chromosomal arrangement frequencies between May 1995 and May 2010 ($\chi^2 = 11.66$; p = 0.0086; df = 3). A comparison of five E chromosomal arrangement frequencies between May 1995 and May 2010 did not show significant differences ($\chi^2 = 8.83$; p = 0.0656; df = 4). For the O chromosome, we also found significant differences in eight arrangement frequencies between May 1995 and May 2010 samples ($\chi^2 = 14.89$; p = 0.0374; df = 7). The O_{st} arrangement showed a non significant frequency decrease between May 1995 and May 2010 ($\chi^2 = 2.49$; p = 0.1145; df = 1, with Yates' correction). Finally, O_{3+4} and all the other O_{3+4+x} chromosomal arrangements derived from it (O_{3+4+1}, O_{3+4+2}, O_{3+4+8}, O_{3+4+22}) showed a trend of increases in frequency ($\chi^2 = 3.25$; p = 0.0714; df = 1, with Yates' correction) in the period May 1995 *vs*. May 2010.

The frequencies of the chromosomal karyotypes observed for the Petnica population are shown in Table 2. The May 2010 sample did not present a significant deviation from Hardy-Weinberg equilibrium for any of the chromosomes: J ($\chi^2 = 0.0$; p

= 1.0; df = 1, with Yates' correction), U (χ^2 = 0.77; p = 0.8566; df = 3), E (χ^2 = 6.44; p = 0.2657; df = 1) and O (χ^2 = 8.98; p = 0.1098; df = 5). However, an excess of some E ($E_{st}/E_{\underline{1+2}}$; $E_{st}/E_{\underline{1+2+9+12}}$; $E_{\underline{1+2+9}}/E_8$) and O ($O_{st}/O_{\underline{3+4}}$; $O_{st}/O_{\underline{3+4}+\underline{2}}$; $O_{\underline{3+4}}/O_{\underline{3+4+8}}$; $O_{\underline{3+4}}/O_{\underline{3+4}+\underline{22}}$) karyotypic combinations was observed. When comparing all three 1995 samples (May *vs.* June; May *vs.* August; June *vs.* August) monthly differences in frequencies were observed for some J, U, E and O chromosomal karyotypes. Between May and June, Jst/J_1 showed a trend of decrease (χ^2 = 3.17; p = 0.0751; df = 1, with Yates' correction), while J_1/J_1 had a significant increase (χ^2 = 4.55; p = 0.0328; df = 1, with Yates' correction). $U_{\underline{1}+\underline{2}}/U_{\underline{1}+\underline{2}}$ presented a trend of increase in August, with regard to May (χ^2 = 3.04; p = 0.0815; df = 1, with Yates' correction) and June (χ^2 = 2.89; p = 0.0892; df = 1, with Yates' correction), while $U_{\underline{1}+\underline{2}}/U_{\underline{1+2+6}}$ showed significant decrease during August with respect to May (χ^2 = 4.02; p = 0.0451; df = 1, with Yates' correction). Furthermore when comparing August to June, E_{st}/E_{st} showed a trend of increase (χ^2 = 2.89; p = 0.0892; df = 1, with Yates' correction). Finally, $O_{\underline{3+4}}/O_{\underline{3+4}+\underline{1}}$ showed significant increase (χ^2 = 4.76; p = 0.0291; df = 1, with Yates' correction) in June with regard to May.

In the old sample (May 1995), 31 different karyotypes were found compared to 28 found in the new (May 2010), and 23 karyotypes were found in both samples. Furthermore, 8 karyotypes observed in May 1995 were not observed in May 2010, while 5 karyotypes from May 2010 sample were not present in May 1995. When comparing May 1995 and May 2010 samples, non significant differences for U karyotype frequencies were observed (χ^2 = 12.01; p = 0.0618; df = 6). However, $U_{\underline{1}+\underline{2}}/U_{\underline{1+8}+\underline{2}}$ and $U_{\underline{1+2+6}}/U_{\underline{1+8}+\underline{2}}$ appeared for the first time in May 2010. The E chromosomal karyotypes did not show significant differences in frequency (χ^2 = 15.77; p = 0.1064; df = 10). However, $E_{st}/E_{\underline{1+2+9+12}}$ and $E_{\underline{1+2+9+12}}/E_8$ were observed for the first time in May 2010. No significant differences were found for O karyotypes between the May 1995 and the May 2010 (χ^2 = 20.64; p = 0.1112; df = 14), but $O_{\underline{3+4}}/O_{\underline{3+4+8}}$ was detected in the May 2010 sample (with a frequency of 14.8%) and was absent in the May 1995 sample.

The index of free recombination (IFR) for the Petnica sample of May 2010 was 78.72±1.84. This value is slightly lower than those obtained fifteen years ago: 81.66±1.18 (May 1995), 80.83±1.31 (June 1995), 84.22±1.84 (August 1995), with a mean of 81.86±0.79. These values fit well with the expectations of a population located in the central area of *D. subobscura* distribution and slightly increase (about 4%) of the amount of inversion polymorphism can be deduced for the May 2010 sample (Krimbas and Loukas, 1980; Krimbas and Powell, 1992; Krimbas, 1993).

Meteorological data for Petnica in the three 1995 months are showed in Table 3. The maximum, minimum and mean temperatures increased in May, June and August 1995. However, the rainfall peaked in June. For the long-term changes in meteorological data (Table 4), the average maximum, minimum and mean temperatures for May 2010 (22.1ºC, 11.9ºC and 16.7ºC) were higher than those recorded in May 1995 (21.4ºC, 10.2ºC and 15.7ºC). However, it is worth to study their variation along years. The time series analyses carried out indicates that for Min. T (Figure 2a) the lineal trend has a sloop of 0.085, presenting a significant increasing tendency (p-value = 0.027). For Max. T (Figure 2b), the lineal trend has a sloop of 0.054, showing an increasing tendency although it is not significant (p-value = 0.337). Finally, for Mean T (Figure 2c) the lineal trend has a sloop of 0.062, presenting an increasing tendency, but not significant (p-value

= 0.197). These temperature increases are small (but in the case of Min. T significant) as expected by the period of time studied (fifteen years) and the order of magnitude of global warming, as explained in the Introduction. Additionally, the average of Petnica's mean temperatures in the period May 2003 – May 2010 was 0.3 ºC warmer than in May 1995 – May 2002. Also, from 1995 some heat waves were recorded: the well documented event of 2003 (Schar and Jendritzky, 2004; Seneviratne, et al., 2012) and other smaller waves as 2000, 2002 and 2009, a phenomenon also described (Kuglitsch et al., 2010). It is worth to note that the Western Europe heat wave of 2006 (Fouillet et al., 2008) had a small effect in Petnica, as expected (Table 4). Finally, the rainfall pattern along these years presents intense droughts with some episodes of heavy rain, as expected by the global climatic change in Southern Europe (Christensen et al., 2007; Wentz et al., 2007). In summary, all results are consistent with global warming predictions (Houghton, 2005; Seneviratne, et al., 2012).

According to the results of the multivariate principal coordinate analysis the 18 Balkan populations analyzed are not distributed at random (Figure 3). The first, second and third axes explain 24.07%, 19.70% and 14.04% of the variability, respectively. Taken together they explain 57.80% of the variability. From Figure 3a, it seems that Apatin and Jastrebac populations are differentiated from the others, possibly due to their particular ecological environments (Zivanovic et al., 1995; Zivanovic and Mestres, 2011a). The remaining populations are not well defined at the two-axes level, but better resolution is observed when the third dimension is used (Figure 3b). In this case, there are two groups of populations: those from the 1990's and those from 2000's (Jastrebac June 1990 is far apart from both groups). In spite of the fact that only 4 years elapsed from the last sample taken during the 1990's (Zanjic June 1997) to the first of the 2000's (Djerdap August 2001), at least one heat-wave occurred in the Balkans in this period. It is worth paying special attention to the climatic data for the populations used in these analyses (Table 5). In general, when comparing one population in the same month but in different years, a light increment in temperatures (maximum, minimum and mean) are detected in the new samples (the cases of Apatin, Avala and Jastrebac). The cluster analysis agrees with the principal component analysis because there is a clear division into two large groups (Figure 4a). All the populations in the first group were collected during the 1990's (with the exception of Avala September 2004), whereas the second group contains all the samples from the 2000's (with the exceptions of Petnica August 1995 and Zanjic June 1997). These latter populations are associated with higher temperatures, which may explain why their chromosomal polymorphism resembles that from the 2000's: the sample from Petnica was collected in August 95 (Table 3), and Zanjic is located at a lower latitude (Figure 1), in which the climate is warmer (Zivanovic et al., 2002). To avoid the seasonal effect, the same study was repeated with all samples collected in the same month, June (11 populations). In the principal coordinate analysis (Figure 5), the first, second and third axes explain 32.33%, 27.11% and 14.10% of the variability, respectively. All axes together explain 73.54% of the variability. In the two dimensions plot (Figure 5a) four groups can be seen: one with Apatin samples, another with Jastrebac 1990 sample, the third contains the remaining 90's samples (Jastrebac 1993 and 1994, Petnica 1995, Kamariste 1996 and Zanjic 1997) and the last one contains samples from the 2000's (Avala 2005 and Djerdap 2001 and 2002). The Apatin population has particular ecological conditions, as described in Zivanovic and Mestres (2011a) and

