Chapter 5

Genetic structure of Leptopilina boulardi populations from different climatic zones of Iran

Abstract

The genetic structure of populations can be influenced by geographical isolation (including physical distance) and ecology. We examined these effects in Leptopilina boulardi, a cosmopolitan parasitoid of Drosophila of African origin and widely distributed over temperate and (sub) tropical climates. We sampled 11 populations of L. boulardi from five climatic zones in Iran. The nuclear genetic variation among these populations was compared using amplified fragment length polymorphism (AFLP). To assess whether these populations had also diverged in their mtDNA, we sequenced part of the cytochrome oxidase (COI) gene. Genetic distances were calculated using Nei and Li’s index (Nei & Li, 1979) and analysed using UPGMA cluster analysis and Principal Coordinates analysis (PCO). The AFLP results demonstrated clear-cut genetic differentiation between populations collected from the central part of Iran and those from north, which are separated by a desert. Both UPGMA and PCO analysis further separated two populations from the very humid western Caspian Sea coast (zone 3) from other northern populations from the temperate Caspian Sea coastal plain (zone 2) which are connected by rain forest. One population from the Caspian coast, Astaneh, was found to be genetically highly diverged from all other populations. An intermediate genetic structure between zone 2 and 3 was found for Nour from zone 2 which indicates some gene flow between these two populations. In all analyses a mountain population, Sorkhabad, was found to be genetically identical to those from the coastal plain (zone 2), which indicates high gene flow between these populations due to short geographical distance. A Mantel test showed a highly significant positive correlation between genetic and geographic distances ($r = 0.47$, $P < 0.001$). The COI gene was found highly conserved among all populations. Our results suggest that both geographic distances and physical barriers contribute to the formation of
genetically distinct populations of *L. boulardi*. Transfer of fruits containing *Drosophila* larvae parasitized by *L. boulardi* may have caused unexpected gene flow and changed the genetic pattern of populations, particularly in urban areas.

**Introduction**

Insect parasitoids have been a favorite model in ecology and evolutionary biology (Godfray and Shimada, 1999). *Drosophila* parasitoids have played a major role in these studies, because of their ease of maintenance in the laboratory, the enormous biological and genetic information on their *Drosophila* host which has been used as model organism for almost a century (Irving et al. 2004) and because of their diversity which allows comparative studies (Chapters 2 & 3). One way to study adaptation in changing environments is to compare populations of a widely distributed organism across contrasting climates. Understanding the genetic structure of the insect populations is an important first step in the study of local adaptation (Mopper and Strauss 1998). Understanding the genetic structure of populations can provide essential insights to interpret the results of ecological studies. Studying genetic population structure of insects is thus essential for comparative studies of behaviour and ecology and evolution of populations (Roderick, 1996). DNA markers provide powerful and efficient tools to study genetic diversity at both inter- and intraspecific level in insects’ populations (Avise, 1994). A wide range of molecular markers has been successfully used to measure genetic diversity of insects, including RFLPs, RAPDs, SSRs and AFLP’s. Among these techniques, Amplified Fragment Length Polymorphism (AFLP) is a useful DNA fingerprinting technique to study genetic diversity within a species (McMichael and Prowel, 1999; Hawthorne, 2001; Wu et al. 2006 and Tao et al. 2009). AFLP is a powerful technique, which is able to detect genetic variation of organisms based on DNA from any source and complexity without prior knowledge of the gene structure or sequences (Vost et al. 1995). In comparison to other methods, AFLP can provide higher resolution whilst needing less DNA (Tao et al. 2009). AFLP has been successfully used to study genetic diversity of insect populations from different geographic regions and belonging to different orders and families (Reineke et al., 1999; Ravel et al., 2001; Lu et al., 2002; Pannebakker et al. 2004; Samara et al. 2008; Almalakala et al. 2009; Tao et al. 2009).

