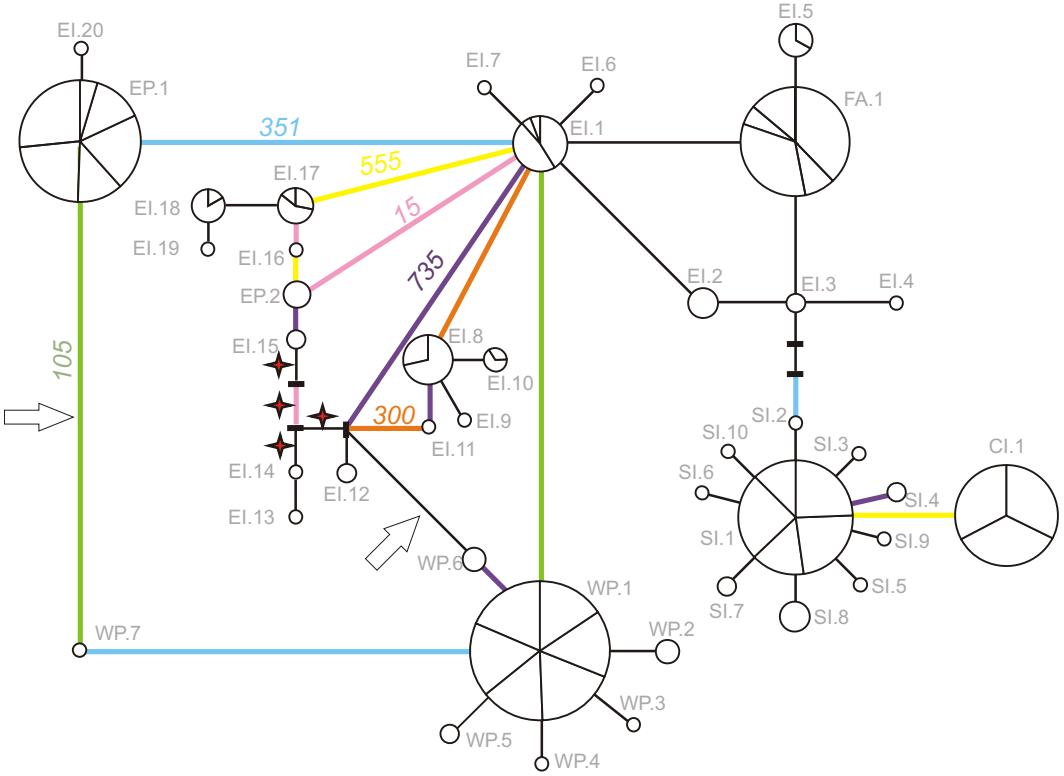
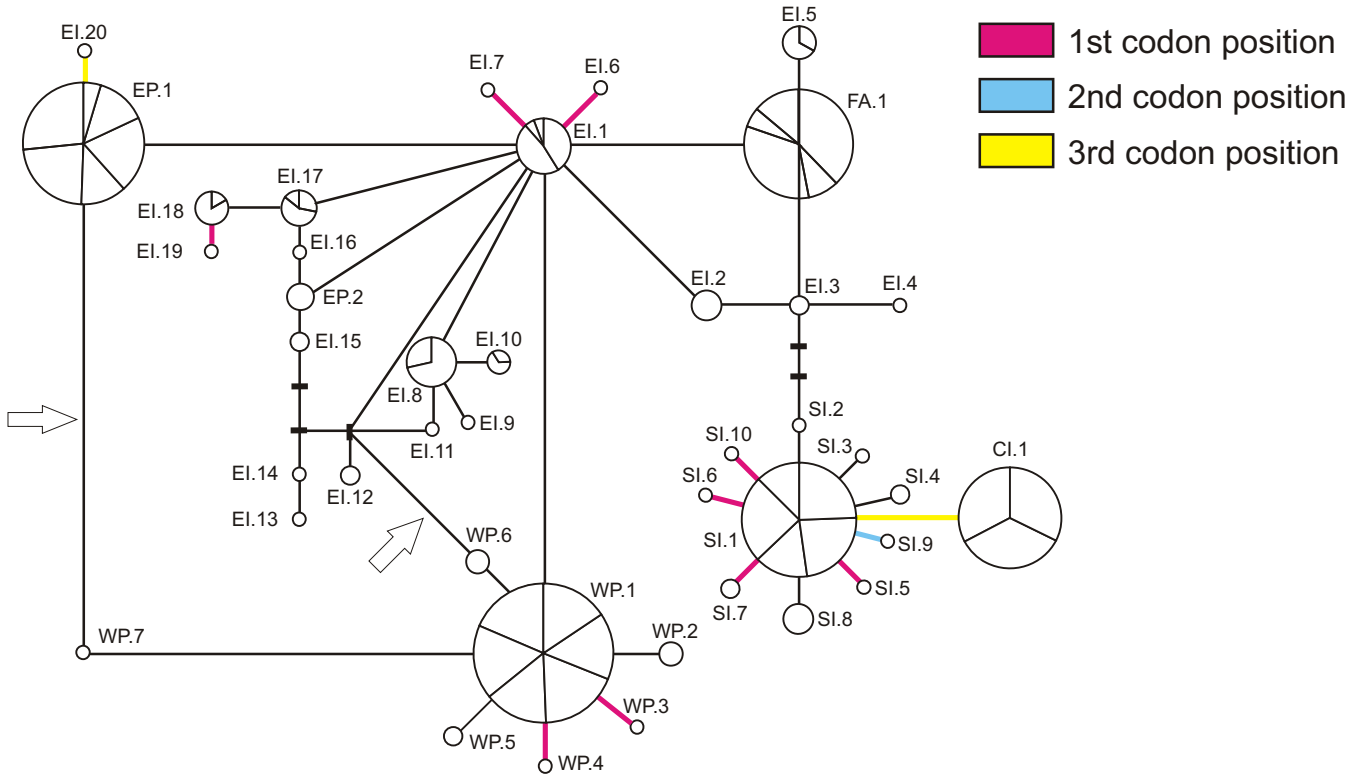
Additional file 2. Details of the 95% Statistical Parsimony network calculated for the 832 bp *COI* fragment from *G. isabellae* (Figure 1b main text).

