Covering the bases: population genomic structure of *Lemna minor* and the cryptic species *L. japonica* in Switzerland

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Abstract

Duckweeds, including the common duckweed *Lemna minor*, are increasingly used to test eco-evolutionary theories. Yet, despite its popularity and near-global distribution, the understanding of its genetic variation is still limited. It is essential that this is resolved, because of the impact genetic diversity has on experimental responses and scientific understanding.

Through whole-genome sequencing, we assessed the genetic diversity and population genomic structure of 23 natural *Lemna* spp. populations from their natural range in Switzerland. We used two distinct analytical approaches, a reference-free kmer approach and the classical reference-based one.
Two genetic clusters were identified across the species distribution, corresponding to species -level divisions. The first cluster contained the targeted *L. minor* individuals and the second contained individuals from a cryptic species: *Lemna japonica*. Within the *L. minor* cluster, we identified a well-defined population structure with little intra-population genetic diversity but high inter-population diversity. In *L. japonica*, the population structure was significantly weaker and genetic variation between a subset of populations was as low as within populations.

This study revealed that *Lemna japonica* is more widespread than previously thought. Our findings empower more robust genotype-to-phenotype analyses in duckweed ecology and evolution, realizing the full potential of *L. minor* in eco-evolutionary research.

Keywords: clonal reproduction, duckweed, genetic diversity, kmers, whole-genome sequencing

Introduction

Species diversity loss from aquatic communities is well characterized (Tickner *et al.*, 2020) but changes in other biodiversity components, namely trait (or functional) and genetic diversity, remain unclear. Intraspecific genetic diversity is a key indicator for any species' potential to adapt under global change because it provides the raw material for natural selection (Hughes & Stachowicz, 2004; Des Roches *et al.*, 2018). It is known that many clonally reproducing plant species retain genetic diversity, i.e., plant populations are often multiclonal and clones are very population-specific (Ellstrand & Roose, 1987). But how this impacts eco-evolutionary findings remains largely unclear, as molecular markers sensitive enough to characterize finer-scale variation within clonal species and populations have only recently become accessible (Ehrlich, 1988; Anisimova, 2019). Thus, our practical knowledge of intraspecific genetic diversity lags far behind our theoretical understanding of its importance. Studies to fill this gap are crucial, especially when they provide a foundation to elucidate the adaptive significance of genetic diversity in changing environments.

Researchers increasingly rely on model systems like duckweed to gain insight into species responses to environmental change. Duckweeds (*Lemnoideae*) are a group of flowering aquatic plants that float on the surface of still or slow-moving freshwater bodies (Landolt, 1986). They are monocotyledonous, represented by 37 species (Sree *et al.*, 2016), including an interspecies hybrid, *Lemna japonica*, with *Lemna turionifera* and *Lemna minor* as parent (Braglia *et al.*, 2021b). *Lemna* spp. often occur at high densities in ponds, streams and ditches in a wide range of environmental conditions (Landolt, 1975), including polluted waters. Duckweeds are of high interest for applied conservation in wastewater treatment, so-called phytoremediation (Sekomo *et al.*, 2012), and are also of commercial interest as a protein source for food products (e.g., replacing soy, Sree *et al.*, 2016) or as biofuel. *Lemna* spp. have the potential to be used for revealing plant defense strategies, genome maintenance and other complex plant biochemical processes (Acosta et al., 2021). Duckweeds, such as *Lemna minor*, also have a long history of use as model organisms in fundamental research and ecological experiments due to their small size, clonal propagation and fast clonal generation time (as little as three days) (Clatworthy & Harper, 1962; Hart *et al.*, 2019). Recently they have increased in popularity as model system for experimental evolution, as well as evolution of species interactions (Laird & Barks, 2018; Hart *et al.*, 2019; O'Brien *et al.*, 2020b,a; Tan *et al.*, 2021; Lanthemann & van Moorsel, 2022).

Lemna minor reproduces vegetatively by budding, although in the wild it also occasionally flowers (Hicks, 1932; Landolt, 1986). Despite the dominance of asexual reproduction, *L. minor* may maintain high levels of intraspecific genetic diversity (Vasseur *et al.*, 1993; Ho, 2018; but see Jordan *et al.*, 1996), with evidence suggesting this impacts fitness within populations (Jewell & Bell, 2023). Specifically, though intraspecific genetic diversity of duckweed is low within populations, allozyme loci between populations can be highly differentiated (Cole & Voskuil, 1996). Populations in close geographic proximity can thus harbour very different genotypes or clones (Vasseur *et al.*, 1993; Ho, 2018; Hart *et al.*, 2019; Tan *et al.*, 2021) and may even be locally adapted to different environmental conditions (e.g. copper pollution, Roubeau Dumont *et al.*, 2019). Recent studies have begun to harness the large phenotypic variation in *L. minor* (Hart *et al.*, 2019; Roubeau Dumont *et al.*, 2019; Tan *et al.*, 2021) but the question remains to what extent the phenotypic variation and fitness

differences are driven by genetic variation (i.e. presence of different alleles or genetic structure).

Importantly, it is widely assumed that while *L. minor* populations are genetically differentiated between sites they are not differentiated within site (Cole & Voskuil, 1996). However, recent evidence found that multiple clones can co-occur in a single pond (Bog *et al.*, 2022). Discrepancies in previous findings may be due to the fact that many genetic studies have examined a limited portion of the species' range, and importantly, used low-resolution genetic markers, which are not intercomparable, such as allozyme loci (Vasseur *et al.*, 1993; Cole & Voskuil, 1996), ISSR markers (El-Kholy *et al.*, 2015), amplified fragment length polymorphisms (Bog *et al.*, 2022) or microsatellite loci (Hart *et al.*, 2019; Kerstetter *et al.*, 2023). Only recently have genomic tools been applied to this model system, a genotyping-by-sequencing study identified three genetically distinct populations of *L. minor* within a circa 160 km-transect in Canada (Senevirathna *et al.*, 2023).