One of the main constraints on local genetic differentiation and adaptation is extensive gene flow between populations. Reduced dispersal of populations can thus accelerate the adaptation process and may lead to genetic subdivision of populations (Slatkin 1987). Environmental or physical barriers may promote isolation of populations. These include geographic distance and physical barriers like mountains, rivers and stretches of unsuitable habitat (Peterson and Denno 1998). AFLP markers
have been used to infer the role of geographical distance and barriers to gene flow in shaping the genetic structure of population in a variety of organisms (e.g. Salvato et al. 2002; Irwin et al. 2005; Clark et al. 2007). In the present study we used AFLP to investigate the role of geographical distance in shaping genetic variation in Iranian L. boulardi populations. *Leptopilina boulardi* (Hymenoptera: Figitidae) is a larval parasitoid of *Drosophila* of African origin (Allemand et al, 2003) which is widely distributed over tropical and warm temperate regions.

Mitochondrial markers have also been used in population genetic studies of insects (e.g. Crozier et al.1997; Olav et al. 2003; Smith 2005). For example, in the ant *Leptothorax rugatulus* (Olav et al.2003) a mitochondrial marker was found to be more informative than microsatellites and discriminated significantly better between populations due to maternal divergence resulting from reduced dispersal of females. Sequencing part of the cytochrome oxidase I gene successfully discriminated between two species of *Homalodisca*, a cicadellid leafhopper and demonstrated genetic differences between *H. coagulate* populations based on haplotype (Smith 2005). Using mitochondrial markers may provide valuable information on the migration of females between populations, because mitochondria are maternally inherited. Being able to trace back migration of female populations could provide crucial information in some insect parasitoids that have established asexual populations after infection with parthenogenesis-inducing *Wolbachia* (Stouthamer et al. 1999).

In this study, we used both AFLP and COI gene sequencing to study the genetic structure among 11 populations of *Leptopilina boulardi* collected from five contrasting climate zones in Iran. The zones were chosen to represent five distinct climates varying in precipitation, length of season and minimum and maximum temperatures. Many studies have combined genetic studies with experimental studies to measure the adaptive value of traits (Roderick, 1996; Kaltz and Shykoff 1998; Mopper and Strauss 1998; Lively 1999; Anderson et al. 2004). The main goal of this study was to describe the genetic structure of these populations, to be used later in a study of local adaptation in life history traits of these populations (chapter 6).

**Material and methods**

**Field sampling**

*Leptopilina boulardi* populations were sampled along a climatic cline stretching from the northern to the central regions of the Islamic Republic of Iran in July 2006. The transect covered five climatic zones:
as follows (Table 1): cool mountains in Damavand (zone 1), wet forests (zone 2) and very wet rain forests along the Caspian sea coast (zone 3), a dry and hot climate with cold winters in the Esfahan region (zone 4) and a Mediterranean climate with very cold winters in Shahre Kord (zone 5). The locations of the sampling sites and climatic zones are shown in Fig. 1. Sampling was conducted in mid-summer to increase the likelihood of collecting adult wasps from all the zones. At each sampling site, we placed 12 traps. The traps consisted of plastic containers (diameter 10 cm; height 7.5 cm) with a 3 cm diameter circular hole in the lid. The lid holes were covered with a plastic mesh with a diameter of 2 mm to prevent large insects, snails, slugs and small rodents from entering the traps. This mesh was wide enough for *Drosophila* flies and their parasitoids to easily enter the traps. Several layers of filter paper were placed in each trap to absorb water and provide pupation sites for *Drosophila* larvae. The traps were baited with a piece of banana cut lengthwise with a smear of live yeast on top that was added to speed up fermentation and to attract fruit flies and parasitoids. The traps were suspended from trees using fishing wire and tilted downward to prevent rain from filling them. Each trap was placed in the shade of a bush or tree to protect it from overheating and its position was recorded by GPS. After a week the traps were collected and all pupae in the traps were wrapped in filter paper and transferred to lab. *Leptopilina boulardi* was the only parasitoid collected from all locations. Partially inbred lines were set up from each sampling site and 20 female wasps per population from early generations were frozen at -80°C for genetic analysis.

