DNA barcoding reveals widespread occurrence of *Leptidea juvernica* (Lepidoptera: Pieridae) in southern Finland

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Abstract

DNA barcoding was used to identify 54 specimens of butterfly genus *Leptidea* collected from various parts of southern Finland in 2011-2013. Results reveal the presence of both the widespread *Leptidea sinapis* and its cryptic congener *L. juvernica* from several locations throughout the southern Finland. Our sampling also reveals different habitat preferences between these species in Finland: specimens collected from open, disturbed habitats were mainly identified as *L. juvernica*, whereas specimens from forest habitats were all found to represent *L. sinapis*. A morphometric analysis revealed that *L. juvernica* and *L. sinapis* hardly differ by their fore wing shape, although males and females seem to differ from each other. Our attempts to DNA barcode selected museum specimens failed and we were not able to verify historical presence of *L. juvernica* in Finland. The recently increased observations of *Leptidea* butterflies in large numbers in unusually open habitats across the southern Finland together with our findings suggests ongoing rapid expansion of *L. juvernica* in Finland.

Introduction

The emergence of molecular systematics and application of DNA barcoding is helping to revise the taxonomy and species delimitation in many Lepidoptera groups (e.g. Hausman et al. 2011). One of the most remarkable examples is in the genus *Leptidea* Billberg (Pieridae). The species *Leptidea sinapis* (Linnaeus, 1758) was considered as a widespread and common species throughout Europe, until it was first splitted into two morphologically cryptic species (Réal 1988), and not long after realised to consist of three genetically distinct species (Dinca et al. 2011). These three species, *L. sinapis*, *L. reali* Reissinger, 1990 and *L. juvernica* (Williams, 1946) can be easily differentiated using DNA sequence data, yet it is very difficult to tell them apart morphologically (Dinca et al. 2011, Mazel 2012). The two widely distributed and largely sympatric species, *L. sinapis* and *L. juvernica* seem to have constant differences in the genitalia (e.g. Sachanowicz 2013), and it has been suggested that the species can be separated by the coloration of the dorsal apical spot (Mazel 2012), or slightly different coloration of wing undersides and more attenuate fore wing apex in *L. juvernica* (Ivonin et al. 2009). In contrast, Solovyev et al. (2015) concluded that neither external nor genital morphology could provide a reliable identification.

At the same time with a taxonomical overhaul the distributions of many lepidopteran species are in flux, apparently due to climate warming (Hill et al. 2002). In Finland, this has resulted in new species records and northward migration of the lepidopteran fauna (Pöyry et al. 2009). One potential such climate driven expansion is *L. juvernica*, although its range is difficult to document because of the morphological similarity with the very common and widespread *L. sinapis*. An opposite trend in the ranges of these two species has been revealed in Poland, where *L. sinapis* has declined and *L. juvernica* expanded during the 20th century (Sachanowicz et al. 2011). In Poland, *L. sinapis* favour woodlands and *L. juvernica* open meadows, and the latter species may therefore be better adapted to human altered habitats (Sachanowicz et al. 2011). In the face of habitat degradation and climate change it is therefore crucial to better understand the distribution and population trends of these two species across their geographical range (Beneš et al. 2003). In Finland, the occurrence of *L. juvernica* was first verified by genital identification from Åland Islands in the year 2000 (Suomen Perhostutkijain Seura ry 2015). The first Finnish DNA barcoded specimens of *L. juvernica* were reported in 2013 from Lappeenranta, eastern Finland (Saarinen et al. 2013). Since then, increasing numbers of *Leptidea* butterflies have been observed from open habitats, previously unoccupied by *Leptidea*, throughout southern Finland (Saarinen 2017). In Sweden, *L. sinapis* is a habitat generalist occurring both in forests and open meadows, in contrast to *L. juvernica*, which is a specialist of open habitats (Friberg et al. 2008a,b). It therefore appears likely that the ongoing expansion of *Leptidea* in open habitats across the southern Finland represents invasion by *L. juvernica*. However, due to the cryptic morphology the actual range and population trends of these two species have remained unverified in Finland.