Jastrebac 1990 is an outlier. When analyzing the three dimensional plot (Figure 5b), there are two main groups: one containing 2000's populations (Apatin 2008+2009, Djerdap 2001 and Djerdap 2002) and Petnica 1995, whereas the second has the 1990's samples (Kamariste 1996, Petnica 1995, Jastrebac 1993 and 1994, Apatin 1994, Zanjic 1997). Only Jastrebac 1990 are clearly separated from both groups. Thus, the general pattern is similar to that obtained using two dimensions. The cluster analysis (Figure 5b) is interesting because both collections from Apatin are separated first, probably due to the ecological conditions. In a second division, Jastrebac 1990 (considered an outlier in the principal component analysis) forms another branch. Finally, two groups remain: one with the 2000's samples (Avala 2004, Djerdap 2001 and 2002) and Zanjic 1997 (probably due to its ecological characteristics previously described) and the other with the 1990's collections (Petnica 1995, Kamariste 1996, Jastrebac 1993 and 1994). These results should also to be interpreted taking into account the analysis of the climatic data. In the principal component analysis (Figure 6), the first and second axes explain 67.27% and 23.17% of the variability, respectively (both axes together accounts for 90.44% of the variability). The first component explains the differences in temperature and rainfall: moving to the right temperature and dryness increase. For instance, this effect can be observed from the Petnica samples of 1995 (May, June and August). The temporal variation in climate is observed between Petnica samples of May 1995 and May 2010. As expected, Zanjic is located to the right of the graph, as well as the August sample from Djerdap. Furthermore, collections of this latter population taken in June (2001 and 2002) are situated more to the left. The sample from Avala (June 04) is to the right of the samples from the same population taken in September. A small differentiation according to the first axis is observed for both samples from Apatin (June 94 and June 08+09). In summary, the climate pattern of these Balkans populations agrees quite well with the global warming model.

DISCUSSION

The adaptive value of chromosomal inversion polymorphism has been extensively demonstrated. The fast establishment of latitudinal clines both in North and South America soon after the beginning of colonization and in the same manner as those described in the Palearctic region is key evidence (Prevosti et al., 1988, 1989; Krimbas, 1992, 1993). For this reason, although historical factors play an important role in the geographical distribution of the chromosomal polymorphism, natural selection seems to be preponderant (Krimbas, 1993). Furthermore, these clines evolved according to global warming expectations (Solé et al., 2002; Balanyà et al., 2004, 2006, 2009), corroborating this phenomenon. The Petnica population of *D. subobscura* has allowed us to study different aspects of the chromosomal polymorphism adaptation to climatic conditions, both at short- and long-term. With regard to the first aspect, variation in the chromosomal polymorphism composition has been observed, although it cannot be related with the gradual increase of maximum, minimum and mean temperatures in May, June and August 1995 (Table 3). Most of the inversions present a peak in June 1995, with the "warm" adapted in general increasing in frequency (A_2 and J_1,) and the "cold" adapted in general decreasing (A_{st}, J_{st}, U_{st} and E_{st}). For the O chromosome, O_{st} ("cold" adapted), as expected, gradually decreases in frequency. On the other hand, O_{3+4}, considered "warm" adapted, maintains invariable its frequency, while the frequency of O_{3+4+22} increases along the studied months. Finally, $O_{\underline{3+4}+\underline{1}}$ arrangement presents an increasing peak in June and $O_{\underline{3+4}+\underline{2}}$ a decreasing peak in the same month. Thus, an expected continuous frequency increase for all $O_{\underline{3+4}+\underline{x}}$ chromosomal arrangements has not been detected. There are many explanations for the behavior of these inversions: they are adaptive and generally react fast to environmental changes but they are not instantaneous. The inversion frequencies in a month are the result of some environmental factors that had acted on *D. subobscura* populations in periods of time before sampling. Detectable seasonal effects on the species chromosomal polymorphism have been observed in quite separate periods of time (Burla and Götz, 1965; Fontdevila et al., 1983, Rodriguez-Trelles et al., 1996, Rodriguez-Trelles, 2003, Zivanovic and Mestres, 2010b). Furthermore, chromosomal polymorphism does not only react to temperature. Laboratory experiments with different *D. subobscura* chromosomal arrangements do not yield the results expected *a priori* (Santos et al., 2005). Temperature should be considered in conjunction with other climatic factors (humidity, insolation and so on) that could modulate the ecological conditions (for instance vegetation or yeast diversity and abundance) that would determine *D. subobscura* success and its population expansion (Zivanovic and Mestres, 2011b). Finally, from the short-term analysis we conclude that chromosomal polymorphism is dynamic, even at the scale of consecutive monthly variation. Thus, these changes are new evidence of "flexible" chromosomal polymorphism in *D. subobscura* (in the sense proposed by Dobzhansky in 1962). Although classic studies classified this species as "rigid" and this was a controversial topic (Ferrari and Taylor, 1981; Sperlich and Pfriem, 1986), data so far accumulated indicate that *D. suboscura* can be defined as "flexible"

(Fontdevila et al., 1983; Rodríguez-Trelles et al., 1996; Rodríguez-Trelles, 2003; Zivanovic, 2007; Zivanovic and Mestres, 2010b).

In Petnica, it has been possible to observe the global warming in a fifteen years period. Long-term changes in Petnica population have been detected and they appear to follow the direction expected by the global warming. All standard chromosomes (A_{st}, J_{st}, U_{st}, E_{st} and O_{st}), considered “cold” adapted arrangements, have shown a frequency decrease. On the other hand, chromosomes considered as “warm” adapted have increased their frequency (A_2, J_1, $U_{\underline{1}+\underline{2}}$ or $O_{\underline{3+4}}$). It is worth pointing out that several arrangements considered “warm” adapted and not present in the May 1995 sample were found in the May 2010 collection: $U_{\underline{1+8}+\underline{2}}$, $E_{\underline{1+2+9+12}}$ and $O_{\underline{3+4+8}}$. Thus, the chromosomal polymorphism changes are in accordance with the observed global warming in Petnica. Furthermore, some heat-waves affected the Balkan peninsula during this period, the well documented event of 2003 (Schar and Jendritzky, 2004) and other less important waves in 2000 and 2009 (Kuglitsch et al., 2010). In Petnica, these heat-waves and the irregular rainfall pattern could have had an important effect on chromosomal polymorphism. According to Balanyà et al. (2006) new *Drosophila subobscura* samples would correspond almost exactly to the composition of samples that in earlier studies were collected from sites 70 miles or 1 degree of latitude closer to the equator. In this study, some chromosomal arrangement frequencies from Petnica are very similar to those from Zanjic (June 1997) (Zivanovic et al., 2002). For example, in Petnica May 2010 population the chromosomal arrangement frequency for $O_{\underline{3+4}}$ and $O_{\underline{3+4}+\underline{x}}$ ($O_{\underline{3+4}+\underline{1}}$, $O_{\underline{3+4}+\underline{2}}$, $O_{\underline{3+4+8}}$, $O_{\underline{3+4}+2\underline{2}}$) is 77.7%, which is very similar to the 79.0% found in Zanjic June 1997 (Zivanovic et al., 2002). Petnica population is located at continental part of Balkan peninsula (latitude 44º14´N), while Zanjic (latitude 42º24´N) is about 300 km to the south and separated by large mountain range up to 2.000m high. This means that Petnica may be evolving to the characteristics of a more Southern population, although migration from Southern latitudes can not be discarded (Balanyà et al., 2004, 2009). In previous studies, similar results were observed for other Serbian populations as Avala or Apatin (Zivanovic and Mestres, 2010a,b and 2011a).