**DNA extraction**

A number of DNA extraction methods were tried using either the whole body of a single female or a group of frozen female wasps. The highest yields of high quality DNA were obtained using an adapted CTAB protocol for insects (Reineke et al. 1998). The DNA extracted by using this method also resulted in the most reproducible AFLP patterns when DNA from the same extractions was amplified multiple times. Five female wasps were pooled per strain for DNA extraction. An extra RNA digestion was carried out by adding RNase A to the final concentration of 20μg / ml and samples were incubated at 37 °C for 30 min. RNase A was then removed from the samples by adding phenol:chloroform:isoamylalcohol (25:24:1). We then suspended the DNA in the upper phase of the solution and transferred the upper phase to a new tube before DNA precipitation. DNA was precipitated using isopropanol (similar to the precipitation step for DNA extraction in CTAB protocol). The quality of the extracted DNA was checked on a 0.8% agarose gel and its quantity measured by a spectrophotometer (ND-1000, www.nanodrop.com ). In the case of low quantity or sheared DNA (smear on the gel) the DNA extraction was repeated using five new females from the same strain.
| Zone ID | Population ID | Climate Description of the location | Vegetation | Elevation (m) |
|---------|---------------|-------------------------------------|-------------|--------------|
| 1       | 1873          | Mediterranean with very cold winter | Close to Shahrak and along Zayandehrod river | 1975        |
| 2       | 1605          | Semi dry desert with cold winter    | Close to Esfahan along the Zayandehrod river | 762         |
| 3       | 1625          | Semi dry desert with cold winter    | Close to Esfahan along the Zayandehrod river | 765         |
| 4       | 485           | Temperate very wet forest          | In Gilan province in coastal Caspian sea | 8.5         |
| 5       | 378           | Temperate very wet forest          | In Gilan province in coastal Caspian sea | 76          |
| 6       | 3             | Temperate very wet forest          | In Gilan province in coastal Caspian sea | 76          |
| 7       | 20            | Temperate very wet forest          | In Mazandaran province in coastal Caspian sea | 75          |
| 8       | 75            | Temperate very wet forest          | In Mazandaran province in coastal Caspian sea | 76          |
| 9       | 1975          | Temperate very wet forest          | In the northern slope of the Damavand mountain, lower Mazandaran province | 765         |

Table 1: Description of the trapping locations of the populations including climate, vegetation and elevation.
Fig 1: Map of the Islamic republic of Iran with climate zones indicated in shades of grey. Sampling points are indicated by the ID name of population, see table 1.

AFLP analysis

To assess the genetic diversity of the *L. boulardi* populations we employed the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) with a slight modification to the standard procedure. The AFLP procedure consisted of four steps. Restriction and ligation were carried out separately overnight. Step 1 restriction digestion: 7 μl containing approximately 500 ng of genomic DNA was incubated with EcoRI and MseI enzymes at 37 °C overnight. Each reaction contained 5U EcoRI, 5U MseI, 4 μl of 10X restriction buffer (react 4) and 10 μg of BSA (bovine serum albumin), all from New England Biolabs (www.neb.com). The total volume was adjusted to 40 μl with autoclaved nanopure water. Step 2, restriction ligation: to each tube containing 40 μl of sample from step 1, 10 μl of adaptor-ligation mix was added, which contained 5 μl EcoRI / MseI adaptor mix (one part 10μM Eco-adaptor and 9 parts 10μM Mse-adaptor; Table 1), 1 μl of 10 mM ATP, 1 μl 10X restriction buffer (react 4), 2U T4 ligase and 2.67 μl autoclaved nanopure water. The restriction-ligation reactions were incubated at 37 °C overnight. Step 3, preamplification: this was the first
of two selection rounds of the fragments aimed at reducing background noise and nonspecific bands (Vos et al., 1995). Two primers, EcoRI and MseI with one base extension (Table 2) were used in the master mix of 16.7 μl per sample. Each reaction contained, 0.2 μl EcoRI+A primer (20 μM), 1.5 μl of Mse+C primer (20 μM), 15 μl AFLP Coremix (Applied biosystem, www.appliedbiosystems.com), and 4 μl of diluted restriction-ligation mix (concentration: 1 part restriction-ligation: 2.5 parts H2O) as DNA template. A touchdown profile amplification was used to increase the optimal primer selectivity in this step. The thermal cycling started with 2 min at 72 ºC to allow the polymerase to repair the nick caused by ligation of adaptors. This was followed by two cycles of 30 sec at 94 ºC, 30 sec at 65 ºC, 1 min 72 ºC, then 12 touchdown cycles during which the annealing temperature was dropped by 0.7 ºC in each cycle, then 23 cycles of 30 sec at 94 ºC, 30 sec at 56 ºC, 1 min 72 ºC and ended with 30 min at 60 ºC. This last step can promote addition of A (adenine) at the 3’ end of the strands by tqa polymerase.