Whole genome sequencing (WGS) is a very powerful tool, capable of revealing complex or fine population structures in species with varying reproductive systems that includes geographic clonality (Stauber *et al.*, 2021) or weak population structure (e.g., Kersten *et al.*, 2021). In this study, we used WGS to quantify the intra-specific genetic diversity of *L. minor*, and, as it turned out ,also the hybrid *L. japonica*, present in Switzerland to understand the structure and patterns in its genetic diversity and gain insights into how this could impact eco-evolutionary research. This is particularly pertinent because the mutation rate for the related duckweed species *Spirodela polyrhiza* is low (Wang *et al.*, 2014; Ho *et al.*, 2019). Though mutation rates remain unknown for *L. minor*, they are thought to be equally low and thus evolution in *L. minor* during experiments will arise from standing genetic variation. Knowing whether this standing genetic variation can be manipulated by using different locally sourced ecotypes (e.g., as done in Hart *et al.*, 2019; van Moorsel, 2022) or even by collecting a large number of individuals from one site, is critical information for duckweed researchers.

Many species of the duckweed family are so morphologically similar they are virtually impossible to distinguish with morphology alone (De Lange & Pieterse, 1973; Landolt, 1975). Consequently, there are also doubts about whether the species has been correctly identified in past experimental studies. A recent screening of 100 clones from the Landolt duckweed collection found that several populations of *L. minor* had been misidentified based on morphological criteria (Braglia *et al.*, 2021a). A study in Canada has revealed the presence of a cryptic species (*L. turionifera*) using DNA barcoding with species-specific primers (Senevirathna *et al.*, 2021). *L. minor* intraspecific diversity may have thus previously been overestimated due to the inclusion of cryptic species (Jordan *et al.*, 1996). Within Switzerland, range maps indicate a prevalence of *L. minor* in Switzerland but very few records of closely related *Lemna* species (e.g. morphologically similar *Lemna japonica*, *Lemna minuta*, *Lemna gibba* and *Lemna turionifera*). Difficulties surrounding correct species identification are likely leading to incorrect distribution maps, and there is a need for large-scale genetic screening of wild *Lemna* populations (Volkova *et al.*, 2023). In line with this, our second research aim was to find out whether there are cryptic species present in Switzerland.

Materials and Methods

Sample collection

L. minor samples were collected opportunistically from 23 populations (hereafter “wild” populations) across a 200 km range within Switzerland and one population from France (Table 1). Sites ranged from 5 km to > 100 km apart, with some separated by mountain ranges. Thus, plant individuals occurring at one site are referred to as population. Within a site/population, several locations (microsites) were sampled (see also Supplemental figure S1). Within such a microsite, we expected to sample only a single clone and thus refer to the microsites as "sample".

For each sample, 80 plant individuals were flash-frozen in liquid nitrogen. In addition to the wild populations, several *Lemna* clones from the Landolt duckweed collection were sampled and sequenced (80 individuals per sample). Each strain represented true clones with a long-recorded history in lab culture and scientific use. To act as reference outgroups, we included the following strains from candidate cryptic species: *Lemna minuta* (strain 9700 collected in Rotterdam, the Netherlands), *Lemna japonica* (strain 9983 collected in Meilen, Switzerland) and *Lemna turionifera* (strain 9478 collected in Raciborz, Poland). Notably, we also aimed to include *Lemna gibba*, but the strain used (9965 collected in St. Gallen, Switzerland) was re-classified during our study as *L. japonica* (Braglia *et al.*, 2021a) based on Tubulin Based Polymorphisms (TBP). Furthermore, as described by Braglia and colleagues (2021), two of the used *L. minor* strains were reclassified as *L. japonica* (strains 9969 and 9978). All samples of the wild *L. minor* populations and the duckweed stock populations were kept at -80°C for downstream use. In total, 87 samples (each based on 80 plant individuals) that yielded high-quality data for genomics were sequenced (see Table 1).

DNA extraction

For DNA extraction, 30 mg of the frozen tissue consisting of several fronds was weighed into 1.5 ml tubes (Eppendorf, Hamburg, Germany) with two 3 mm metal beads and ground into a fine powder with a TissueLyser II (Qiagen, Germany) at 30 Hz for 2 min. The extraction was performed using the Norgen Plant and Fungi genomic kit (Norgen Biotek, Thorold, ON, Canada) with minor modifications. Specifically, 750 µL of lysis buffer supplemented with 1 µL RNAse A (DNase-free, 100,000 units/mL in 50 % glycerol) and 3 µL Proteinase K (>600 u/ml ~ 20 mg/ml in 10 mM Tris-HCl pH 7.5, containing calcium acetate and 50% v/v glycerol) was added to each tube and vortexed vigorously. Then, 150 µL of Binding Buffer I was added to each tube, vortexed and incubated for 5 min on ice. The rest of the extraction procedure was conducted according to the manufacturer’s protocol. Finally, the purified DNA was eluted in 100 µl of elution buffer and stored at -20°C. Quantification and quality control of the purified DNA was performed by Qubit dsDNA HS Assay Kits (Thermo Fisher Scientific) and gel electrophoresis. Library preparation and sequencing was conducted by Novogene (UK) using Illumina NovaSeq PE150 paired-end sequencing (Novogene, Cambridge, UK).

Data processing

Short read processing

Short reads were first de-duplicated with NGSReadsTreatment (version 1.3, Gaia *et al.*, 2019) and quality trimmed with fastp (version 0.20.0, Chen *et al.*, 2018).

K-mer based approach

To process samples for population genetic analysis in a reference free manner, we used a recently described pipeline (Voichek & Weigel, 2020) following the publicly accessible manual (github.com/voichek/kmersGWAS). Analyses were done using the default k-mer size of 31. The kinship matrix generated with the pipeline was converted to a distance matrix (1-kinship) to enable comparability across analyses. It should be noted that we did not intend to provide a full comparison between k-mer and alignment-based pipelines but to use a reference-free approach to assist with species misassignments.