Here, we have DNA barcoded 54 *Leptidea* specimens in order to estimate the range of *L. juvernica* in southern Finland. The specimens were collected from several locations from Åland Islands through Finnish southern coast to East Finland during 2011-2013. It is possible that *L. juvernica* has been part of Finnish fauna already for a long time, or that the species have had short-living populations during favourable years. We therefore investigated historical collections in order to test the hypothesis that *L. juvernica* is a new member of Finnish fauna. Furthermore, we ran a morphometric analysis to test numerical support for the perceived difference in the fore wing shape. Our results revealed widespread occurence of *L. juvernica* throughout this region and suggests that the two species cannot be reliably identified by their fore wing shape.

Material and methods

Sampling of natural history collections

The butterfly collections located at the Zoological Museum of the University of Turku (also holding collections of Åbo Akademi) were visually inspected by AT considering characteristics that have been suggested useful in telling *L. juvernica* apart from *L. sinapis*, specifically the shape and darkness of the dorsal apical spot and the fore wing shape (Ivonin et al. 2009, Mazel 2012). Six specimens that in these characteristics approached the suggested phenotype of *L. juvernica* were found, these were all collected from SW Finland (AAT-2015-004 Ab: Paimio 1932; AAT-2015-001 & AAT-2015-005 Ab: Kakskerta 1973; AAT-2015-003 Ab: Kakskerta 1976; AAT-2015-006 Ab: Kakskerta 1982; AAT-2015-002 Ab: Turku 1992). One leg was detached from each of these specimens and used for DNA extraction, but we failed to produce any sequence data from them.

Field sampling

During 2011-2013 we repeatedly observed *Leptidea* specimens in open habitats generally avoided by *L. sinapis* in Finland. These were suspected to belong to *L. juvernica* instead, and we opportunistically sampled specimens from these open habitats as well as from more forested sites using a butterfly sweep net. The sampling localities are described below.

Finland: Al *Eckerö*, Torp. In total 14 first generation specimens were collected as *L. juvernica* from a west-posing slope having a gradient of humid to dry meadow-like vegetation in 2012. DNA barcoding revealed that one of these was actually *L. sinapis* (see below).

Finland: Ab *Pargas*, Lemlaxön. Most specimens observed in this location were found flying at forest edges and were suspected to belong to *L. sinapis*. For five sampled specimen this identification was confirmed by DNA barcoding. However, in May 2013 one individual was collected from an unplowed harvested field, flying close to the forest edge. This specimen was assumed and later confirmed to represent *L. juvernica*. All these specimens represent the first generation.

Finland: Ab *Salo*, Tupuri. A single second generation specimen of *L. juvernica* was collected from a humid grass-dominated meadow surrounded by forests.

Finland: N *Helsinki*, Vuosaari. About 20 first generation individuals of *L. juvernica* were observed in May and early June 2012 in the neighbourhood of a landfill hill on open habitats, both on dry and moist meadow, grassland and wasteland areas. In 2013, four specimens were observed in May.

Finland: N *Sipoo*. A single first generation specimen of *L. sinapis* was collected near the village of Helgträsk in May 2013. The area is mostly spruce-dominated mixed forest with some logging areas.

Finland: Sa *Imatra*, Räikkölä. In total 12 specimens of *L. juvernica* were collected from a relatively dry meadow in 2012-2013, most of them representing the second generation. The surrounding area is a mosaic of forests and cultivated fields.

Finland: Sa *Lappeenranta*, Joutseno, Kiukas. Three specimens of *L. juvernica* were collected from a set-aside field with abundant flowers in 2013.

Finland: Sa *Lappeenranta*, Joutseno, Anola. Three second generation specimens of *L. juvernica* were collected from a relatively open, dry meadow in 2011.

Finland: Sa *Lappeenranta*, Joutseno, Myllymäki. One *L. juvernica* was collected from a hot and dry ski slope having abundant flowers in 2012.

Finland: Sa *Lappeenranta*, Joutseno, Korvenkylä. Six specimens of first and second generation *L. juvernica* were collected during 2012-2013 from a relatively large field that had not been cultivated for several years.

Finland: Sa *Lappeenranta*, Vainikkala. A single first generation specimen of *L. sinapis* was collected from a grassy wasteland area with sparsely growing saplings, flying close to a forest edge.

Finland: Ka *Virolahti*, Ala-Pihlaja. A single first generation specimen of *L. juvernica* was collected from a set-aside field with meadow flowers in 2012.

Finland: Ta *Heinola*. A single first generation specimen of *L. sinapis* was collected by a road-side cutting through a mixed forest in 2013.

