Whole-Gene Positive Selection, Elevated Synonymous Substitution Rates, Duplication, and Indel Evolution of the Chloroplast \textit{clpP1} Gene

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Background. Synonymous DNA substitution rates in the plant chloroplast genome are generally relatively slow and lineage dependent. Non-synonymous rates are usually even slower due to purifying selection acting on the genes. Positive selection is expected to speed up non-synonymous substitution rates, whereas synonymous rates are expected to be unaffected. Until recently, positive selection has seldom been observed in chloroplast genes, and large-scale structural rearrangements leading to gene duplications are hitherto supposed to be rare. Methodology/Principle Findings. We found high substitution rates in the exons of the plastid \textit{clpP1} gene in \textit{Oenothera} (the Evening Primrose family) and three separate lineages in the tribe \textit{Sileneae} (Caryophyllaceae, the Carnation family). Introns have been lost in some of the lineages, but where present, the intron sequences have substitution rates similar to those found in other introns of their genomes. The elevated substitution rates of \textit{clpP1} are associated with statistically significant whole-gene positive selection in three branches of the phylogeny. In two of the lineages we found multiple copies of the gene. Neighboring genes present in the duplicated fragments do not show signs of elevated substitution rates or positive selection. Although non-synonymous substitutions account for most of the increase in substitution rates, synonymous rates are also markedly elevated in some lineages. Whereas plant \textit{clpP1} genes experiencing negative (purifying) selection are characterized by having very conserved lengths, genes under positive selection often have large insertions of more or less repetitive amino acid sequence motifs. Conclusions/Significance. We found positive selection of the \textit{clpP1} gene in various plant lineages to be correlated with repeated duplication of the \textit{clpP1} gene and surrounding regions, repetitive amino acid sequences, and increase in synonymous substitution rates. The present study sheds light on the controversial issue of whether negative or positive selection is to be expected after gene duplications by providing evidence for the latter alternative. The observed increase in synonymous substitution rates in some of the lineages indicates that the detection of positive selection may be obscured under such circumstances. Future studies are required to explore the functional significance of the large inserted repeated amino acid motifs, as well as the possibility that synonymous substitution rates may be affected by positive selection.

INTRODUCTION
The circular chloroplast genome is in general expected to be a non-recombining unit where large within-genome duplications are rare. Most of its genes are single-copy, occurring in large and small single-copy regions, that are intervened by inverted repeat regions [1]. Substitution rates of chloroplast DNA (cpDNA) are held to be relatively slow and not very variable, although not constant, among lineages [2–5]. The gene content is likewise thought to be well conserved [6].

Most reports of positive selection are from the human genome or other model organisms [7,8], and documented significant positive bias of non-synonymous (dN) over synonymous (dS) substitutions from non-model organisms and/or entire genes are rare [but see [9] and [10]]. From an evolutionary perspective it is to be expected that some genes (e.g., those involved in the immune system) can have specific sites that are under positive selection [11], but genes that exhibit positive selection as a whole and with non-synonymous substitutions more or less evenly distributed over the entire length of the gene are clearly more enigmatic and of greater general evolutionary interest.

The chloroplast-encoded \textit{clpP1} (caseinolytic peptidase, ATP-dependent, proteolytic subunit) is part of a gene family encoding \textit{clpP} proteases with six members in \textit{Arabidopsis} of the mustard family \textit{Brassicaceae} [12]. The other five members are encoded in the nucleus (\textit{clpP2–clpP6}) [12]. The main function of the protein is to degrade polypeptides, but the \textit{clpP} proteases are involved in a variety of processes, ranging from developmental changes to stress tolerance [13]. It has been suggested that the \textit{clpP1} gene is essential for plant cell viability [14,15]. The gene is found in the chloroplast genome of all higher plants and most eukaryotic algae [12]. The structure of the gene and the amino acid composition are highly conserved with 196 amino acid residues distributed over three exons and with two intervening introns, but \textit{Omphalodes}, grasses, and the conifer genus \textit{Pinus} lack the two introns. Most green algae also lack introns, but they have roughly the same number of amino acid residues as land plants. However, \textit{Chlamydomonas} and \textit{Scenedesmus} (both \textit{Chlorophyceae}) have large insertions resulting in a total of 525 and 538 amino acids,

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RESULTS

In all 25 flowering plant species sequenced for this study (21 Sileneae, 4 Oenothera), there is one copy of the clpP1 gene located between the rps12 and petB genes. None of these had frame shifts or premature stop codons, and thus appeared functional. This is in accordance with all published land plant chloroplast genomes. Additional clpP1 copies were found in two of the species (Fig. 1). Several of the clpP1 sequences lack introns (Table 1).