Reference-based approach

Reads were aligned to the reference genomes of either *L. minor* strain 7210 or the diploid *L. japonica* strain 9421 (Ernst *et al.*, 2023) using bowtie2 (version 2.4.5, with a minimal mapping quality of 10, Langmead & Salzberg, 2012). Runs of samples with more than one sequencing run were merged with samtools (version 1.18, Danecek *et al.*, 2021). SNPs were identified with bcftools (version 1.18, Danecek *et al.*, 2021) and filtered for a minimal quality of 20. SNPs were further filtered for a coverage between 10 and 1,000 within at least 80 % of all samples. Pairwise genetic distances were calculated as the fraction of alleles that differed between two individuals (ignoring SNPs with pairwise missing data). After identification of the *L. minor* and *L. japonica* subgroups (see below), the subgroups were divided and treated separately. Each cluster was re-analysed as described above using the species-specific genome.

Non-plant DNA

To assess whether the samples contained sequences from other organisms, we scanned all reads with the metagenome classifier tool CLARK (version 1.2.6.1, Ounit *et al.*, 2015). We used the family-level databases of fungi, bacteria, and viruses (set_targets.sh options: bacteria viruses fungi --family) and a k-mer size of 31 (classify_metagenome.sh options -k 31 -m 2 --ldm --light). Abundances were obtained with default settings (estimate_abundance.sh). The fraction of reads that were assigned during the process was extracted from the output of classify_metagenome.sh. We filtered for families with at least 10 sequence counts in 10 samples and used DESeq2 (version 1.24.0, Love *et al.*, 2014) to obtain log2(x+1) transformed normalized counts that were used to calculate pairwise Manhattan distances between the samples. Differential abundance of families between *L. minor* and *L. japonica* individuals was done as described previously (Schmid *et al.*, 2019) and families with an FDR < 0.05 and a log2 fold-change of at least 2 were considered to be differentially abundant.

Data analysis and visualization

SNP-wise test for association with genetic clusters ("assignment markers")

To test for associations of a genetic variant with a group of samples (i.e., the two genetic clusters which corresponded to the two species *L. minor* and *L. japonica*), we ran an ANOVA for each individual SNP using R (version 4.2.3, i.e., we ran a GWAS using a simple GLM). The genetic variants in case of bi-allelic SNPs have three possible levels corresponding to “homozygous reference”, “heterozygous”, and “homozygous alternative”: 0/0, 0/1, and 1/1. Besides this three-level factor, we also used two two-level factors for comparisons of one homozygous state to the other two states (factor A: 0/0 *vs.* 0/1 and 1/1; factor B: 1/1 *vs.* 0/1 and 0/0). We then only considered the factor with the best fit. Factorial data was tested with

anova(glm(formula, family = quasibinomial("logit")), test = "F")) and the effect size was calculated as the percent of deviance (%-DEV) explained by the variant. *P*-values were adjusted for multiple testing to reflect false discovery rates (FDRs). Associations with an FDR below 0.05 and %-DEV of at least 80% were considered significant. We chose the high %-DEV cut-off to only include cases that were clearly and consistently different between the two species. Given the relatively large sample size, only filtering by FDR would also yield SNPs that are specific to for example one population and not the "entire" species.

Differences in variation between populations

Differences in variation within populations was analyzed using the function betadisper() from the R-package vegan (version 2.5.7, Dixon, 2003). The function was also used to extract the distances to species and population centroids.

Isolation by distance

Isolation by distance, i.e. correlation between genetic distance matrices and correlation of genetic distances with physical distances, was tested with Mantel tests using 9,999 permutations with the R-package vegan (version 2.5.7, Dixon, 2003). Correlations between genetic and physical distances were also tested with an ANOVA (anova(lm()). In both cases, physical distances in meters were log10 transformed.

Visualizations

Heatmaps were generated using the distance matrices and the function gl.plot.heatmap() from the R-package "dartR" (version 1.0.2, Gruber *et al.*, 2018). Supplemental figure S4B was done using the function heatmap.2 from the R-package gplots (version 3.1.1, Warnes *et al.*, 2024). All other plots were done in R using base graphics.

Results

Three different approaches recovered two species

The kmer approach identified 1,357,142,099 variants in the kmer table. Alignment to the *L. minor* reference genome (strain 7210) and SNP calling with bcftools (Danecek *et al.*, 2021) yielded 10,910,630 SNP's in total, out of which 1,343,857 SNPs had a coverage of at least 10 in at least 80 % of all individuals. Alignment to the *L. japonica* reference genome (strain 9421) and SNP calling yielded 10,067,012 SNPs, out of which 1,511,714 SNPs passed the coverage filter. We created kinship matrices based on all three approaches (Fig. 2A) and recovered two distinct genetic clusters. The included *L. minor* sample from the Landolt duckweed collection with known species attribution (based on Tubulin polymorphism, Braglia et al 2021) clustered together with one subgroup, whereas the collections' *L. japonica* samples clustered with the other subgroup (see grouping in Fig. 2A). To test whether these two clusters could be attributed to two species, we used three additional approaches: 1) alignment rate comparison, 2) identification of cluster-specific SNPs, and 3) comparison of alternative allele depths using also publicly available data (Ernst *et al.*, 2023).

Differences in alignment rates

Alignment rates for the *L. japonica* reference genome were around 50-90 % (except for the *L. minuta* sample with an alignment rate of 2.9 %). Rates for the *L. minor* genome were generally lower for all samples, but there was a marked difference among samples (Supplemental figure S2). While the alignment rates of the *L. minor* samples were on average only 10.2 % lower (range: 5.6 – 13.9 %), alignment rates of *L. japonica* samples were about 26.1 % lower (range: 16.3 - 32.4 %). As a comparison, the alignment rate of the *L. turionifera* sample, whose genome is only represented in the *L. japonica* reference, was 66.1 % lower.