Finally, long-term changes in the chromosomal polymorphism in *D. subobscura* and their relation with the global warming have been studied at continental level, both in Europe (Solé et al., 2002; Balanyà et al., 2004) and America (Balanyà et al., 2006). However, we attempted to ascertain whether the chromosomal polymorphism also changed at regional level. The principal coordinates and cluster analyses have shown that the populations are separated according to their chromosomal composition. This separation is due to two main aspects: their habitat particularities and temporal changes. These temporal changes may be related to global warming. The results of the principal components analysis using the climatic data of the Balkan populations support this conclusion. However, this relation could be direct or indirect, i. e., global warming could affect certain ecological conditions that influence the species' habitat. In this context, it is of interest to comment many results of the multivariate analyses. For instance, samples from the Apatin population are separated according to the temporal changes (global warming effect), but they are also set apart from the other populations, possibly because they have particular environmental conditions (a swampy region located on the left bank of Danube river). The Jastrebac population, located on a mountain in the South-east of Serbia, is also differentiated ecologically from the remaining populations (Zivanovic et al., 1995). The Balkan peninsula is very close to the Mediterranean region, which is one of world areas most vulnerable to global climate warming.

The results obtained in the Balkan populations are in agreement with those from other *D. subobscura* populations, where an increase in the frequency of “warm” adapted arrangements and a decrease in the frequency of “cold” adapted arrangements has generally been observed (de Frutos and Prevosti, 1984; Gosteli, 1990; Orengo and Prevosti, 1996; Rodriguez-Trelles et al., 1996; Solé et al., 2002; Balanyà et al., 2004, 2006, 2009). Although in this species, genetic flow (a factor producing a decrease in genetic differentiation between populations) has been observed (Pascual et al., 2001; Zivanovic et al., 2007; Araúz et al. 2011), natural populations are clearly differentiated with regard to chromosomal polymorphism. Thus, natural selection is generally accepted as the main mechanism responsible for the evolution of this chromosomal polymorphism in a scenario of global warming (Anderson et al. 2005; Hoffman and Rieseberg, 2008; Balanyà et al., 2006, 2009; Zivanovic and Mestres, 2010a, 2011a).

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FIGURE CAPTIONS:

Fig. 1. Geographical location of the populations used in this research: Petnica, Apatin, Kamariste, Avala, Djerdap, Jastrebac and Zanjic (in Montenegro). Belgrade appears also as a geographical reference.

Fig. 2. Results of time series analysis on temperature. Rhombus and squares mean original temperatures and smoothing values (smoothing window k=3), respectively. (a) Min. T. The lineal trend has a sloop of 0.085 (p-value = 0.027). (b) Max T. The lineal trend has a sloop of 0.054 (p-value = 0.337). (c) Mean T. the lineal trend has a sloop of 0.062 (p-value = 0.197).

Fig. 3. Principal coordinate analysis using the inversion chromosomal polymorphism of the following samples: Pm95 (Petnica May 1995), Pj95 (Petnica June 1995), Pa95 (Petnica August 1995), Pm10 (Petnica May 2010), Aj94 (Apatin June 1994), Aj08 (Apatin June 08+09), Kj96 (Kamariste June 1996), Aj04 (Avala June 2004), As03 (Avala September 2003), As04 (Avala September 2004), As05 (Avala September 2005), Dj01 (Djerdap June 2001), Dj02 (Djerdap June 2002), Da01 (Djerdap August 2001), Jj90 (Jastrebac June 1990), Jj93 (Jastrebac June 1993), Jj94 (Jastrebac June 1994) and Zj97 (Zanjic June 1997). (a) Two axes representation. (b) Three axes representation.

Fig. 4. GEVA-Ward clusters obtained using the inversion chromosomal polymorphism of the following samples: (a) Pm95 (Petnica May 1995), Pj95 (Petnica June 1995), Pa95 (Petnica August 1995), Pm10 (Petnica May 2010), Aj94 (Apatin June 1994), Aj08

(Apatin June 08+09), Kj96 (Kamariste June 1996), Aj04 (Avala June 2004), As03 (Avala September 2003), As04 (Avala September 2004), As05 (Avala September 2005), Dj01 (Djerdap June 2001), Dj02 (Djerdap June 2002), Da01 (Djerdap August 2001), Jj90 (Jastrebac June 1990), Jj93 (Jastrebac June 1993), Jj94 (Jastrebac June 1994) and Zj97 (Zanjic June 1997). (b) Pj95 (Petnica June 1995), Aj94 (Apatin June 1994), Aj08 (Apatin June 08+09), Kj96 (Kamariste June 1996), Aj04 (Avala June 2004), Dj01 (Djerdap June 2001), Dj02 (Djerdap June 2002), Jj90 (Jastrebac June 1990), Jj93 (Jastrebac June 1993), Jj94 (Jastrebac June 1994) and Zj97 (Zanjic June 1997).

Fig. 5. Principal coordinate analysis using the inversion chromosomal polymorphism of those samples collected in the month of June: Pj95 (Petnica June 1995), Aj94 (Apatin June 1994), Aj08 (Apatin June 08+09), Kj96 (Kamariste June 1996), Aj04 (Avala June 2004), Dj01 (Djerdap June 2001), Dj02 (Djerdap June 2002), Jj90 (Jastrebac June 1990), Jj93 (Jastrebac June 1993), Jj94 (Jastrebac June 1994) and Zj97 (Zanjic June 1997). (a) Two axes representation. (b) Three axes representation.

Fig. 6. Principal components analysis computed with the climatic information (maximum, minimum and mean temperatures, as well as rainfall) of the following samples: Pm95 (Petnica May 1995), Pj95 (Petnica June 1995), Pa95 (Petnica August 1995), Pm10 (Petnica May 2010), Aj94 (Apatin June 1994), Aj08 (Apatin June 08+09), Kj96 (Kamariste June 1996), Aj04 (Avala June 2004), As03 (Avala September 2003), As04 (Avala September 2004), As05 (Avala September 2005), Dj01 (Djerdap June 2001), Dj02

(Djerdap June 2002), Da01 (Djerdap August 2001), Jj90 (Jastrebac June 1990), Jj93 (Jastrebac June 1993), Jj94 (Jastrebac June 1994) and Zj97 (Zanjic June 1997).

Table 1

Frequencies of chromosomal arrangements for the Petnica natural population of *D. subobscura*.

Chrom. arrangements	May 1995		June 1995		August 1995		May 2010	
	n	%	n	%	n	%	n	%
A_{st}	30	56.6	26	50.0	17	60.7	11	44.0
A_1	18	34.0	17	32.7	9	32.2	9	36.0
A_2	5	9.4	9	17.3	2	7.1	5	20.0
Total	53		52		28		25	
J_{st}	29	27.4	15	14.4	16	28.6	8	16.0
J_1	77	72.6	89	85.6	40	71.4	42	84.0
Total	106		104		56		50	
U_{st}	14	13.2	13	12.5	10	17.9	6	12.0
U_1	/	/	3	2.9	/	/	/	/
$U_{\underline{1+2}}$	40	37.7	35	33.7	23	41.1	24	48.0
$U_{\underline{1+2+3}}$	/	/	1	0.9	/	/	/	/
$U_{\underline{1+2+6}}$	52	49.1	52	50.0	21	37.5	16	32.0
$U_{\underline{1+8+2}}$	/	/	/	/	2	3.5	4	8.0
Total	106		104		56		50	
E_{st}	43	40.6	34	32.7	32	57.1	15	30.0
$E_{\underline{1+2}}$	4	3.8	3	2.9	3	5.4	3	6.0
$E_{\underline{1+2+9}}$	41	38.7	41	39.4	13	23.2	17	34.0
$E_{\underline{1+2+9+12}}$	/	/	/	/	/	/	3	6.0

E_8	18	16.9	26	25.0	8	14.3	12	24.0
Total	106		104		56		50	
O_{st}	38	35.9	28	26.9	13	23.2	12	22.2
O_6	1	0.9	2	1.9	/	/	/	/
O_{22}	1	0.9	1	1.0	/	/	/	/
$O_{\underline{3+4}}$	42	39.6	42	40.4	22	39.3	23	42.6
$O_{\underline{3+4}+\underline{1}}$	15	14.2	21	20.2	8	14.3	6	11.1
$O_{\underline{3+4}+\underline{2}}$	3	2.8	1	1.0	3	5.4	1	1.9
$O_{\underline{3+4}+\underline{6}}$	/	/	1	1.0	/	/	/	/
$O_{\underline{3+4+8}}$	/	/	/	/	2	3.5	4	7.4
$O_{\underline{3+4}+\underline{22}}$	6	5.7	8	7.6	8	14.3	8	14.8
Total	106		104		56		54	

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Table 2

Frequencies of chromosomal karyotypes for the Petnica natural population of *D. subobscura*.