Table2: List of primers and adaptors used for the AFLP analysis

| Type               | Name            | Sequence         | 5’-3’ |
|--------------------|-----------------|------------------|-------|
| Adaptor            | EcoRI adaptor/F| CTCGTAGACTGCGTGACC|       |
|                    | EcoRI adaptor/R| AATTGGTACGCAGTCTAC|       |
|                    | MseI adaptor/F  | GACGATGAGTCCTGAG  |       |
|                    | MseI adaptor/R  | TACTCAGGACTCAT   |       |
| Primer, pre select.| EcoRI Preamp.   | GAC TCGTGACCAATTCA* |     |
| amp.               | MseI Preamp.    | GATGAGTCTGAGTAAC* |     |
| Primer, select. amp.| EcoRI Select.  | GACTCGTGACCAATTCAANN * |   |
|                    | MseI Select.    | GATGAGTCTGAGTAACN * |     |

*Nucleotides in bold are the fixed extended base in the 3’ end of the primers and N are the variable extension to provide different primer combination, see above.

The quality of amplified product was checked by loading 4 μl of PCR product on a 0.8% agarose gel prior to next step. Step 4, selective amplification: this last selection step was performed using one of three EcoRI primers (fluorescently labeled) and one of three MseI with three and two base extensions in each primer respectively. Different combinations of these primers resulted in 9 useful primer combinations (Table 3). Each PCR reaction contained 0.25 μl MseI C+X (20μM), 1.0 μl EcoRI A+XX (1 μM), 15 μl AFLP Coremix, 0.75 μl of autoclaved nanopure water, and 3 μl of PCR product from step 3 (diluted 1/10) in a total of 20 μl. Thermal cycling conditions were identical to those used in the preamplification, except that it started with 1 min at 94 ºC instead of 2 min at 72 ºC.
Table 3: List of selective amplification primers and fluorescent labels used in nine different combinations and the number of scorable and polymorphic bands generated by each combination.

| Combination | Primers and labels | Scored band | Polymorphic band |
|-------------|--------------------|-------------|-----------------|
| A           | EcoRI+ACA (labeled Fam), MseI+CA | 117         | 22 (18.8%)      |
| B           | EcoRI+AGG (labeled Joe), MseI+CA | 63          | 20 (31.74%)     |
| C           | EcoRI+AAC (labeled Ned), MseI+CA | 65          | 12 (1834%)      |
| D           | EcoRI+ACA (labeled Fam), MseI+CT | 101         | 15 (14.85%)     |
| E           | EcoRI+AGG (labeled Joe), MseI+CT | 65          | 25 (38.46%)     |
| F           | EcoRI+AAC (labeled Ned), MseI+CT | 58          | 11 (18.96%)     |
| G           | EcoRI+AGG (labeled Joe), MseI+CG | 80          | 14 (17.5%)      |
| H           | EcoRI+AGG (labeled Joe), MseI+CG | 69          | 16 (23.19%)     |
| I           | EcoRI+AGG (labeled Joe), MseI+CC | 52          | 12 (23.08%)     |