Species assignment markers

We tested all SNPs that passed the filter for significant association with either of the clusters (i.e. species) using generalized linear models (GLMs) followed by multiple testing corrections and stringent filtering (FDR < 0.05 and %-deviance explained > 80 %). While using the *L. minor* reference, 5.95 % of all SNPs were highly differentiated between the two clusters. In contrast, with the *L. japonica* reference only 0.13 % of all SNPs showed significant differentiation (Fig. 2B). Meeting with theoretical expectations for allele frequencies between species, most differentiated markers were homozygous fixed differences (99.76 % *L. minor* reference, and 82.30 % *L. japonica* reference). The difference in the fraction of significant SNPs was probably due to the fact that parts of the genome from *L. japonica* (i.e., the copy from the parent that is not *L. minor,* hence *L. turionifera*) wrongly aligned to the *L. minor* reference sequence, and that this was corrected when using the *L. japonica* reference genome. The limited difference between the two clusters with the *L. japonica* reference genome further suggested that the copy of the *L. minor* parent in the *L. japonica* individuals was similar to the genome of the *L. minor* individuals.

Alternative allele ratio

To verify the difference between the two clusters and to assess the genetic composition of the *L. japonica* individuals, we extracted the "alternative allele ratio" at each SNP using the *L. minor* reference genome data (Fig. 2C). For example, one SNP might be covered with 30 reads that suggest that the base is "A" like in the reference genome, whereas another 30 reads suggest an alternative base "T". Hence, the ratio would be 0.5 and indicate that the individual is heterozygous at this position. However, if there were only 15 reads suggesting the base "T", the ratio would be 1:2 and indicate that the individual might be triploid with one copy being different (or hexaploid with two copies being different). Doing this for all SNPs, we indeed found an enrichment of the alternative allele ratio of 50 % in all *L. minor* individuals and an enrichment of an alternative allele ratio around 33 % in all *L. japonica* individuals (Fig. 2C). To verify this approach, we added data from the previously published *Lemna* reference genomes (Ernst *et al.*, 2023), Supplementary Figure S3). Among them were three different *L. japonica* hybrids with different mixtures of the parental genomes M (*L. minor*) and T (*L. turionifera*): 1) a diploid M/T, 2) a triploid M/M/T, and 3) a triploid M/T/T hybrid. These three hybrids matched the order of the expected alternative allele ratios with the lowest in M/M/T, intermediary in M/T, and highest in M/T/T. While the expected ratios would be in theory 33, 50, and 66%, the observed ratios were slightly lower (see Supplementary Figure S3). It is unclear why this was the case. Nonetheless, all *L. japonica* in our study were closest to the triploid M/M/T hybrid, indicating that *L. japonica* in Switzerland also comprises triploid M/M/T hybrids. Additional support for this hypothesis came from the average coverage of the two

sub-genomes, *L. minor* and *L. turionifera*, in the *L. japonica* reference genome. While the *L. minor* samples had practically zero coverage on the *L. turionifera* sub-genome, the *L. japonica* samples exhibited approximately a 2:1 ratio in genome coverage between the *L. minor* and the *L. turionifera* sub-genomes (Supplemental figure 3C). Taken together, our analyses suggest the widespread presence of a triploid *L. japonica* hybrid with two copies of the *L. minor* genomes and one copy of the *L. turionifera* genome in Switzerland. We will, therefore, refer to the two genetic clusters as *L. minor* and *L. japonica* groups.

Finer population structure differed depending on the used approach

The population structure depicted in Figure 2 revealed general agreement among the three datasets. However, we highlight a few differences to demonstrate the need for caution when it is uncertain whether all individuals in a study belong to the same species. While correlations between pairwise distances were clearly significant ($P_{Mantel} = 0.0001$ with 9,999 permutations), they were still markedly distinct. Correlation between the kmer and the *L. minor* reference genome data sets was relatively high (Pearson correlation coefficient = 0.9). However, the *L. japonica* reference and the *L. minor* reference were less correlated (Pearson correlation coefficient = 0.71). The *L. japonica* reference and the kmer dataset correlated only weakly (Pearson correlation coefficient = 0.56). This discrepancy was also visible in the inconsistent placement of some *L. japonica* individuals in the three dendrograms (Fig. 2A). We ultimately used the assignment from the kmer and alignment to *L. minor* reference genome approaches, which we could verify as described above.

Furthermore, the distance values (see dendrogram and color-gradient in Fig. 2A), suggest that contrasting conclusions could be drawn. The kmer data set suggests considerable intrapopulation and interpopulation variation. The *L. minor* reference genome dataset indicates practically no intrapopulation variation but significant interpopulation genetic variation for the *L. minor* samples. Meanwhile, for the *L. japonica* group it would suggest intermediate variation within and between populations (ignoring for now the large group of similar *L. japonica* individuals which comprises multiple populations). Again, in contrast, the *L. japonica* reference genome dataset would suggest very little intrapopulation variation and large interpopulation variation for both species. The different output resulting from the kmer approach might have been in part due to the presence of environmental DNA (note that we did not remove the reads assigned to eDNA prior to the kmer analysis). The difference between the two reference genomes was solely due to the presence of the *L. turionifera* sub-genome in the *L. japonica* assembly. Given that half of the samples were *L. minor* and we filtered SNPs that were not covered by at least 80 % of all samples, this filter masked much variation in the *L. japonica* group on the *L. turionifera* sub-genome while the presence of the *L. turionifera* sub-genome during the alignment lead to fewer errors in the *L. minor* sub-genome. While conclusions would not be much different for the *L. minor* samples, they would be distinct for the *L. japonica* samples. Hence, to assess the population structure of the two species individually, we split the data into two sets: 1) *L. minor* samples using the *L. minor* reference and 2) *L. japonica* samples using the *L. japonica* reference.

Population genomic structure differs between *L. minor* and *L. japonica*

To assess the wild population structure for *L. minor* and *L. japonica*, we kept the two species separate and excluded the samples from the Landolt collection. We further excluded three samples in the analyses of the within- and between-population distances (R4, GR6, and BA1) because they were genetically implausibly far apart from their population of origin. R4 grouped with samples from another population, suggesting a mislabelling during processing. The other two samples might indeed be markedly different genotypes within their population, but at least for BA1 it seems highly unlikely because this population originated from an isolated, tiny pond in a garden surrounded by large buildings. Thus, both should be verified independently.