Molecular identification

DNA extraction, PCR, and sequencing of COI (Cyto-chrome oxidase subunit I) barcode marker were carried out as in Sorvari et al. (2012). The resulting sequences were uploaded to the project Barcoding International and Finnish Fauna Independently (BIFFI) project in the Barcode of Life Data System (Ratnasingham & Hebert 2007) with process ID's as in Table 1. The trace files and pictures of the samples are also uploaded into the BIFFI project.

In total 54 sequences were produced and initially identified using the Barcode of Life Data System (Ratnasingham & Hebert 2007). Some of these sequences were of rather low quality and not analysed further, but the 37 best quality sequences were included in a phylogenetic analysis together with 102 sequences downloaded from GenBank and BOLD databases representing seven different *Leptidea* species and three outgroup taxa. The sequences used for phylogenetic analyses were aligned using MUSCLE (Edgar 2004). Then, a maximum likelihood analysis using GTR substitution model with 100 bootstrap replications was carried out and consensus tree was extracted from this analysis. The phylogenetic analysis was done using PHYML plugin (Guindon & Gascuel 2003; plugin was developed by V. Lefort, J. Heled, S. Guindon and the Geneious team) with default settings in software Geneious (version 6.1.8; Kearse et al. 2012).

Morphometric analysis

We quantitatively evaluated the fore wing shapes of DNA identified specimens by running an Elliptic Fourier analysis (Kuhl & Giardiana 1982). First, we digitized the fore wing outlines using photographs taken from the pinned specimens. These outlines were then inputted into computer program package SHAPE (Iwata & Ukai 2002). Using this software package we automatically extracted two-dimensional contours of wing outlines and derived normalized Elliptic Fourier Descriptors (EFD) for the wing shapes. We then performed a principal component analysis of the coefficients of the EFDs with the same software package. Due to the limited number of specimens, we did not differentiate between the generations. Most of our *L. sinapis* samples represent the first generation; the *L. juvernica* specimens are more evenly distributed between the first and second generation.

Results

Molecular analysis and habitat choice

Our own samples clearly clustered together with either *Leptidea juvernica* or *L. sinapis* (fig. 1). Specimens widely collected from Åland Islands, archipelago of Turku (Pargas), Salo, Helsinki and

East Finland undoubtedly group with *L. juvernica* reference samples. Our study also included several *L. sinapis* specimens throughout the same area. However, all the *L. juvernica* specimens observed in this study were found from open habitats with low vegetation, in contrast to *L. sinapis*, which was mostly confined to forest edges and semi-open forests (fig. 2). Unfortunately, none of the sampled historical museum specimens yielded any DNA and their identity therefore remains uncertain.

Morphometric analysis

The first two principal components explained 37.6% and 35.3% of the total variance in fore wing shape, respectively (cumulatively 72.9%). The third principal component explained another 10.5%. We restrict our discussion in the results based on the first two principal components only (fig. 3). Fore wing shapes of the females clearly clustered together with no difference between the species. In contrast, the male specimens of *L. juvernica* seem to generally differ from females of both species in their wing shape. The males of *L. sinapis* were intermediate and their wing shape overlapped with both the *L. juvernica* males and females of both species. The significant shape variation appears to be linked with the general roundedness of the fore wing: the first principal component explained shape variation mainly at the base of costal margin and at the distal part of the inner margin, thus, largely explaining variation in the general wing breadth (fig. 4). The second principal component explained variation at the distal part of the costal margin, at wing apex, and at outer margin (fig. 4). This variation largely determines how rounded or pointed the wing appears.

Discussion

Our study confirmed the widespread occurrence of *L. juvernica* in southern Finland. We did not perform systematic sampling and cannot therefore confirm continuous presence of *L. juvernica* throughout this area, but we expect the species to occupy far more sites than we have sampled here.