a) Colours and numbers besides connections indicate the nucleotide where each mutation occurred. Stars point to ambiguous assignations as from software TCS 1.21.



b) Non-synonymous changes along the alignment. We calculated the dN/dS ratio (1000 bootstrap replications were used to estimate variances) and then tested the null hypothesis of no selection (H0: dN=dS) versus the purifying selection hypothesis (H1: dN<dS) using a one-tailed codon based Z-test: $Z = (dN-dS)/\sqrt{(Var(dS)+Var(dN))}$ and the "Kumar method (Kimura 2-para)" as substitution model. All these calculations were performed using MEGA5 [1].



c) Discussion.

There were twelve non-synonymous substitutions (out of 34) scattered all along the mitochondrial *COI* sequences obtained from *G. isabellae*. All of them occurred at terminal haplotypes, particularly as part of the "southern" lineage (clusters SI-CI). The ratio of within-lineages non-synonymous and synonymous nucleotide polymorphisms of the *COI* gene pointed to purifying selection to be acting on clusters EI, WP, EP and FA, whereas the null-hypothesis $d_N/d_S = 1$ could not be rejected for the SI-CI group. This result should be taken with caution, as the use of d_N/d_S ratios to test for selection at intraspecific level is controversial [2]. In addition, the no rejection of the null hypothesis is usually interpreted as due to largely neutral amino-acid substitutions (in this case on the southern lineage). However, such a result may be also due to positive selection cancelling purifying selection. Mutation, selection, demography and even statistical artefacts may have led to the observed non-synonymous substitutions (revised by [2]). Surveying the process causing the accumulation of amino-acid changes on one of the lineages of *G. isabellae* is beyond the scope of this work. However, it is worth noting that the SI cluster, using *P. nigra* and perhaps *P. halepensis/P. pinaster* as host-species, showed evidence of population fluctuations (Tables 1 and 2, Figures 1b and 2b). Therefore, plausible hypotheses accounting for the prevalence of non-synonymous changes on the SI cluster would be (i) positive selection (revised by [3]), caused by the hypothetically different southern environmental conditions; (ii) the release of strong evolutionary constraints during a population expansion (e.g. [4]), a result reported for the star-like SI cluster and (iii) the fact that non-synonymous changes are expected to occur more frequently in small populations, as slightly deleterious variants may be fixed by genetic drift then [5]. The population-size effect on the fixation of deleterious mutations is more severe in genes under strong purifying selection [6], which was

proved to act on the mitochondrial *COI* gene and conditioned the evolution of three divergent haplogroups of *Drosophila simulans* [7]. Although inconclusive, our results prepare the ground for future research confirming purifying selection on the *COI* gene and testing whether the northern lineage maintained a more stable environment keeping the populations of *G. isabellae* large enough to remove slightly deleterious mutations, whereas the demographic and/or environmental changes suffered by the southern lineage led to the fixation of such "disadvantageous" variants (e.g. [8]).

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