Kinship matrices and dendrograms (Fig. 3A) indicated that *L. minor* populations were well structured. In contrast, *L. japonica* was split into one large cluster comprising multiple populations, one group comprising two populations, and two remaining populations. Thus, several of the originally sampled *L. japonica* populations were clearly not distinct from each other. We therefore used the grouping inferred from the kinship matrices and dendrograms to compare within- to between-population distances (Fig. 3B). In both species, within-population differences were much smaller than between-population differences ($P < 10^{-10}$). Distances within groups were on average 0.0147 (*L. minor*) and 0.0126 (*L. japonica*). In contrast, distances between groups were on average 0.2779 (*L. minor*) and 0.298 (*L. japonica*). We then used the original population labels to test whether variation within populations differed and found that within population variations were significantly different in *L. japonica* ($P = 0.0001$ with 9,999 permutations) but not in *L. minor* ($P = 0.1611$, Fig. 3B). We finally tested whether genetic distances correlated to physical distances (Fig. 3C). The correlation was significant in both species (both $P_{\text{Mantel}} < 0.01$ and both $P_{\text{ANOVA}} < 10^{-10}$) but much higher in *L. minor* (Pearson correlation of 0.83) compared to *L. japonica* (Pearson correlation of 0.24).

Considering that *L. japonica* was a hybrid with the genomes of both parents available in the assembly, we could compare the variation within these two "sub-genomes". We, therefore, split the SNPs according to the sub-genome of origin, re-calculated the pairwise distances, and compared the distances to each other. If the two were markedly different from each other, it might indicate that the data contained samples with different parents. Due to differences in coverage (Supplemental figure S3), the number of SNPs was very different: 516,007 and 4,081 in the *L. minor* (M) and the *L. turionifera* (T) sub-genome, respectively. Nonetheless, pairwise distances were very similar to each other (Pearson correlation of 0.95, $P_{Mantel} = 0.0001$ with 9,999 permutations). It is therefore possible that the *L. japonica* samples have a common hybrid ancestor and that the differences we observed were gained after the original hybridization. However, it is also still possible that there were multiple hybridization events but that the dissimilarity between the parents was similar.

In summary, we found clear population structures that also reflected the physical distance between the populations, especially in *L. minor*. In *L. japonica*, the structure was less pronounced, and several populations were similar enough to be grouped into one large group exhibiting "within-population-level" genetic differences even though they were physically well separated from each other ("various populations" in Fig. 3A).

Non-plant eDNA associated with collected samples

On average, about 6.19 % of all reads were classified as bacterial, viral, or fungal and the two species were alike (Supplemental figure S4A). In total, 555 different fungi/bacteria/virus families passed the filters. Correlations between samples using the eDNA were between 0.67 and 0.99. Samples were not grouped by eDNA according to populations, but to some extent by species (Supplemental figure S4B). Likewise, correlations between pairwise distances from the eDNA data to the three approaches above were limited (but $P_{Mantel} < 0.01$): with the *L. minor* genome: 0.17, with the *L. japonica* genome: 0.20, and with kmers: 0.09. Thus, the eDNA in the samples did not reflect the population structure seen with the plant data. Averaged *L. minor* and *L. japonica* samples were highly similar to each other (correlation of 0.98, Supplemental figure S4C). Out of the 555 families, five were differentially abundant in *L. japonica* compared to *L. minor.* All five families were more abundant in *L. japonica* samples: *Desulfuromonadaceae*, *Fastidiosibacteraceae*, *Paludibacteraceae*, *Prolixibacteraceae*, and *Marseilleviridae*. While little is known about these families, there are differences for some families and the distances in eDNA did not simply reflect those obtained from the plant data. This suggests that there was a proportion of reads that came from organisms other than the studied plants. While this may be used to study the microorganisms associated with the plants, it also indicates that the kmer approach should be used carefully as it does not inherently filter against sequences coming from non-target organisms. An option might be to filter reads matching to eDNA before running the kmer analysis.

Discussion

Higher between- than within population genetic variation in *L. minor*

L. minor generally displayed very low within population genetic diversity. It is noteworthy that the genetic distances reflect the fraction of alleles that differ between individuals and that these are calculated only on positions that have at least one individual with an alternative allele. Thus, within population distances are likely overestimates. The low levels of intrapopulation differences (~1.5 %), signal that it is likely that the populations were in general clonal. However, we could not confirm this with replication of samples as planned because several samples failed during processing. Differences between populations were much larger than those within populations, indicating *L. minor* shows spatial genetic structure with signals of isolation by distance. These findings are in line with previous research based on genetic markers (Cole & Voskuil, 1996; Hart *et al.*, 2019; Bog *et al.*, 2022). However, more genetic variation was found here relative to the comparable geographical range in Senevirathna *et al*., (2023). These results indicate that when including populations from multiple sites in an experimental setting, chances are high that these combined populations contain multiple genotypes and therefore some standing genetic variation on which evolution can act. However, when including only individuals from one site (i.e., a single population), there is a high chance that the individuals are genetically very similar to each other, if not clonal. This difference in baseline will likely impact results obtained, especially for evolutionary studies, ultimately indicating this should not be ignored.

Presence of cryptic species revealed

Correctly attributing species within the *Lemna* family is challenging (Volkova *et al.*, 2023). Indeed, we showed that *L. minor* and *L. japonica* are both present in Switzerland. This was surprising, as current species range maps of *L. minor*, such as for example provided by Info Flora (https://www.infoflora.ch/de/flora/lemna-minor.html), indicate a broad distribution of *L. minor* across Switzerland but no records of *L. japonica*. The presence of *L. japonica* in Switzerland was recently inferred based on the re-analysis of strains from the Landolt duckweed collection that showed a number of species misassignments (Braglia *et al.*, 2021b). However, the large geographical extent of *L. japonica's* distribution was unknown prior to our study. The substantial geographical range shown here indicates duckweed research should molecularly confirm their focal species to ensure accurate conclusions are drawn.