Chrom. arrangements	May 1995		June 1995		August 1995		May 2010	
	n	%	n	%	n	%	n	%
J_{st}/J_{st}	3	5.7	1	1.9	3	10.7	/	/
J_{st}/J_1	23	43.4	13	25.0	10	35.7	8	32.0
J_1/J_1	27	50.9	38	73.1	15	53.6	17	68.0
Total	53		52		28		25	
U_{st}/U_{st}	/	/	/	/	1	3.6	/	/
$U_{st}/U_{\underline{1+2}}$	8	15.1	4	7.7	2	7.2	4	16.0
$U_{st}/U_{\underline{1+2+6}}$	6	11.3	9	17.3	4	14.2	2	8.0
$U_{st}/U_{\underline{1+8}+\underline{2}}$	/	/	/	/	2	7.2	/	/
$U_1/U_{\underline{1+2+6}}$	/	/	3	5.8	/	/	/	/
$U_{\underline{1}+\underline{2}}/U_{\underline{1}+\underline{2}}$	7	13.2	7	13.4	9	32.1	5	20.0
$U_{\underline{1}+\underline{2}}/U_{\underline{1}+\underline{2+3}}$	/	/	1	1.9	/	/	/	/
$U_{\underline{1}+\underline{2}}/U_{\underline{1+2+6}}$	18	34.0	16	30.8	3	10.7	8	32.0
$U_{\underline{1}+\underline{2}}/U_{\underline{1+8}+\underline{2}}$	/	/	/	/	/	/	2	8.0
$U_{\underline{1+2+6}}/U_{\underline{1+2+6}}$	14	26.4	12	23.1	7	25.0	2	8.0
$U_{\underline{1+2+6}}/U_{\underline{1+8}+\underline{2}}$	/	/	/	/	/	/	2	8.0
Total	53		52		28		25	
E_{st}/E_{st}	9	17.0	7	13.5	9	32.1	1	4.0
$E_{st}/E_{\underline{1+2}}$	2	3.8	/	/	2	7.2	3	12.0

$E_{st}/E_{\underline{1+2+9}}$	16	30.1	15	28.8	5	17.8	6	24.0
$E_{st}/E_{\underline{1+2+9+12}}$	/	/	/	/	/	/	2	8.0
E_{st}/E_{8}	7	13.2	5	9.6	7	25.0	2	8.0
$E_{\underline{1+2}}/E_{\underline{1+2+9}}$	1	1.9	2	3.9	1	3.6	/	/
$E_{\underline{1+2}}/E_{8}$	1	1.9	1	1.9	/	/	/	/
$E_{\underline{1+2+9}}/E_{\underline{1+2+9}}$	9	17.0	7	13.5	3	10.7	2	8.0
$E_{\underline{1+2+9}}/E_{8}$	6	11.3	10	19.2	1	3.6	7	28.0
$E_{\underline{1+2+9+12}}/E_{8}$	/	/	/	/	/	/	1	4.0
E_{8}/E_{8}	2	3.8	5	9.6	/	/	1	4.0
Total	53		52		28		25	
O_{st}/O_{st}	8	15.1	3	5.8	3	10.7	1	3.7
O_{st}/O_{6}	/	/	2	3.9	/	/	/	/
O_{st}/O_{22}	1	1.9	/	/	/	/	/	/
$O_{st}/O_{\underline{3+4}}$	16	30.1	10	19.2	4	14.2	8	29.7
$O_{st}/O_{\underline{3+4}+\underline{1}}$	4	7.5	7	13.5	1	3.6	1	3.7
$O_{st}/O_{\underline{3+4}+\underline{2}}$	1	1.9	/	/	1	3.6	1	3.7
$O_{st}/O_{\underline{3+4}+\underline{22}}$	/	/	3	5.8	1	3.6	/	/
$O_{6}/O_{\underline{3+4}+\underline{1}}$	1	1.9	/	/	/	/	/	/
$O_{22}/O_{\underline{3+4}}$	/	/	1	1.9	/	/	/	/
$O_{\underline{3+4}}/O_{\underline{3+4}}$	10	18.8	8	15.3	5	17.8	2	7.4
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{1}}$	2	3.8	10	19.2	3	10.7	1	3.7
$O_{\underline{3+4}}/O_{3+4+\underline{2}}$	1	1.9	1	1.9	/	/	/	/
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{6}}$	/	/	1	1.9	/	/	/	/

$O_{\underline{3+4}}/O_{\underline{3+4+8}}$	/	/	/	/	1	3.6	4	14.8
$O_{\underline{3+4}}/O_{\underline{3+4}+\underline{22}}$	3	5.7	3	5.8	4	14.2	6	22.2
$O_{\underline{3+4}+\underline{1}}/O_{\underline{3+4}+\underline{1}}$	3	5.7	2	3.9	1	3.6	1	3.7
$O_{\underline{3+4}+\underline{1}}/O_{\underline{3+4}+\underline{2}}$	1	1.9	/	/	/	/	/	/
$O_{\underline{3+4}+\underline{1}}/O_{\underline{3+4+8}}$	/	/	/	/	1	3.6	/	/
$O_{\underline{3+4}+\underline{1}}/O_{\underline{3+4}+\underline{22}}$	1	1.9	/	/	1	3.6	2	7.4
$O_{\underline{3+4}+\underline{2}}/O_{\underline{3+4}+\underline{2}}$	/	/	/	/	1	3.6	/	/
$O_{\underline{3+4}+\underline{22}}/O_{\underline{3+4}+\underline{22}}$	1	1.9	1	1.9	1	3.6	/	/
Total	53		52		28		27	

Table 3

Meteorological data for the Petnica population in the three 1995 analyzed months.

Month	Max. T (ºC)	Min. T (ºC)	Mean T (ºC)	Rainfall (mm)
May	21.4	10.2	15.7	73.4
June	24.3	14.2	18.7	189.1
August	25.9	15.2	19.8	104.6

Max. T and Min. T stand for maximum and minimum temperatures, respectively.

Table 4

Meteorological data for the Petnica population for the month of May from 1995 to 2010.

Year	Max. T (ºC)	Min. T (ºC)	Mean T (ºC)	Rainfall (mm)
1995	21.4	10.2	15.7	73.4
1996	23.8	11.4	17.5	122.3
1997	23.8	9.9	16.9	31.6
1998	21.2	10.3	15.5	80.2
1999	22.3	10.7	16.8	71.3
2000	25.2	11.1	18.3	67.6
2001	23.6	10.9	17.4	43.4
2002	24.1	11.7	18.1	83.7
2003	26.4	12.5	19.7	56.6
2004	20.6	9.4	14.9	71.5
2005	22.4	10.3	16.6	70.9
2006	22.3	10.1	16.2	46.4
2007	23.9	12.6	18.0	125.0
2008	24.5	11.5	18.0	72.9
2009	25.1	12.0	18.5	32.2
2010	22.1	11.9	16.7	117.8

Max. T and Min. T stand for maximum and minimum temperatures, respectively.

Table 5

Meteorological data of Balkan populations of *Drosophila subobscura* used in the joint analyses.

Population	Max. T (ºC)	Min. T (ºC)	Mean T (ºC)	Rainfall (mm)
Apatin June 94	26.3	13.8	20.3	55.8
Apatin June 08+09	26.4	14.3	20.6	99.4
Kamariste June 1996	27.5	13.6	21.0	22.9
Avala June 2004	25.3	15.1	19.9	113.3
Avala Sept. 2003	22.9	13.3	17.6	54.4
Avala Sept. 2004	22.3	12.5	17.0	46.5
Avala Sept. 2005	22.8	14.4	17.9	48.2
Djerdap June 2001	24.4	12.5	18.2	225.7
Djerdap June 2002	27.9	14.8	21.7	122.5
Djerdap August 2001	30.6	16.2	22.9	43.8
Jastrebac June 1990	-	-	-	-
Jastrebac June 1993	27.0	13.2	19.8	88.1
Jastrebac June 1994	25.5	13.3	19.1	90.7
Zanjic June 1997	26.6	18.7	22.6	24.8

Apatin 08+09 values are an average from climatic data of 2008 and 2009. Data from Jastrebac June 1990 were not available.

Max. T and Min. T stand for maximum and minimum temperatures, respectively.