Total band 670 147

For capillary electrophoresis, the PCR products from the selective amplification were diluted 10-fold. DNA was purified to remove the extra salt from the solution by following the MegaBacel purification and precipitation protocol. DNA pellets were dissolved in 20 μl distilled water at 40 ºC for 30 min. Each sample contained 3.75 μl Megabace loading solution, 0.25 μl ET-500 R Size standard (both from Amersham Bioscience (www.amersham.com) and 2 μl of clean PCR product. Samples were denatured at 95 ºC for 3 min, immediately placed on ice and injected into LAP matrix (Amersham) capillaries at 4 kV for 54 sec and run on a DNA sequencer MegaBACE 1000 system (Amersham Pharmacia USA, www.amersham.com) at 10 kV for 75 min. The sequencer automatically captured and stored electrophoretic profiles for each sample. The fluorescent profiles were loaded into Fragment Profiler software ver1.2 (Amersham, Biosciences, www.amersham.com) using specific peak filters and were manually checked for correct alignment of the size standard. The positions of polymorphic markers between 80 and 500bp were scored and exported as a binary matrix in an excel sheet which ‘1’ indicating presence and ‘0’ absence of a band at a particular locus.

Amplification and sequence alignment for COI

A 638 bp portion of the cytochrome oxidase subunit I (COI) gene was amplified to evaluate the mitochondrial variation among the 11 Leptopilina boulardi populations from Iran. A. L. boulardi population from Sospel (France) was used as out-group. PCR amplification was performed using a forward primer designed for a herbivorous hymenoptera from Torymidae family (Scheffer & Grissell 2003), COI-1775-F 5’-CGAATAAATAATATAAGATTG-
and a reverse primer designed for *Leptopilina clavipes*, another species of the same genus (K. Kraaijeveld, unpublished), COI-2413-R, 5'-TCATCTAAAAATTITTAATCCCATGT-3'. The amplification was carried out on a Thermocycler PTC-2000 using the following thermal cycles: denaturing for 3 min at 92ºC followed by a touchdown with one degree drop in annealing temperature per cycle from 53-40 ºC (10 sec at 92 ºC ,10 sec at 53-40 ºC and 2 min at 72 ºC), then 25 cycles of 10 sec at 92 ºC,10 sec at 40 ºC and 2 min at 72 ºC, and ended with 5 min extension at 72 ºC. Each PCR reaction contained 1.2μl of 2.5mM DNTPs, 0.5μl of 10 μM of each primer, 0.15μl of Taq DNA polymerase(5 U), 1.5μl of 10X Buffer and 0.45μl of 15mM MgCl2 μl; all Qiagen products and was adjusted to 15μl with autoclaved nanopure water. The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, www.promega.com) following the manufacturer’s protocol. The amplified bands were sequenced both forward and reverse on MegaBACETM 1000 sequencer. The Sequencer software Version 4.2 (Gene Codes Corp.) was used to assemble the contigs and obtain consensus sequences. The sequences were aligned using pairwise-alignment in MacClade 4.08 (Maddison and Maddison 2005) and edited manually.

**Data analysis**

Pair-wise genetic distances between populations were calculated from the AFLP data using Nei and Li’s index (Nei & Li, 1979) in Treecon version 1.3b for windows (van de Peer and de Wachter, 1994). UPGMA cluster analysis (Sokal and Michener, 1958) with 1000 replications of bootstraps was performed in Treecon and genetic distances were visualized in a dendrogram format. The geographic distances between collecting sites were calculated from their GPS locations and combined with the genetic distance values into a pair-wise genetic and geographic distance matrix. Isolation by distance was investigated with a Mantel test (Mantel, 1967) using the web-based program Isolation by distance web service (IBDWS) version 3.16 (Jensen et al. 2005). The combined binary AFLP data matrix was used to perform Principal Coordinate analysis (PCO) with GenAlex 6 (Peakall and Smouse, 2006) using Nei and Li’s coefficient (Nei & Li, 1979) for calculating similarities, three first coordinates were used to graphically depict genetic variation among populations.