Population genomic structure of *L. japonica*

To the best of our knowledge, no other study has investigated multiple clones of wild *L. japonica* populations, although some *L. minor* studies may have accidentally included *L. japonica* clones. In contrast to *L. minor*, the population genomic structure of *L. japonica* is less clearly defined. Many populations are highly similar to each other (on the level of intrapopulation variation). Nonetheless, we found groups that were genetically similarly differentiated as the *L. minor* populations. This may add further evidence to the hypothesis that hybridization between *L. minor* and *L. turionifera* has occurred more than once, resulting in independent lineages of *L. japonica* with different ploidy or chromosome rearrangements (Braglia *et al.*, 2021a; Ernst *et al.*, 2023). Broader geographical comparisons of *L. japonica* individuals with same ploidy level and configuration (M/M/T) are now needed to confirm if multiple hybridization events lead to different *L. japonica* lineages or if all the *L. japonica* individuals of the M/M/T configuration have a single common ancestor. It might also be worth to consider studying DNA methylation as this can resolve intraspecific phylogenies with a high temporal resolution (Yao *et al.*, 2023).

It remains to be tested whether this *L. japonica* population genomic structure is ubiquitous to the species or specific to the unique geographical structure or colonisation history of Switzerland. Recent interest in this species for technical applications (Liang *et al.*, 2023) certainly warrants a deeper investigation.

Polyploidy in *L. japonica* but not in *L. minor*

Polyploidization is assumed to be one of the major mechanisms of plant speciation and evolution (Herben *et al.*, 2017). The relative importance may be greatest for plant species which reproduce mostly vegetatively, and thus experience less genome mixing via sexual reproduction. Duckweeds mostly reproduce vegetatively but do have the capability to flower (Hicks, 1932). For *L. japonica*, we found evidence that all samples were likely triploid. Being triploid is frequently associated with infertility and vegetative propagation (Ramsey & Schemske, 1998), suggesting that *L. japonica* is more likely to be clonal than its diploid sister species. This may explain the weaker population structure in *L. japonica*.

Conclusions

We used whole-genome sequencing to elucidate the population genomic structure of *L. minor* in Switzerland. Our analyses revealed the presence of *L. japonica* in Switzerland. In line with previous studies, we conclude that the morphological analysis of *Lemna* spp. in the field

may not be enough for correct species attribution. Future studies using Lemnaceae as a model system in experimental population genetics (Acosta et al., 2021), community ecology (Laird and Barks, 2018) and eco-evolutionary dynamics (Hart et al., 2019) or applied research should ensure that they are indeed dealing with *L. minor*. Our study provides genomic resources for other researchers working on Lemna species or strains. Whole-genome sequencing has become a cost-effective and powerful tool to describe duckweed population structure and should be used in future efforts to map species and genotype presence of Lemna species.

DATA AVAILABILITY

Short reads were deposited on SRA (accession number PRJNA1069743). Additional data (SNP data and sample annotation) are publicly available on Zenodo (10.5281/zenodo.10599894).

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AUTHOR CONTRIBUTIONS

Marc W. Schmid: Software, Formal analysis, Data Curation, Writing - Original draft, Writing - Review & Editing, Visualization **Aboubakr Moradi**: Conceptualization, Methodology, Investigation, Writing – Review & Editing **Deborah M. Leigh:** Conceptualization, Writing: Review & Editing **Meredith Schuman**: Methodology, Funding acquisition, Writing – Review & Editing **Sofia van Moorsel:** Conceptualization, Methodology, Investigation, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition

COMPETING INTERESTS

The authors declare no conflict of interest.

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Table1. Sampled sites, collection dates and number of samples included in the data analyses.

Site	Location (canton)	Lat	Lon	Date collected	*N*
Kob	Koblenz, AG	47.60092	8.225556	07.08.2021	3
Yve	Yverdon, VD	46.79745	6.633209	07.08.2021	5
Mot	Motto, TI	46.42877	8.967596	08.08.2021	6
Ram	Ramosch, GR	46.83374	10.40119	08.08.2021	3
Reh	Rehalp, ZH	47.3524	8.583125	18.08.2021	3
Fae	Fällanden, ZH	47.3524	8.651295	19.08.2021	2
Kap	Kappel, ZH	47.2276	8.525658	26.08.2021	2
Moe	Mönchaltorf, ZH	47.3118	8.706101	09.09.2021	5
Zun	Zunzgen, BL	47.45058	7.790417	07.09.2021	3
Lie	Liestal, BL	47.49902	7.739983	08.09.2021	6
Bas	Basel, BL	47.53847	7.61535	09.09.2021	3
Len	Lenzburg, AG	47.36212	8.173317	17.09.2021	1
Fri	Fribourg, FR	46.7946	6.992633	19.09.2021	1
Lon	Longes Rayes, VD	46.38983	6.892783	15.10.2021	3
Cha	Châtelet, VD	46.37607	6.907367	03.10.2021	5
GC	Grand Canal, VD	46.3814	6.889267	03.10.2021	1
Gra	Grangettes, VD	46.39573	6.900383	15.10.2021	3
Tol	Tolochenaz, VD	46.49357	6.482117	14.10.2021	1
Cre	Creux de Terre, VD	46.71997	6.567183	14.10.2021	5
Gor	Gordola, TI	46.1676	8.864586	27.10.2021	5
Fra	Chateau, France	49.06114	1.738231	03.07.2022	1
Blu	Blümlisalpstrasse, ZH	7.388018	8.547354	19.09.2022	2
Kre	Kreuzlingen, TG	47.65712	9.180429	19.09.2022	1
Sch	Schwamendingen, ZH	47.39552	8.581181	21.09.2022	6
Landolt collection samples					
Strain No.	**Species**	**Source location**	**Lat**	**Lon**	**N**
9967	*Lemna minor*	Bätzimatt, SZ	47.21544	8.941524	1
9978	*Lemna japonica**	Oberegg, AI	47.41857	9.549893	1
9969	*Lemna japonica**	Heiden, AR			1
9983	*Lemna japonica*	Meilen, ZH			1
9965	*Lemna japonica**	St. Gallen, SG			1
9700	*Lemna minuta*	Rotterdam, NL			1
9478	*Lemna turionifera*	Raciborz, POL			1

Note: Where possible, coordinates for Landolt duckweed collection were estimated based on geographical names. *species annotation sensu Braglia et al. (2021).