Figure 1

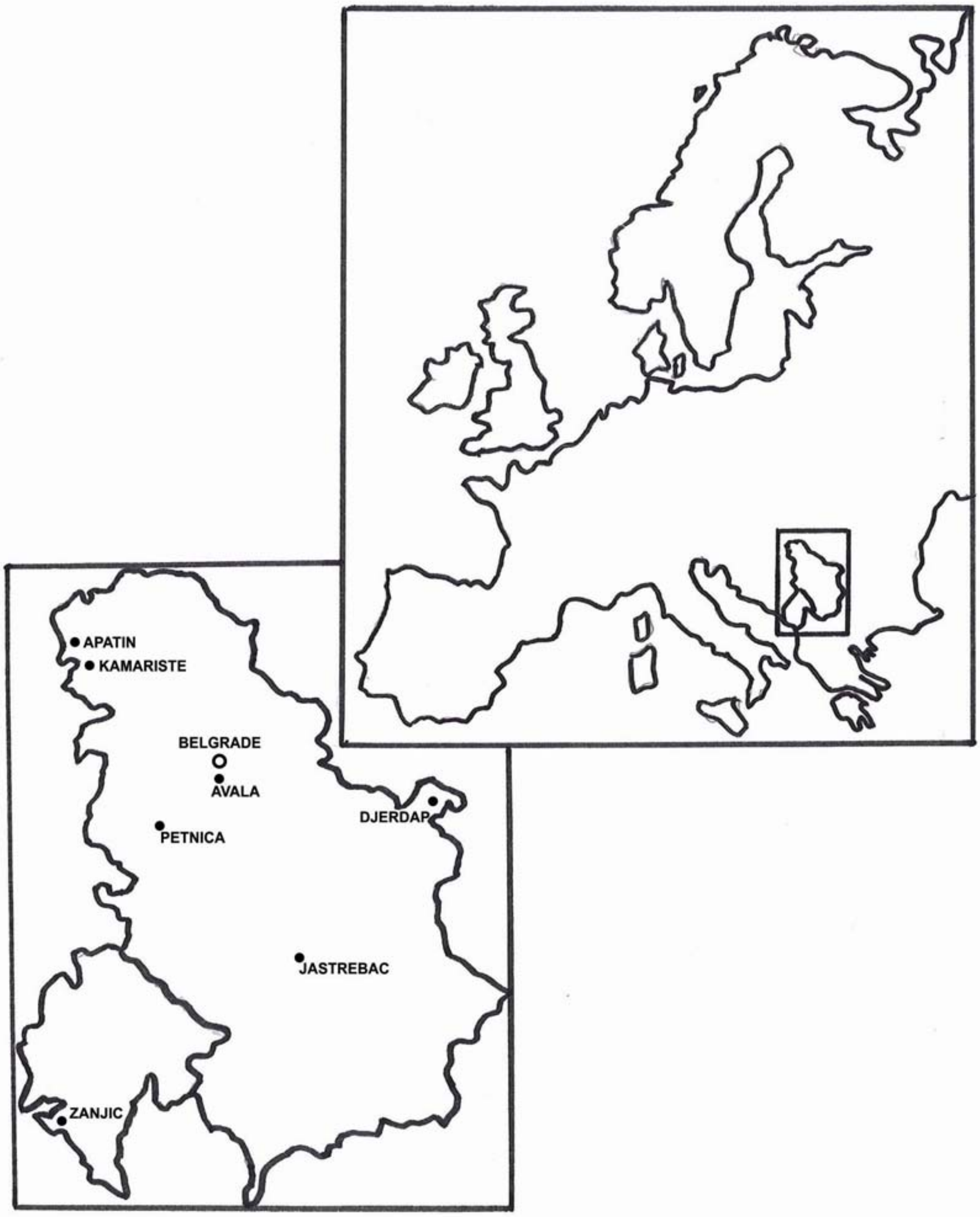

APATIN
KAMARISTE
BELGRADE
AVALA
PETNICA
DJERDAP
JASTREBAC
ZANJIC

Figure 2a. Min. T

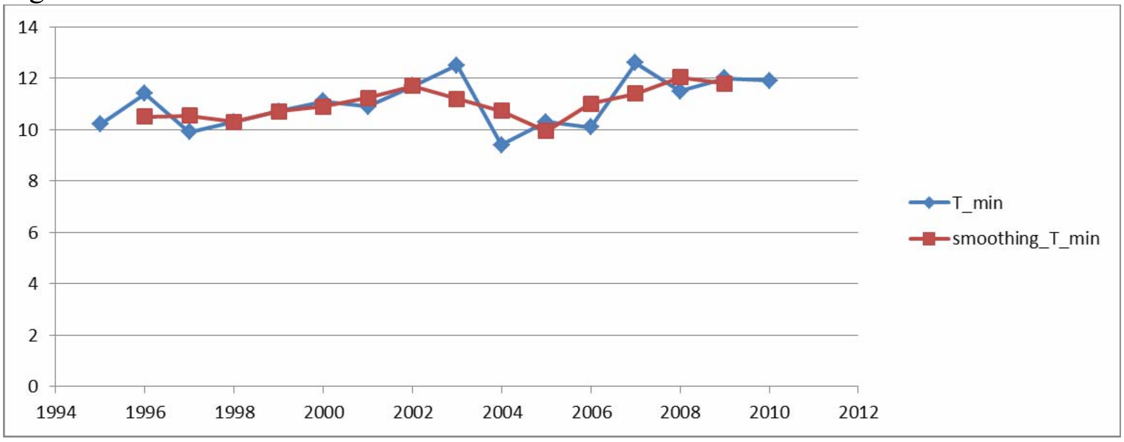


Figure 2b. Max T.

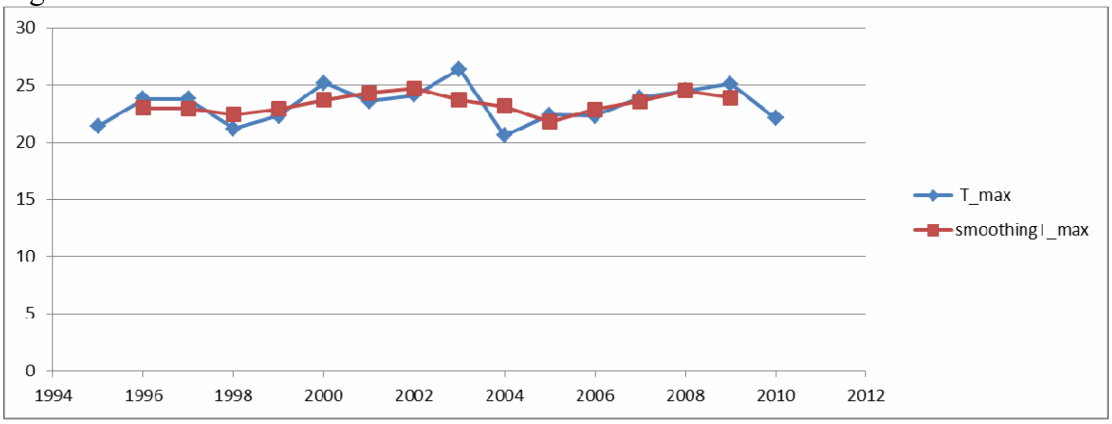


Figure 2c. Mean T.