Six of 21 investigated species of Sileneae showed signs of elevated branch lengths in the clpP1 exons (Fig. 2B). These taxa are distributed in three phylogenetically distinct lineages [17–20]: two closely related species in Silene subgenus Behen (S. conica and S. conoidea), one species in Silene subgenus Silene (S. fruticosa), and three of four investigated Lychinis species (L. chalcedonica, L. flo-cuculi, and L. abyssinica). Three of the six species (S. conica, S. fruticosa, and L. chalcedonica) have been extensively sequenced (>25 kb) also for other, both coding and non-coding chloroplast regions, but do not display such extreme branch lengths elsewhere in the plastid genome [18]. Surprisingly, the synonymous substitution rates in two of these three species are clearly elevated (up to five times) in clpP1 when compared with other cpDNA genes (Fig. 3). All four investigated species of Oenothera had elevated substitution rates. Only O. fascia possess introns of the four, and it is also the one with the shortest branch length (Fig. 2B). Synonymous substitution rates in the clpP1 gene of O. elata are 2.5–3.2 times higher than other chloroplast genes (Fig. 3).

The position and length of the clpP1 exons are generally conserved in Sileneae, like in most other angiosperms (Table 1), but exceptions were found in the six Sileneae species with long branches and the Oenothera sequences. Silene conica and S. conoidea share seven indels, of which the two largest insertions differ in length between the two species. Several of the species had long insertions partially consisting of amino acid repeats in their exons (Table 1).

Analysis of all available seed plant clpP1 sequences together with the five nuclear encoded gene family members (clpP2–clpP6) from Arabidopsis and Oryza strongly indicated (posterior probability 1.00) that the divergent sequences found in this study are of chloroplast origin (Fig. S1). The relationships within Sileneae in the gene family analysis, where all the long branches group together, are at odds with all previous cpDNA analyses (e.g. [18]). The analysis of only third positions from the clpP1 exons did, however, result in a topology compatible with other analyses of sequences from other cpDNA regions (Fig. 2B). For example, despite its very long branch, the Silene conica and S. conoidea clade grouped together with other members of Silene subgenus Behen (Fig. 2B).

The Sileneae phylogenies based on clpP1 exon (Fig. 4A) and intron (Fig. 4B) sequences were substantially different. Intron sequences from Lychinis chalcedonica and Silene fruticosa did not show signs of elevated substitution rates relative to other cpDNA regions, and the phylogeny (Fig. 4B) is compatible with other Sileneae phylogenies based on cpDNA [18].

We used the method of Yang [21] to investigate the ratio of non-synonymous to synonymous substitutions (dN/dS, ω) and found that this ratio varies significantly among lineages (P<0.0001), both under a generally accepted eudicot phylogeny (Fig. 2A) and under the tree topology from Bayesian analysis of only third positions from the clpP1 exons (Fig. 2B). Several branches in both Sileneae and Oenothera resulted in ω>1 (Fig. 2). In addition, the branches in Solanaceae have high ω-values, despite shorter branch lengths. Three branches have ω that are significantly higher than one after Bonferroni correction (table 2, Fig. 2A). The second topology (Fig. 2B) gives very similar ω-values, but the values of the three branches with significant positive selection are higher still, and two internal branches not resolved in the first topology (Fig. 2A) receive ω-values>2.0 (Fig. 2B).

Figure 1. Multiple Copies of the clpP1 Gene Region Found in Two Species. DNA fragments sequenced from the clpP1 region (thick black bars) for Lychnis chalcedonica (Lc) and Silene fruticosa (Sf). Shown in gray is the corresponding region, with the genes (boxes) and introns (dotted lines), in Spinacia. Thick white bars indicate non-homologous flanking regions (Lc2 is located differently in the chloroplast genome). Fragments with arrows on the left side consist of a continuous sequence from rbcl to petB (c. 18 kb). Thin lines mark lack of introns. ? indicates stop codon. ? indicates unknown flanking regions.