**Results**

**AFLP analysis**

The 9 primer combinations yielded 670 scorable bands of which 147 (21.94%) were polymorphic. UPGMA tree and bootstrap analysis indicated that the three populations from the central part of Iran (Dorcheh, Khairabad and Zamankhan) clustered together, but were differentiated from the northern populations sampled along the Caspian
Sea coast (bootstrap value = 88%, Fig. 2). One population from the very wet Caspian Sea coast (Astaneh) was considerably different from all other populations. UPGMA tree separated this population strongly from all other collected populations with high bootstrap value (bootstrap = 100). The Sorkhabad population, collected from a mountainous region on the slope of the Damavand mountain near Mazandaran clustered together with the wet Caspian Sea coastal populations.

Principal coordinates analysis revealed informative separation of populations. The first three coordinates explained 69.32% of AFLP variation. By plotting the first two coordinates (which together explained 52.73 % of the AFLP variation) the three populations from the dry central region of Iran clustered separately from the northern populations. These two principal coordinates also separated the populations from the two northern zones (zone 2 and 3), except Nour - a population from zone 2 clustered as zone 3 and Astaneh - the most divergent population which stood apart from all others. The other two PCO plots showed further evidence for divergence among the populations along the Caspian Sea coast. By plotting first and third coordinates and second and third coordinates (which in total explained 46.67% and 39.26% of AFLP variation respectively) two clusters were evident among the populations from the Caspian Sea coast, while Astaneh, the most divergent population, again stood apart from all other groups. Furthermore, the Zamankhan population from a Mediterranean climate (zone 5) appeared separated from the two other central populations from zone 4 (Khairabad and Dorcheh). Consistent with the UPGMA analysis, the Sorkhabad population from the mountain region clustered with populations from the geographically close, but ecologically different wet Caspian Sea coast.

![Dendogram derived from the UPGMA (unweighted pair group methods of arithmetic averages) analysis of 147 polymorphic AFLP bands. Shown are the genetic distances of 11 population of Iranian *Leptopilina boulardi* and their outgroup (a population of *L. boulardi* from France). Scales indicate genetic distances (Nei & Li, 1979) and the numbers at nodes represent bootstrap value (1000 replication).](image)
Isolation of populations by distance

A significant positive correlation between genetic and geographic distance was observed among the *L. boulardi* populations (Mantel test; \( r = 0.47, P < 0.001, \) Fig. 4a). The result of this test showed that a considerable part of the genetic variation was explained by geographic distance and supported the UPGMA and Principal Coordinate analyses since all geographically-close populations resembled each other. The only exception was the population from Astaneh, which was highly distinct from all others populations, even from those collected from a distance of less than 15 km. By excluding the Astaneh population from the Mantel test, the correlation coefficient increased substantially (mantel test; \( r = 0.73, P < 0.001, \) Fig. 4b).

Variation in cytochrome oxidase I (COI)

COI sequence was found to be highly conserved among the Iranian *L. boulardi* populations. Only two bases were found to be different in two Caspian Sea coast populations and four insertions and one replacement were found in the out-group compared to the Iranian populations (Table 4).