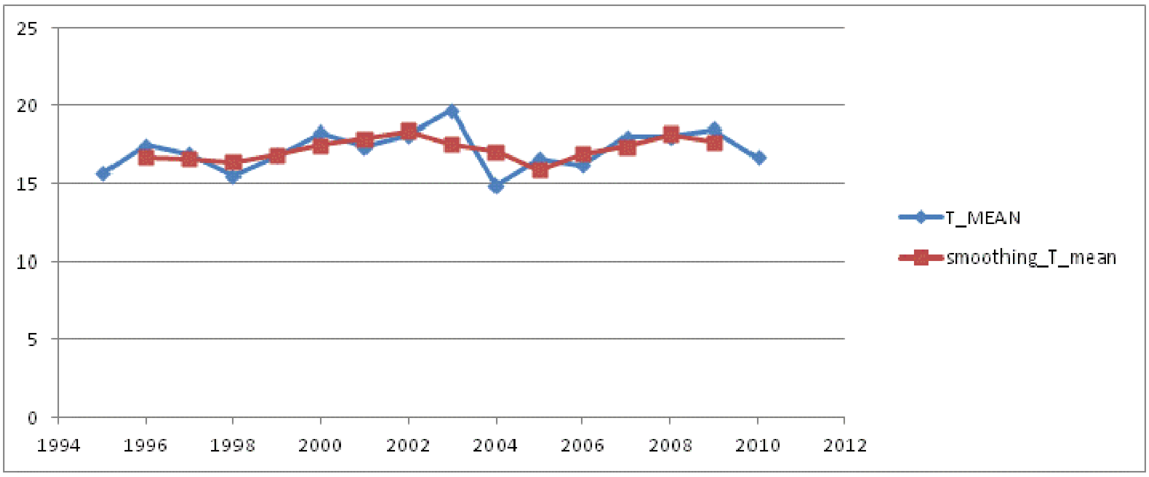
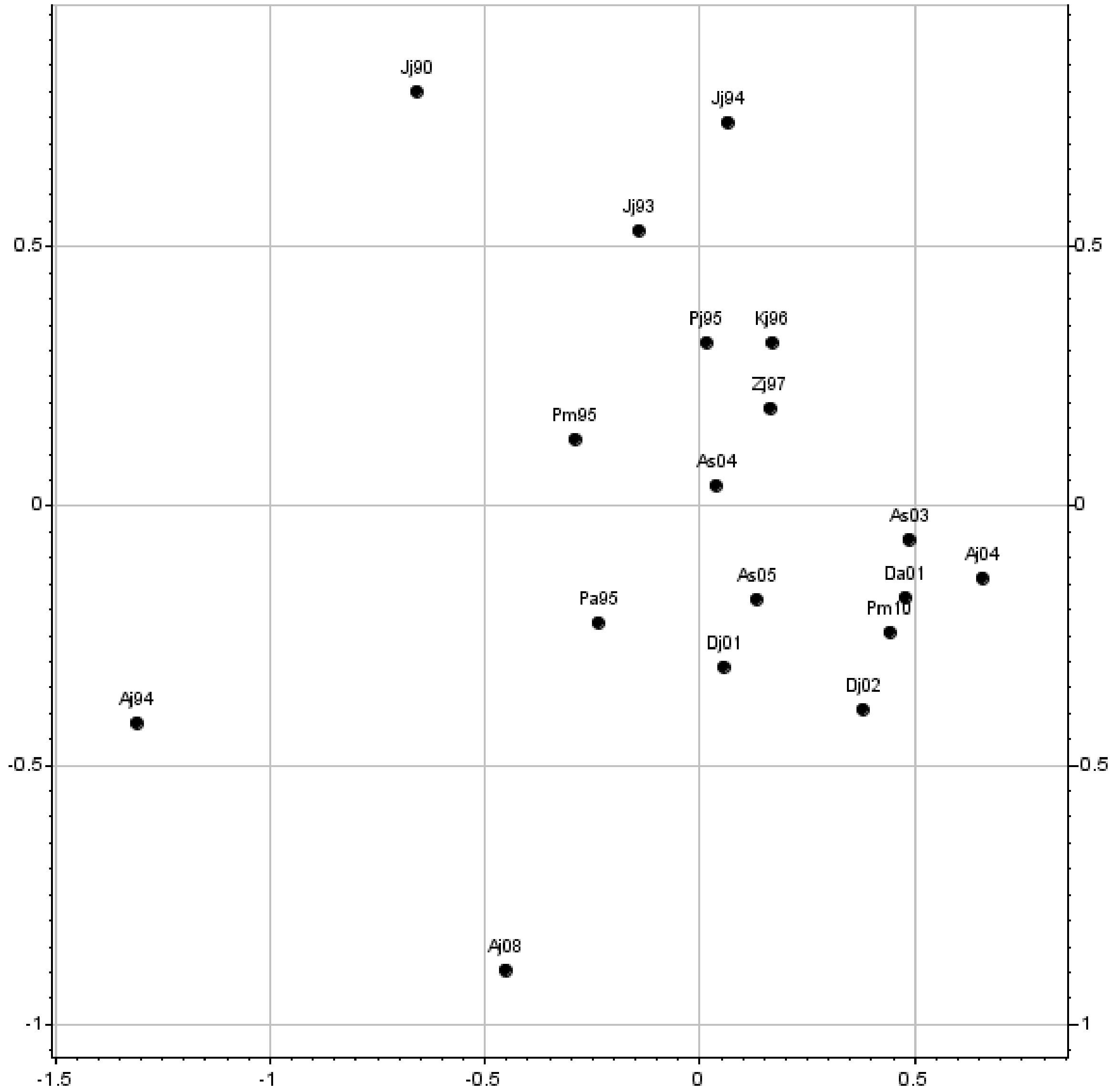


Figure 3a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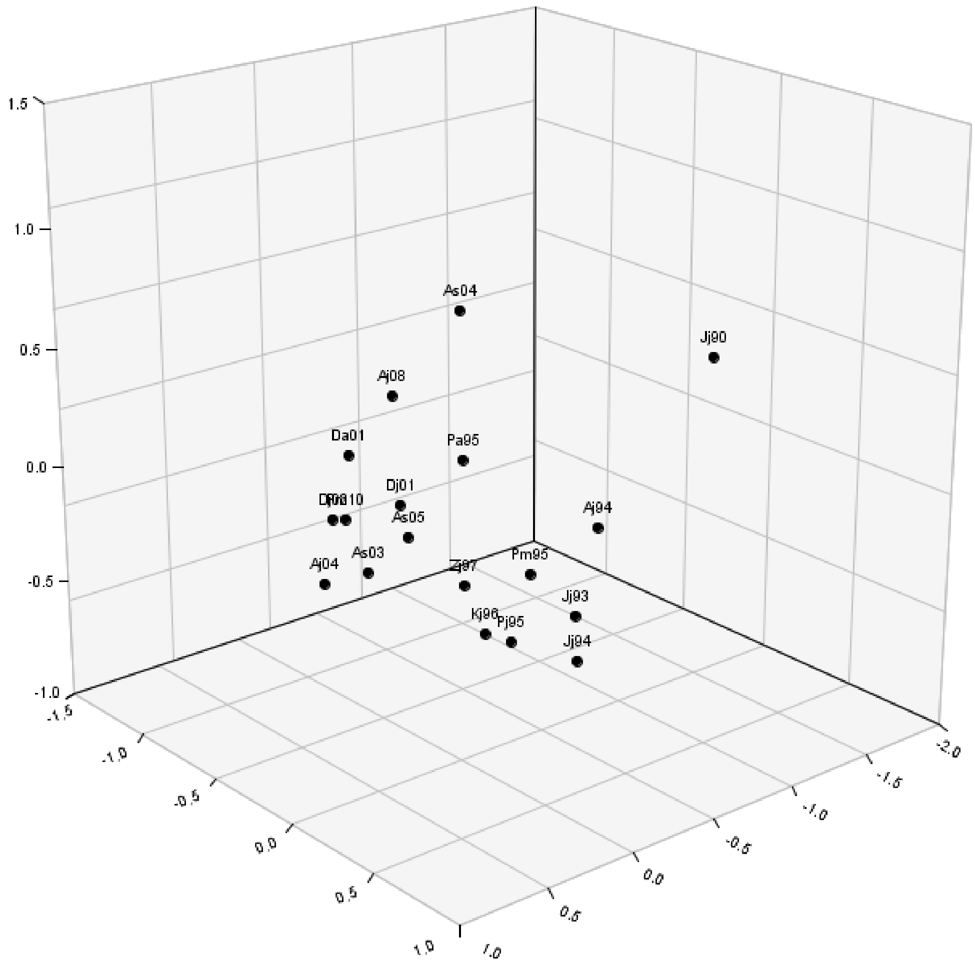
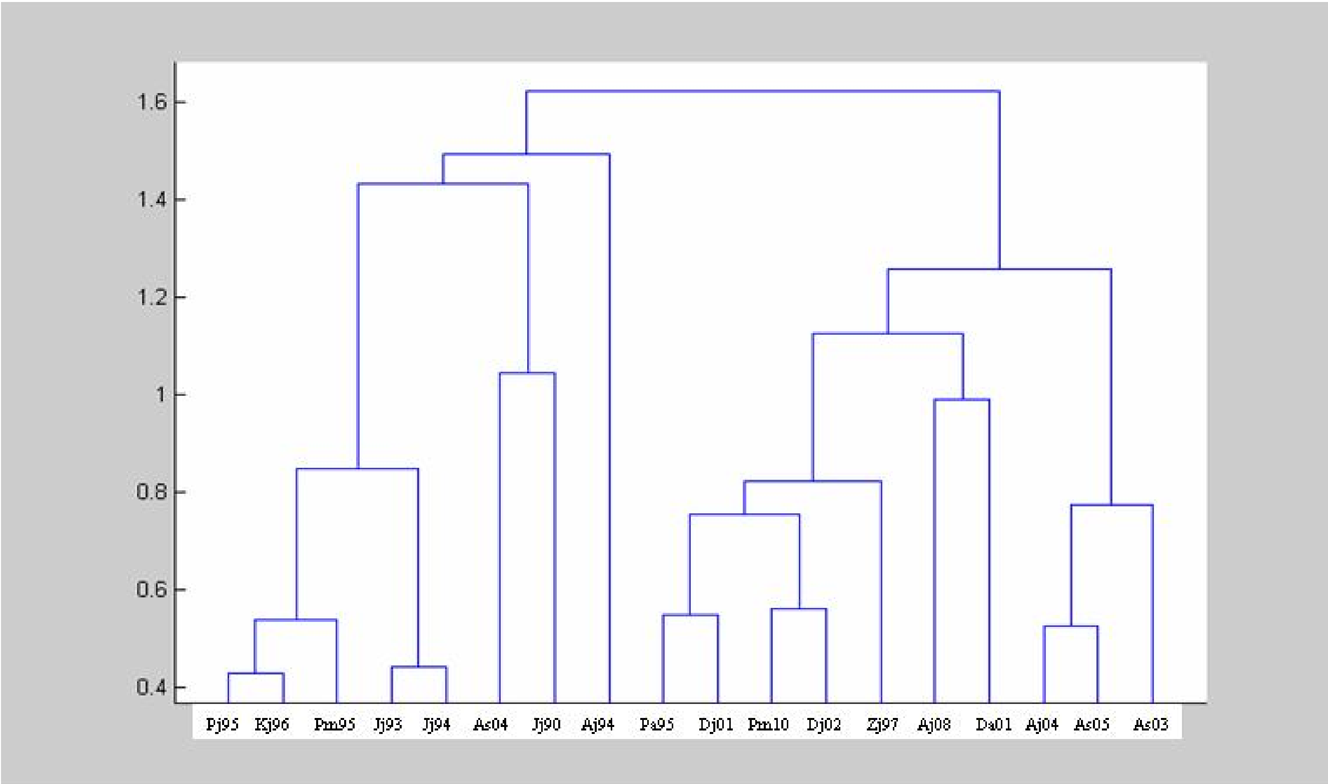
Jj94
Jj93
Pj95
Kj96
Zj97
Pm95
As04
As03
Aj04
Da01
As05
Pa95
Pm10
Dj01
Dj02
Aj94
Aj08
0.5
0
-0.5
-1
-1.5
-1
-0.5
0
0.5