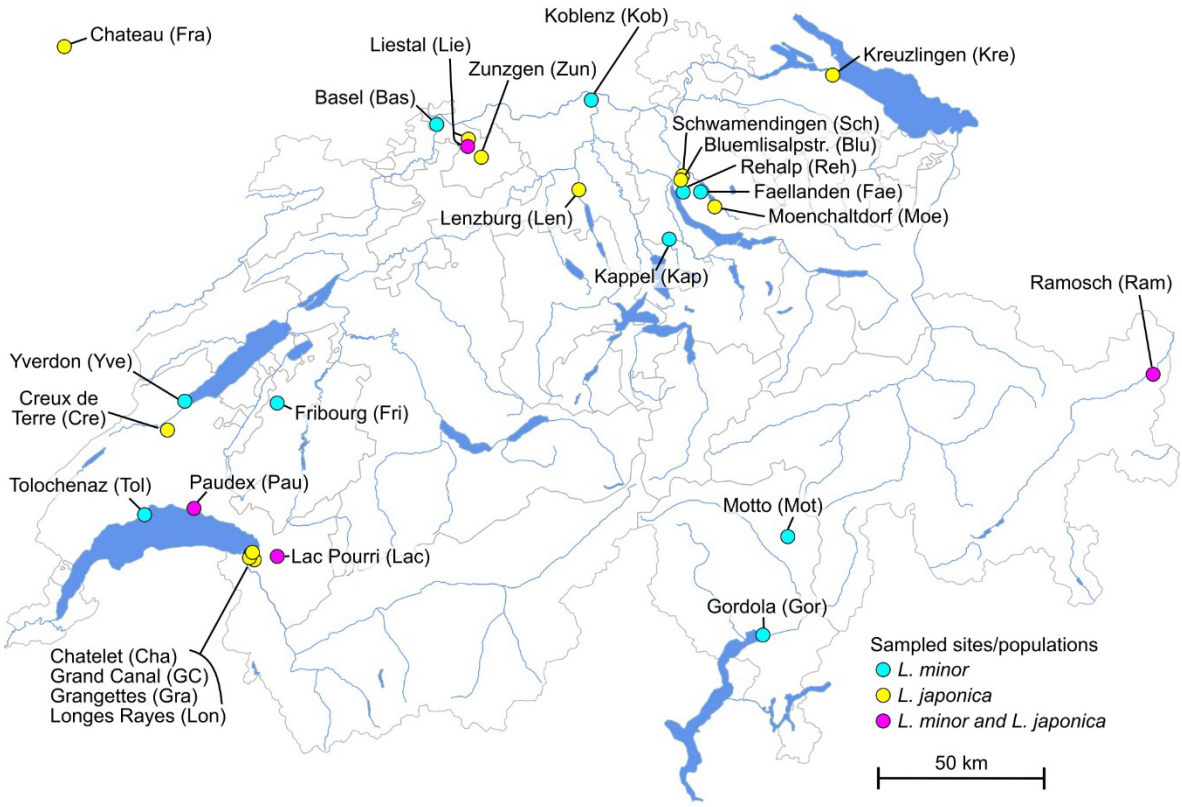

Figure 1. Sampling sites. Cyan for sites with only *L. minor* individuals, yellow for sites with only *L. japonica* individuals, magenta for sites with individuals from both species. Species assignment based on the kmer-approach.