Over the past couple of years the number of *Leptidea* specimens observed in unusually open habitats has dramatically increased across the southern Finland (Saarinen et al. 2013, Saarinen 2017) and it is now evident that these butterflies mostly represent *L. juvernica*. This study reveals that in Finland these species partition their habitats in a similar way as in Sweden, where *L. juvernica* is also a specialist of open habitats (Friberg 2008a,b). Our results support the view that *L. juvernica* is currently spreading in Finland and is probably a relatively new addition to Finnish fauna, although we cannot rule out its previous presence either sporadically or in low numbers. This observation is in line with similar trend observed in Poland, where expansion of *L. juvernica* is furthermore associated with a decline of *L. sinapis* (Sachanowicz et al. 2011). It remains to be documented if similar replacement will take place in Finland. Due to their different ecological preferences it has been assumed that opposing population trends in these species are related to anthropogenic habitat changes rather than direct competition between them (Sachanowicz et al. 2011). Our observations are congruent with this view – we only observed *L. juvernica* in heavily modified open habitats, but most *L. sinapis* were observed in forested landscape (fig. 2). It therefore seems likely that *L. juvernica* benefit from anthropogenic disturbance, possibly at the expense of *L. sinapis*. Population trends and habitat selection in these species may also be driven by courtship pressure on less abundant species (Friberg et al. 2008c, 2013). The current situation in southern Finland provides an excellent opportunity to study the factors and possible interactions causing the opposite population trends in these two species.

For population monitoring purposes a clear morphological character distinguishing the two species would be desired. The presumably more attenuated fore wing apex in *L. juvernica* has been suggested to be a diagnostic character (Ivonin et al. 2009), although this has been questioned (Solovyev et al. 2015). To our knowledge, the wing shape variation has not been quantified or numerically analysed in *Leptidea* before. Our morphometric analysis revealed that the main difference in the fore wing shape seems to distinguish females from males. *Leptidea sinapis* males have fore wing shape intermediate and to some degree overlapping between females in general and *L. juvernica* males. The first principal component of shape variation was related to the general wing breadth and roundedness; females have more rounded wings than males also based on visual judgement. The second principal component was more related to the shape of outer margin and wing apex attenuation, giving mathematical support to the idea that *L. juvernica* has more pointed wings. However, this only applies to males and even then the wing shape is broadly overlapping with *L. sinapis* males. Our mixing of first and second generation specimens in the analysis and limited sampling of *L. sinapis* does not allow us to conclude how much the wing shape of males in these two species actually overlaps, but it seems likely that the wing shape has very limited, if any, use in practical identification.

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TABLE LEGENDS

Table 1. Specimens analysed in this study.

Process ID	Collection locality	Collection date	Species
BIFFI001-12	Southern Savonia, Lappeenranta	01-Jun-2012	*Leptidea juvernica*
BIFFI002-12	Southern Savonia, Lappeenranta	01-Jun-2012	*Leptidea juvernica*
BIFFI003-12	Southern Savonia, Lappeenranta	01-Jun-2012	*Leptidea juvernica*
BIFFI004-12	Southern Savonia, Lappeenranta	01-Jun-2012	*Leptidea juvernica*
BIFFI005-12	South Karelia, Virolahti	31-May-2012	*Leptidea juvernica*
BIFFI006-12	Southern Savonia, Lappeenranta	23-Jul-2011	*Leptidea juvernica*
BIFFI007-12	Southern Savonia, Lappeenranta	23-Jul-2011	*Leptidea juvernica*
BIFFI008-12	Southern Savonia, Lappeenranta	23-Jul-2011	*Leptidea juvernica*
BIFFI009-12	Southern Savonia, Imatra	25-Jul-2012	*Leptidea juvernica*
BIFFI010-12	Southern Savonia, Imatra	25-Jul-2012	*Leptidea juvernica*
BIFFI011-12	Southern Savonia, Imatra	25-Jul-2012	*Leptidea juvernica*
BIFFI012-12	Southern Savonia, Imatra	25-Jul-2012	*Leptidea juvernica*
BIFFI013-12	Southern Savonia, Imatra	02-Aug-2012	*Leptidea juvernica*
BIFFI014-12	Southern Savonia, Imatra	02-Aug-2012	*Leptidea juvernica*
BIFFI015-12	Southern Savonia, Imatra	02-Aug-2012	*Leptidea juvernica*
BIFFI016-12	Southern Savonia, Imatra	02-Aug-2012	*Leptidea juvernica*
BIFFI017-12	Southern Savonia, Imatra	02-Aug-2012	*Leptidea juvernica*
BIFFI018-12	Southern Savonia, Imatra	02-Aug-2012	*Leptidea juvernica*
BIFFI019-12	Southern Savonia, Lappeenranta	02-Aug-2012	*Leptidea juvernica*
BIFFI026-13	Uusimaa, Helsinki	15-May-2012	*Leptidea juvernica*
BIFFI027-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
BIFFI029-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
BIFFI030-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*