Figure 3 b



1.5
1.0
0.5
0.0
-0.5
-1.0
As04
Jj90
Aj08
Da01
Pa95
Dj01
As05
Aj94
Aj04
As03
Pm95
Jj93
Kj96
Pj95
Jj94
-1.5
-1.0
-0.5
0.0
0.5
1.0
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0

Figure 4 a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1.4
1.2
1
0.8
0.6
0.4
Pj95 Kj96 Pm95 Jj93 Jj94 As04 Jj90 Aj94 Pa95 Dj01 Pm10 Dj02 Zj97 Aj08 Da01 Aj04 As05 As03

Figure 4 b

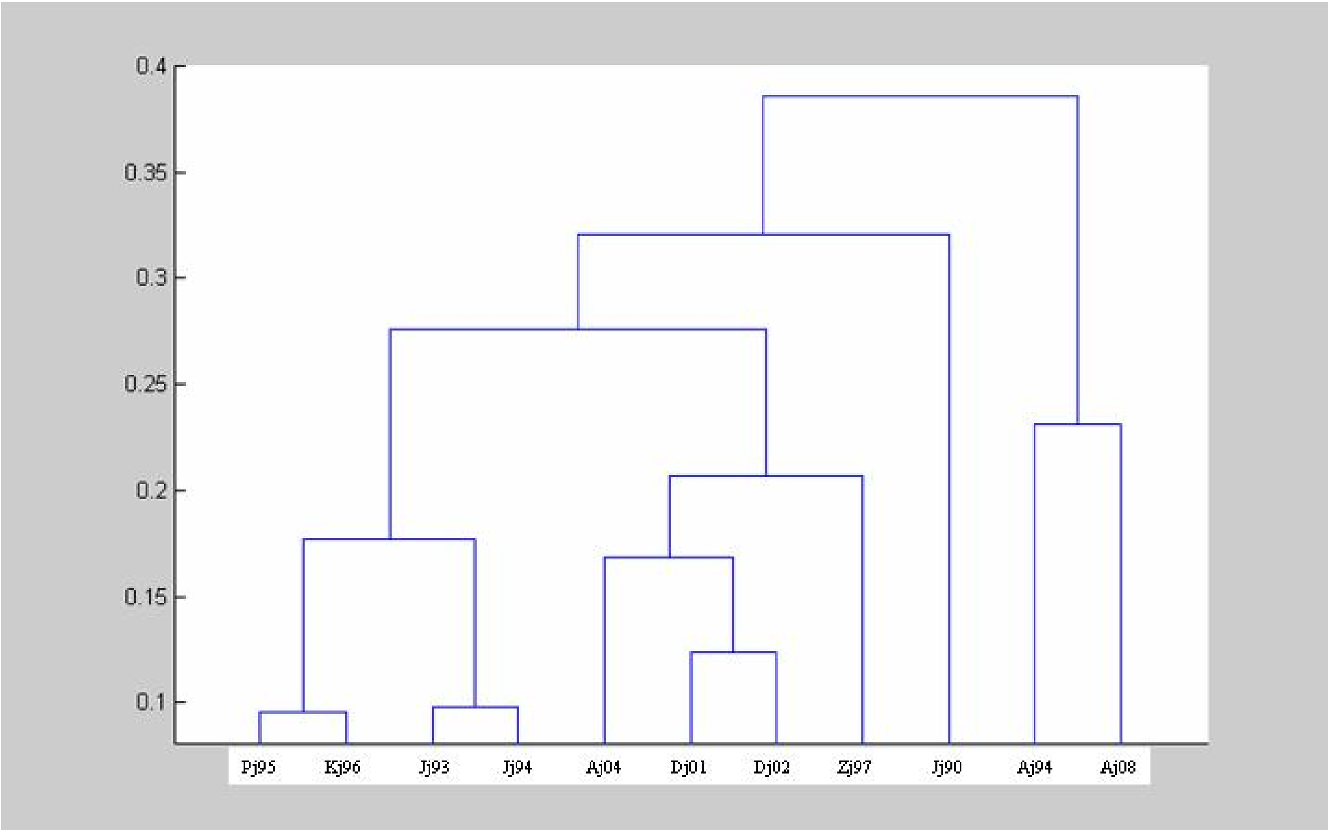

0.4
0.35
0.3
0.25
0.2
0.15
0.1
Pj95
Kj96
Jj93
Jj94
Aj04
Dj01
Dj02
Zj97
Jj90
Aj94
Aj08