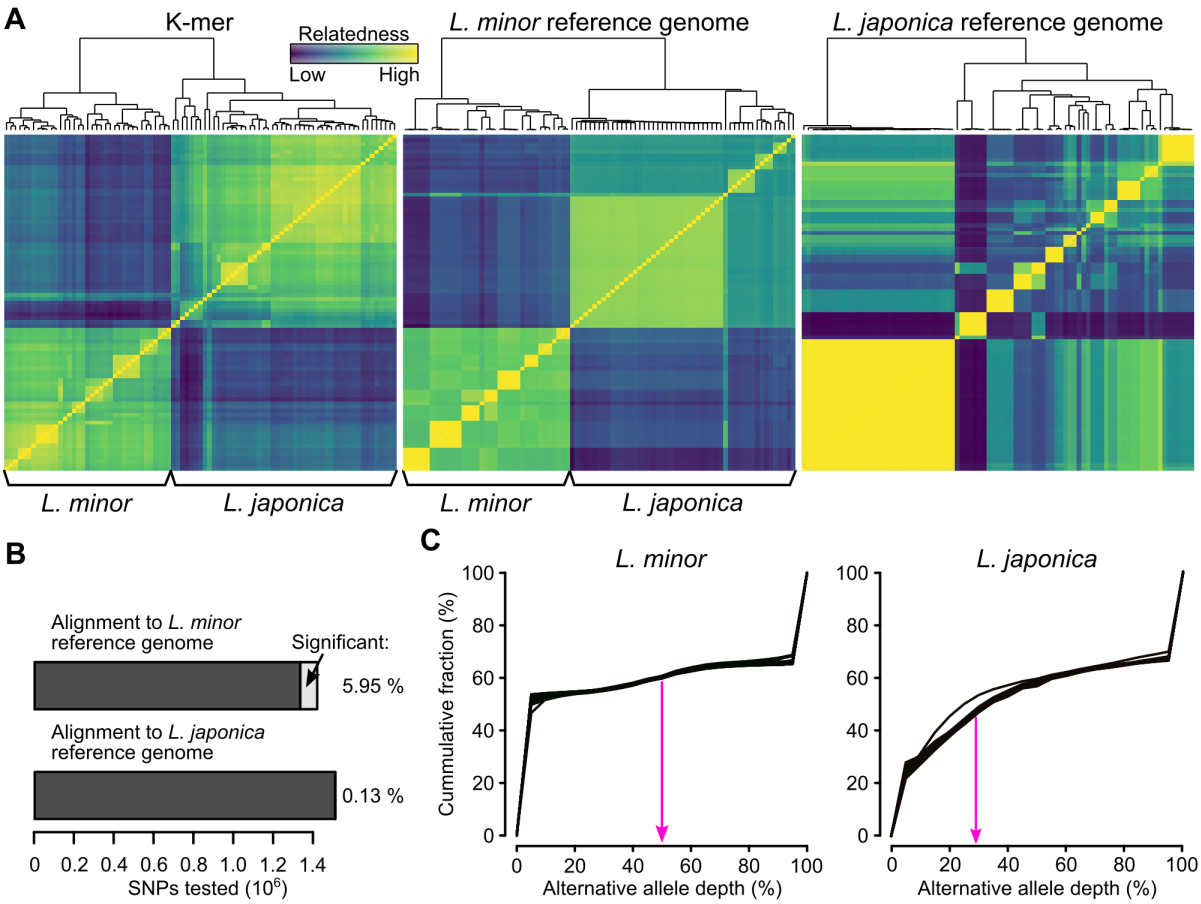

Figure 2. Analysis with all samples. A) Kinship matrices based on kmers, alignment with *L. minor* reference genome, and alignment with *L. japonica* reference genome. Species were assigned based on the kmer-approach and match to the approach with *L. minor* as reference genome. B) Test for genetic differentiation between *L. minor* and *L. japonica* on the level of individual SNPs. Significance level was set to FDR < 0.05 and %-deviance explained by the species > 80 %. Most differences were homozygous *versus* heterozygous differences: 99.73 % and 80.69 % in case of the *L. minor* and the *L. japonica* reference genome, respectively. C) Allele frequencies in *L. minor versus L. japonica* individuals using the *L. minor* reference genome. *L. minor* individuals have an enrichment of non-homozygous frequencies at 50 %. In contrast, *L. japonica* individuals have an enrichment of non-homozygous frequencies around 33 % (see also Supplemental figure S3 with data from the publicly available reference genomes).

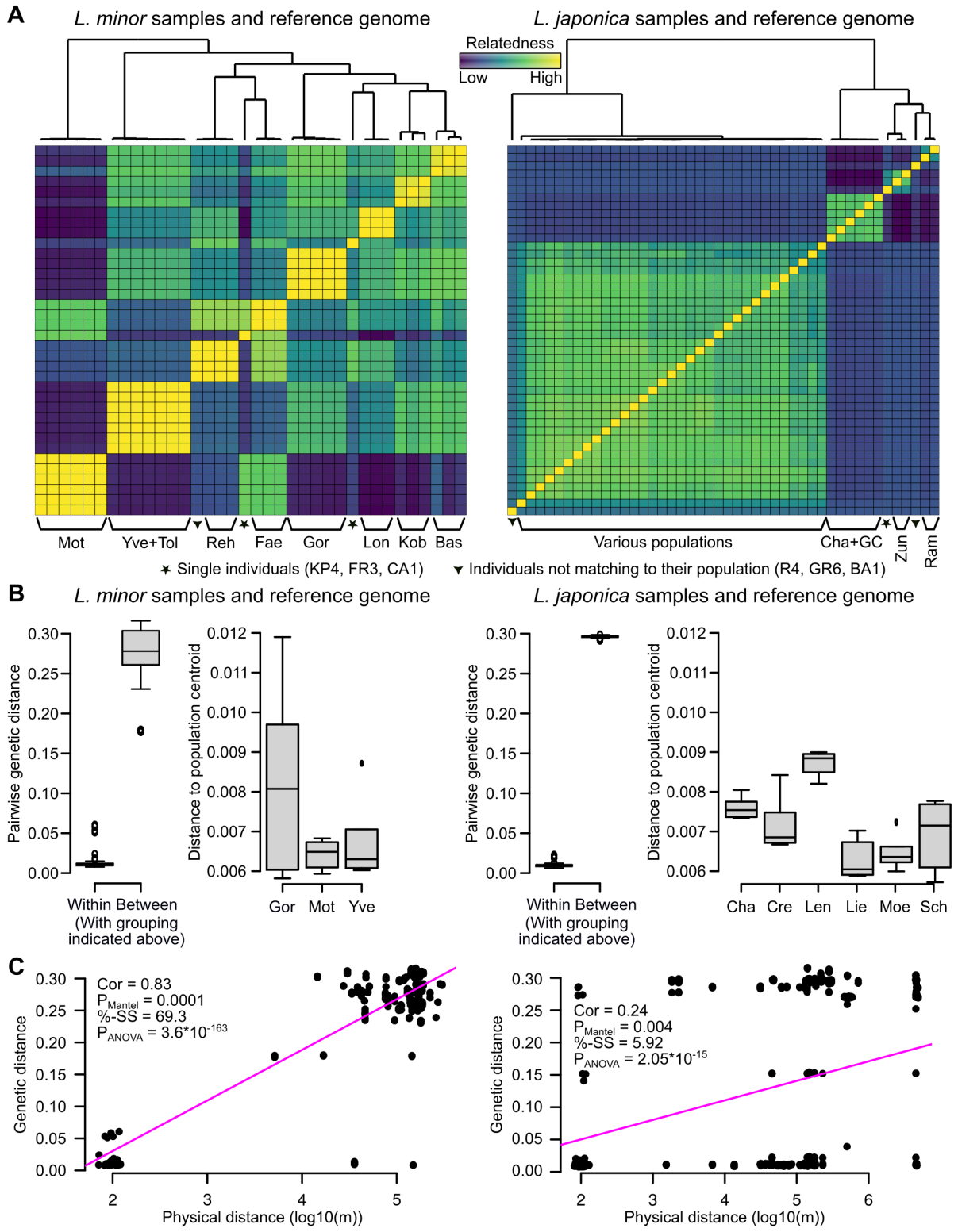

Figure 3. Per-species analysis. A) Kinship matrices for *L. minor* (left) and *L. japonica* (right). Whereas the populations of *L. minor* are generally well separated from each other, several populations of *L. japonica* are forming a single group. B) Left panels with pairwise genetic distances summarized within and between groups using all groups with at least three individuals. Note that the groups were formed according to the grouping indicated in the

kinship matrices above. I.e., especially in case of *L. japonica*, one group comprises multiple populations. Right panels show within population distances to the population centroid (only populations with at least four individuals are shown). C) Correlation between genetic and physical distances. The correlation was strong and highly significant in case of *L. minor*. Even though still significant in *L. japonica*, the correlation is much weaker.