327	BIFFI031-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
328	BIFFI032-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
329	BIFFI033-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
330	BIFFI034-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
331	BIFFI035-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
332	BIFFI036-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
333	BIFFI037-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
334	BIFFI038-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
335	BIFFI039-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
336	BIFFI040-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
337	BIFFI041-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea juvernica*
338	BIFFI044-14	Southern Savonia, Imatra	01-Jun-2013	*Leptidea juvernica*
339	BIFFI045-14	Southern Savonia, Lappeenranta	01-Jun-2013	*Leptidea juvernica*
340	BIFFI046-14	Southern Savonia, Lappeenranta	01-Jun-2013	*Leptidea juvernica*
341	BIFFI047-14	Southern Savonia, Imatra	29-Jul-2013	*Leptidea juvernica*
342	BIFFI048-14	Southern Savonia, Lappeenranta	30-Jul-2013	*Leptidea juvernica*
343	BIFFI049-14	Southern Savonia, Lappeenranta	30-Jul-2013	*Leptidea juvernica*
344	BIFFI050-14	Southern Savonia, Lappeenranta	29-Jul-2013	*Leptidea juvernica*
345	BIFFI060-14	Finland Proper, Pargas	19-May-2013	*Leptidea juvernica*
346	BIFFI061-14	Finland Proper, Salo	28-Jul-2013	*Leptidea juvernica*
347	BIFFI064-15	Uusimaa, Helsinki	26-May-2012	*Leptidea juvernica*
348	BIFFI065-15	Uusimaa, Helsinki	27-May-2012	*Leptidea juvernica*
349	BIFFI028-13	Åland Islands, Eckerö	09-Jun-2012	*Leptidea sinapis*
350	BIFFI052-14	Tavastia australis, Heinola	02-Jun-2013	*Leptidea sinapis*
351	BIFFI053-14	Finland Proper, Pargas	06-Jun-2013	*Leptidea sinapis*

BIFFI054-14	Finland Proper, Pargas	06-Jun-2013	*Leptidea sinapis*
BIFFI055-14	Finland Proper, Pargas	06-Jun-2013	*Leptidea sinapis*
BIFFI057-14	Finland Proper, Pargas	06-Jun-2013	*Leptidea sinapis*
BIFFI058-14	Finland Proper, Pargas	28-May-2013	*Leptidea sinapis*
BIFFI059-14	Southern Savonia, Lappeenranta	02-Jun-2013	*Leptidea sinapis*
BIFFI066-15	Uusimaa, Sipoo	28-May-2013	*Leptidea sinapis*

Table 1.

FIGURE CAPTIONS

Figure 1. Relationships within *Leptidea* species based on ML analysis of COI sequences. Specimens examined in this study are marked with dots in the phylogeny and map (blue dots = *L. juvernica*, red dots = *L. sinapis*, notice that in the westernmost location at the Åland Islands both species were observed at the same site). The grey dots mark the general distribution of the genus *Leptidea* in Finland based on the records accessed through the Global Biodiversity Information Facility (GBIF; http://gbif.org accessed 28th October 2015 GBIF Occurrence Download http://doi.org/10.15468/dl.9dnmx8). Sequence data for other specimens in the phylogeny were taken from GenBank and BOLD (see Materials and Methods for details).

Figure 2. Typical habitats of the two cryptic *Leptidea* species in Finland. On the left, open landfill area in Helsinki Vuosaari. *Leptidea juvernica* occured on this site in 2012-2013. On the right, harvested spruce-dominated moist forest with abundant herbs on the forest floor in Pargas, Lemlaxön. *Leptidea sinapis* was abundant on this site in 2013. Butterflies from left to right: *L. juvernica* male and female, *L. sinapis* male and female, all DNA identified first generation individuals.

Figure 3. The *Leptidea* specimens DNA identified in this study plotted based on the first two principal components explaining 72.9% of the observed variation in the fore wing shape.

Figure 4. Visualization of the variation in *Leptidea* fore wing shape accounted for by the first two principal components. The mean contour lines and +/- 2 standard deviation contour lines are shown separately and, on the leftmost panel, plotted on top of each other.