Figure 5 a

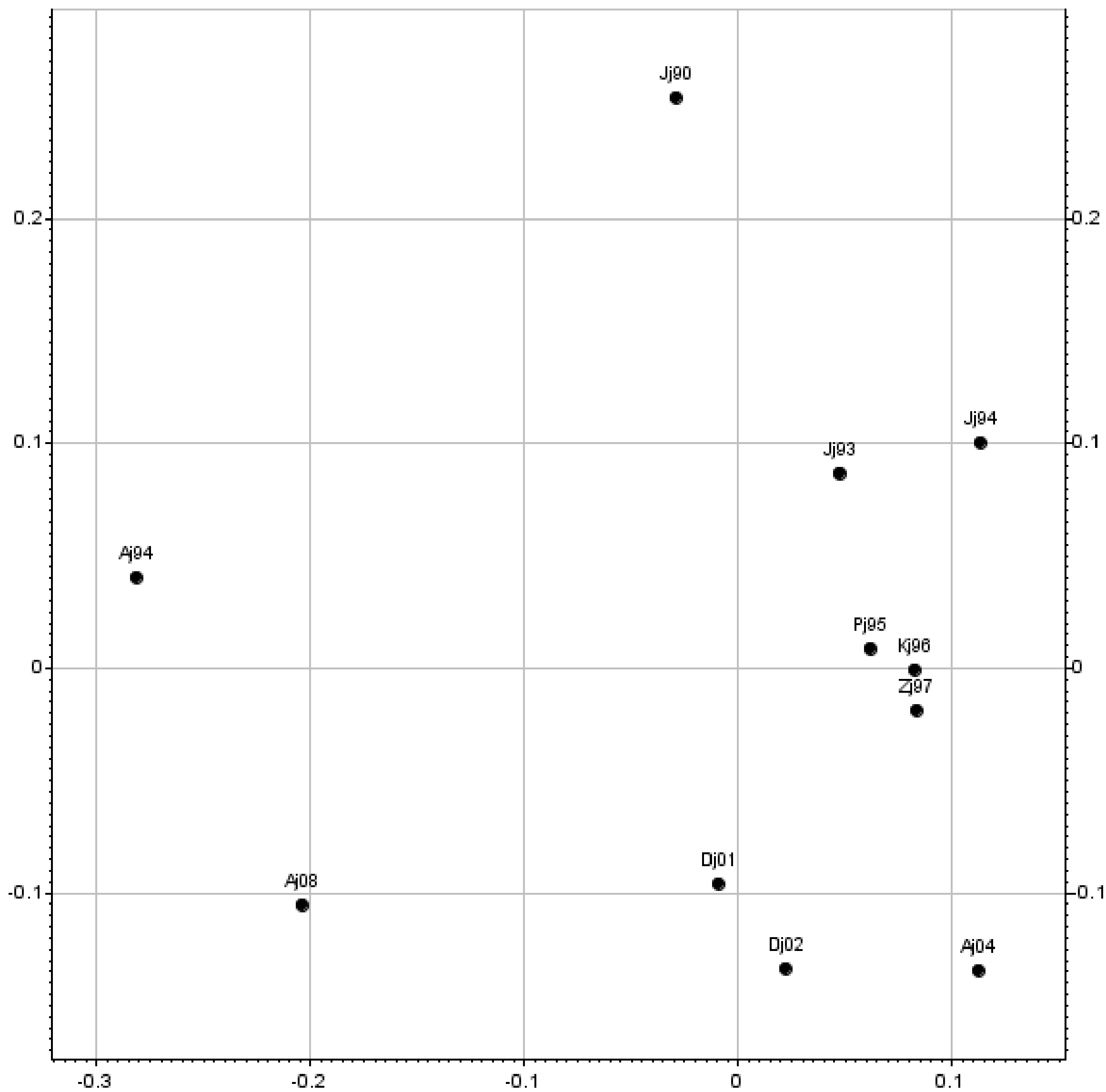

Jj90
Jj94
Jj93
Aj94
Pj95
Kj96
Zj97
Dj01
Aj08
Dj02
Aj04
0.2
0.1
0
-0.1
-0.3
-0.2
-0.1
0
0.1

Figure 5 b

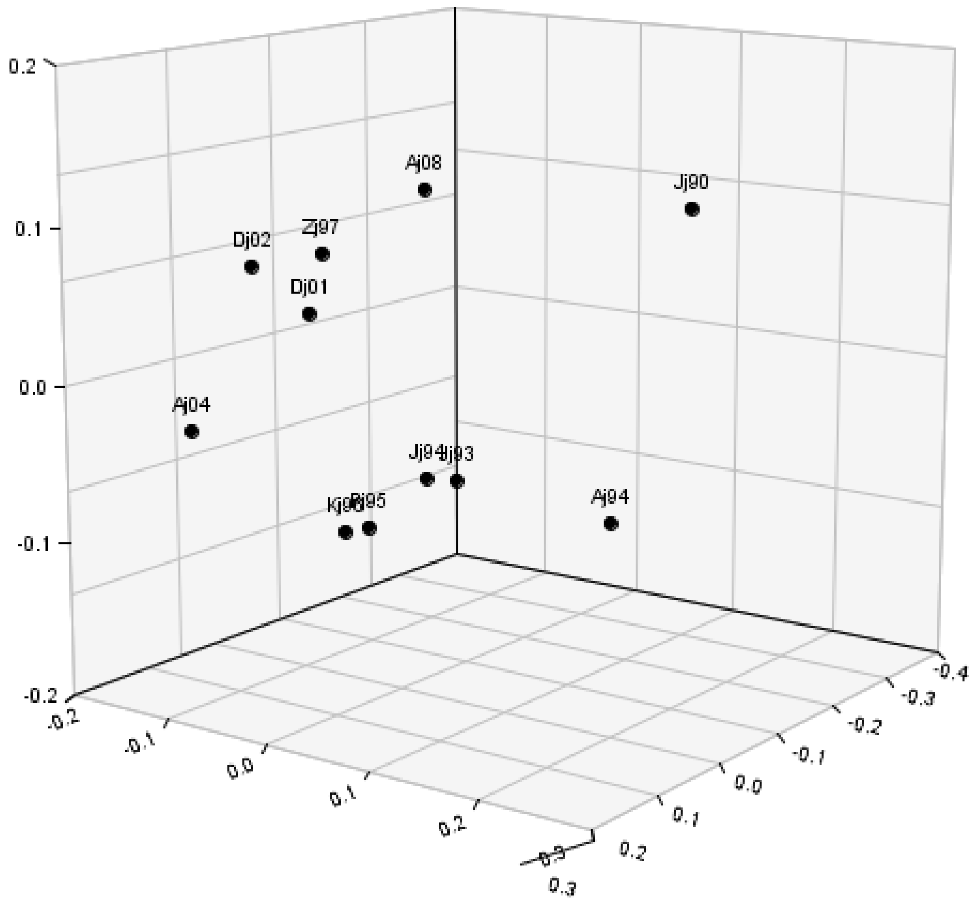
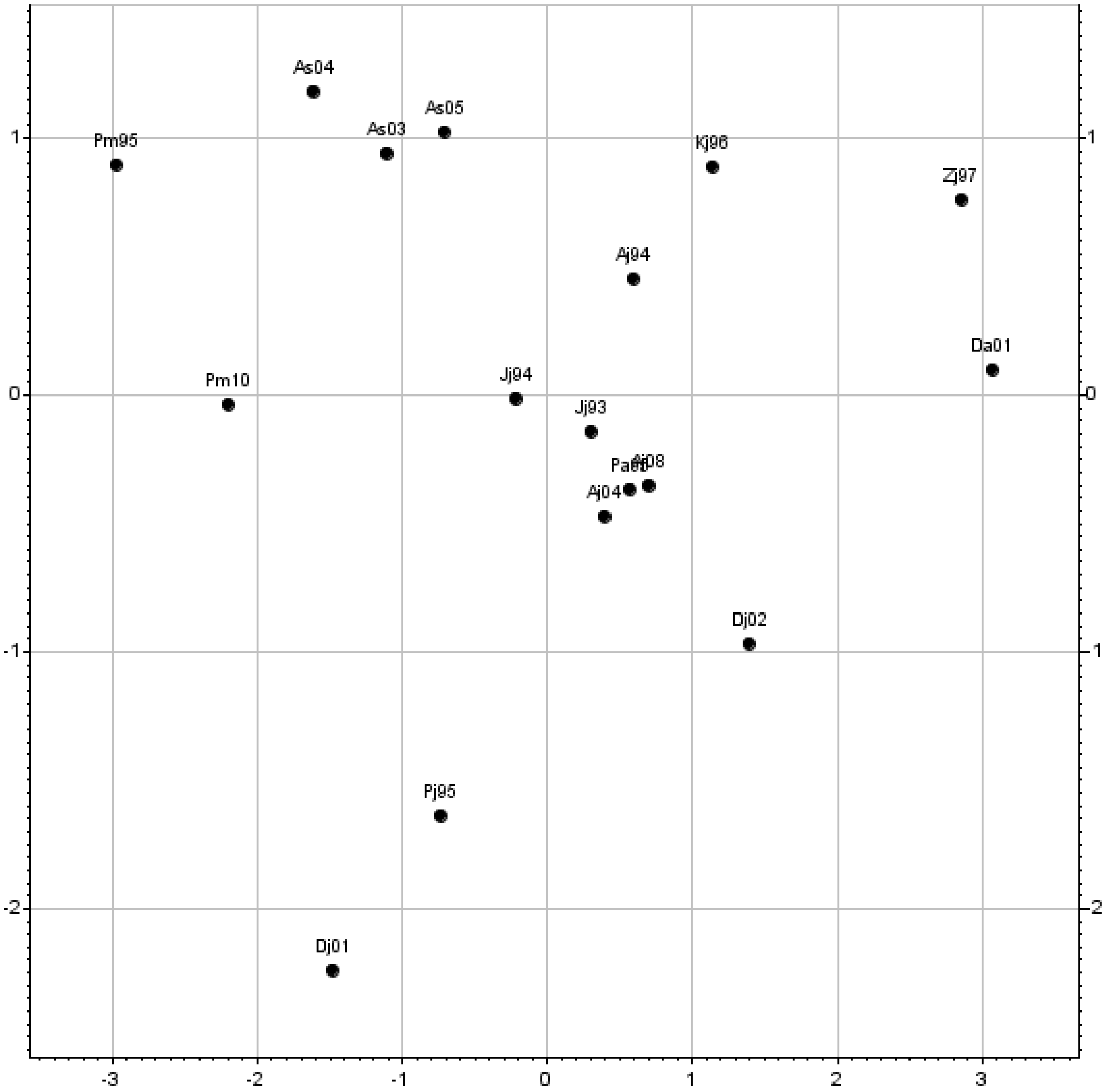

Aj08
Jj90
Dj02
Zj97
Dj01
Aj04
Jj94
Aj94
0.2
0.1
0.0
-0.1
-0.2
-0.2
-0.1
0.0
0.1
0.2
0.3
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3

Figure 6



As04
As05
As03
Pm95
Kj96
Zj97
Aj94
Da01
Pm10
Jj94
Jj93
Aj08
Aj04
Dj02
Pj95
Dj01
1
0
-1
-2
-3
2
3