**Table 4:** Polymorphic site of the COI gene in 638 bp sequenced for 11 Iranian *Leptopilina boulardi* aligned with their out-group, Sospel - a population of *L. boulardi* from France.

| Population | Position 10 | Position 60 | Position 131 | Position 321 | Position 338 | Position 440 | Position 459 |
|------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Sospel     | -           | G           | C           | G           | A           | A           | C           |
| Zamankhan  | -           | -           | C           | -           | -           | G           | -           |
| Qaemshahr1 | -           | -           | C           | -           | -           | G           | -           |
| Qaemshahr2 | -           | -           | C           | -           | -           | G           | -           |
| Khairabad  | -           | -           | C           | -           | -           | G           | -           |
| Dorche     | -           | -           | A           | -           | -           | G           | -           |
| Nour       | -           | -           | C           | -           | -           | G           | -           |
| Astaneh    | -           | -           | C           | -           | -           | G           | -           |
| Seyahkal   | -           | -           | C           | -           | -           | G           | -           |
| Lunak      | -           | -           | C           | -           | -           | G           | -           |
| Sorkhabad  | -           | -           | C           | -           | -           | G           | -           |
| Chalus     | C           | -           | C           | -           | -           | G           | -           |
Chapter 5

Discussion

The AFLP analysis provided 147 polymorphic loci out of 670 reproducible and scorable bands in nine primer combinations. This provided enough information to allow a clear distinction among *L. boulardi* populations originating from different climatic zones in Iran. One of the main challenges in AFLP analysis for insects is generating enough reproducible and scorable bands and this is highly dependent on the quality of DNA (Reineke et al. 1998). In population genetic studies, pooling a number of individuals instead of extracting DNA from a single organism is a possibility to get enough DNA from small organisms and this solution has been suggested in previous studies (Pannebakker et al. 2004). A CTAB protocol modified for insects resulted in high quality DNA with highly reproducible bands in AFLP analyses in comparison with other extractions methods (Reineke et al. 1998). Pooling a number of female wasps and using the CTAB protocol modified for insects in the present study resulted in highly reproducible and scorable bands. The number of polymorphic bands among the populations depends on the primer combination and the genetic divergence between populations and can vary from very high to relatively low (e.g. high; Samara et al. 2008; Tao et al. 2009 and low; Najimi et al.2002; Pannebakker et al. 2004). Similar to the results of a previous study on genetic diversity in a different *Leptopilina* species, *L. clavipes* (Pannebakker et al. 2004) we found a relatively low number of polymorphic bands per primer combination (21.94% in total). By increasing the number of primer combinations (nine combinations) we obtained a high number of polymorphic bands (147 scorable polymorphic bands), providing highly informative data on the genetic structure of these populations. Similar to other AFLP studies, the consistency of scorable bands over all populations, the reproducibility of results and the presence of polymorphisms were the three criteria to choose the proper primer combination (Samara et al. 2008; Clark et al. 2007). As AFLP markers are dominant, the main assumption in the analysis is presence or absence of identical fragments (which are considered as homologous). To calculate the genetic distance between populations with Nei and Li’s index (Nei & Li, 1979) only shared-presence bands were used. AFLP results showed the lowest distance values for two populations (Qaemshar1 and Qaemshar 2) collected at short range from each other (1.5 km) which was expected because of the high probability of gene flow and in agreement with a population genetic study using AFLP in a hymenopteran parasitoid (Samara et al. 2008). Excluding the Astaneh population, which was the most divergent population in both the UPGMA cluster analysis and the Principal coordinate Analysis (PCO), other northern populations clustered close together in two clades, with relatively high bootstrap value (76%). Nour, a population from zone 2 remained as an unresolved branch in UPGMA cluster-analysis between two northern populations (zone 2 and 3). PCO analysis provided a similar
pattern and supports the results of the UPGMA cluster-analysis. Based on PCO analysis, Nour grouped with populations from zone 3, based on the first two coordinates plot. By plotting the first and third coordinates in PCO, this population separated from all other northern populations and in plotting second and third coordinates of PCO, Nour grouped with populations from zone 2, the zone it originated from. The different results obtained with different plotting options in PCO analysis supported the unresolved position of this population found in the UPGMA cluster analysis. Genetically, it is intermediate between populations from zone 2 and 3. A clear distinction between northern and central populations resulted from both analyses (UPGMA; bootstrap value = 88%). A distinct difference between Zamankhan, a Mediterranean population, and the other two dry region populations, Khairabad and Dorche (zone 4) was also observed in UPGMA (bootstrap = 85%) and partially supported by PCO. Sorkhabad, the mountain population was almost identical to the populations from the Caspian coastal plain (zone 2) in both analyses. Both analyses resulted in strong separation of the Astaneh population, which was divergent from all other populations (UPGMA; bootstrap = 100%).