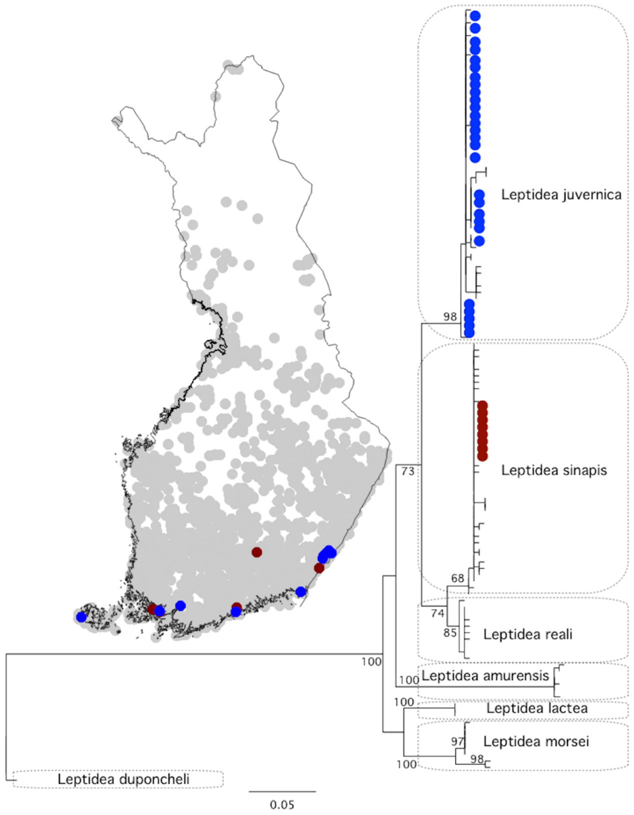
Leptidea juvernica
98
Leptidea sinapis
73
68
74
85
Leptidea reali
100
100
Leptidea amurensis
100
Leptidea lactea
97
98
100
Leptidea morsei
Leptidea duponcheli
0.05

Fig. 1.

Fig. 2.

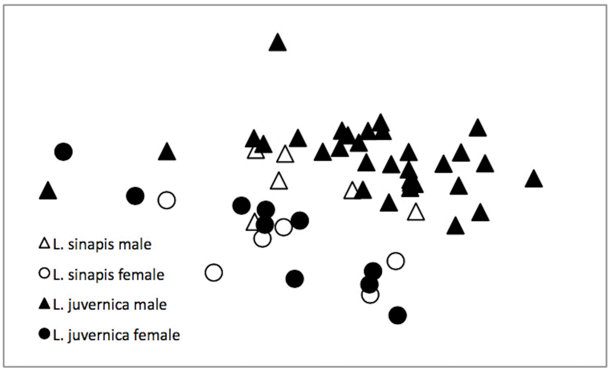
L. sinapis male
L. sinapis female
L. juvernica male
L. juvernica female

Fig. 3.

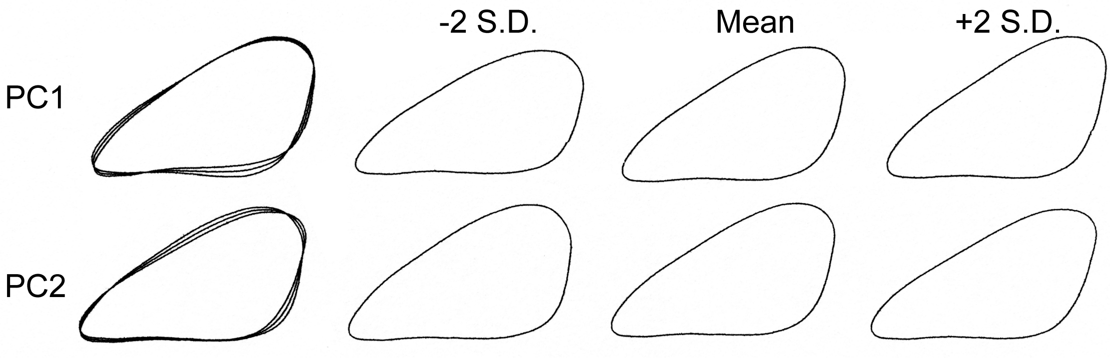
-2 S.D.
Mean
+2 S.D.
PC1
PC2

Fig. 4.