Isolation of insect populations by distance or physical barriers has been shown in several studies (Salvato et al. 2002; Kerdelhue et al. 2006; Clark et al. 2007). Spatial distribution of organisms may prevent genetic differentiation of populations e.g. by massive gene flow between populations (Slatkin 1987). When suitable habitat connects distant sites, Drosophila parasitoids may be able to disperse over long distances. Barriers of unsuitable habitat or limited dispersal capabilities of parasitoids may hamper gene flow and cause the formation of divergent populations. Parasitized fruit flies may be transported with fruits and give rise to populations dissimilar from the neighbouring ones, in particular in urban areas. We found unexpected genetic divergence in two northern populations Astaneh (which was highly divergent from the neighbouring) and Nour (which showed intermediate structure between tow Caspian Coast zones). Both populations originated from urban areas and could have arrived with fruit transports.

Apart from these exceptions, our results demonstrate a highly positive correlation between genetic and geographic distance, which explained a large part of the genetic variation among populations in this study. To our knowledge studies on the genetic structure of Drosophila parasitoid populations are rare. A comparison of L. clavipes populations in western Europe found distinct genetic differentiation between sexual and asexual populations, but no correlation between geographic and genetic distances (Pannebakker et al. 2004). The positive correlation between genetic and geographic distances in our study is consistent with several other genetic structure studies on insect populations (Salvato et al. 2002; Kerdelhue et al. 2006; Clark et al. 2007).
Chapter 5

**Fig. 3:** Separation of Iranian *L. boulardi* populations by the first three coordinates in Principal Coordinates analysis. The first three coordinates explained 69.23 % of AFLP variations among Iranian *L. boulardi* populations. For the origin zone of populations see table 1.

*Life-history evolution in hymenopteran parasitoids: the roles of host and climate*
Genetic structure of Leptopilina boulardi populations from different climatic zones of Iran

The high genetic divergence over relatively short geographic distance between central populations than northern populations implies the role of a geographic barrier formed by unsuitable habitat (dry desert). Populations in the central part of Iran are from isolated locations surrounded by dry desert.

Sequence data on the cytochrome oxidase I gene were less informative in our study than ALFPs. Mitochondrial markers are potentially informative markers in genetic studies of insect populations (Crozier et al. 1997; Olav et al. 2003; Smith 2005), sensitive to differences in the rates of male and female migration (Olav et al. 2003). However, most studies using CO I and II as molecular markers in insects have used them in phylogenetic and taxonomic contexts to discriminate between higher taxa than populations (Lunt et al. 1996; Caterino and Sperling 1999; Zaldívar-Riverón et al. 2006). Our results demonstrate a highly conserved pattern for COI among *L. boulardi* populations, as expected for sexually reproducing populations.

Our study adds to the evidence that AFLPs are convenient markers to study genetic diversity of populations within species (Yan et al., 1999; Sharbel et al., 2000; Schneider et al., 2002; Alamalakala et al., 2009) and can be used successfully for population genetic studies on hymenopteran parasitoids (e.g. Schneider et al. 2002; Pannebakker et al. 2004; Samara et al. 2008).

**Fig. 4:** Correlation between genetic and geographic distances, (a) including all populations and (b) excluding Astaneh, the most divergent population in all analyses. 

![Fig. 4: Correlation between genetic and geographic distances](image-url)