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We reject the idea that the observed open reading frames in the clpP1 sequences of Silene conica and S. conoidea have persisted by chance alone under a neutral model of evolution, because only 0.69% of simulated sequences with a branch length of 0.25 from the S. latifolia sequence appear to have remained functional (98.66% had premature stop-codons and an additional 0.65% lacked a proper start-codon). The average number of premature stop codons per simulated sequence was 4.3.

DISCUSSION

Our results indicate highly elevated substitution rates in the chloroplast clpP1 exons in Sileneae and in Oenothera. Although long branch lengths also could indicate high ages, we argue that this is unlikely because the comparisons with other genes (Fig. 3) which, unless acquired by horizontal transfer, must be of the same age. Even if the topological relationships sometimes are ambiguous, any resolution of the trees in Fig. 2 will show that the sister group has a significantly shorter branch. As sister groups by definition has the same age, either the short branch has undergone a substitution rate decrease or vice versa. This supports the explanation for the long clpP1 branches as likely to have been the result of a substitution rate increase.

In Sileneae, there are at least three independent increases in substitution rates. Loss of introns in clpP1 appears correlated with...
Figure 2. Positive Selection in the Chloroplast clpP Gene. A) $dN/dS$ ratios calculated on the Eudicot tree topology. Numbers on nodes indicate classification as follows: 1 Myrtales, 2 Eurosids II, 5 Eurosids I, 13 Caryophyllales, 17 Euasterids I, 19 Euasterids II, 20 Asterids [36]; 3 Hologalegina, 4 Fabaceae [37]; 7, 8, 9 subgenus *Behen*, 10 subgenus *Silene*, 11, 12 *Sileneae* [17]; 14 Solaneae, 15 Solanoideae, 16 Solanaceae [38]. Values on branches are the $dN/dS$ ratios, $S$: $dN = 0$, $N$: $dS = 0$. Ratios significantly above one (Bonferroni-corrected) are indicated by asterisks: ***$P < 0.001$, *$P < 0.05$. B) $dN$- and $dS$-branch (in grey and black, respectively) lengths imposed on topology from Bayesian analysis of third codon positions from the *clpP1* exons. Numbers on nodes are Bayesian posterior probabilities (Bpp). Only Bpp values $> 0.50$ are shown.

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Figure 3. dS and dN Values of Four Chloroplast Genes. Diagram shows the pairwise dS values (top) and dN values (bottom) between eleven species of Sileneae and the outgroup (Heliosperma alpestre) for four chloroplast genes (clpP1; 591 bp, psbB; 1527 bp, cemA; 639 bp, and petA; 963 bp). To the right of the vertical gray line is the pairwise comparison between Oenothera elata and Eucalyptus globulus, for the same genes.

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elevated substitution rates in several lineages. In two of the Sileneae lineages, high substitution rates are accompanied with both gene duplications and significant positive selection.

Figure 4. Bayesian Consensus Phylograms of the Tribe Sileneae. A) The clpP1 exon sequences, and B) the clpP1 intron sequences. Branches in bold have Bayesian posterior probabilities (Bpp) of 1.00. Only Bpp values above 0.90 are shown at nodes. Note that A and B are drawn at different scales. ** indicates taxa that lack introns in clpP1.

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Table 2. Branches with dN>dS in the phylogeny in figure 1A.

| Branch leading to: | dN/dS | -lnL | \( \chi^2 = 2 \lnL \) | P\(_{\text{uncorrected}} \) | P\(_{\text{Bonferroni}} \) |
|-------------------|-------|------|-----------------|-----------------|-----------------|
| O. fruticosa      | 1.717 | 7019.4213 | 4.0108 | 0.0452 * | n.s. |
| O. elata          | 2.392 | 7020.8512 | 6.8706 | 0.0087 ** | n.s. |
| O. macrocarpa     | 4.101 | 7027.0673 | 19.3028 | 0.0001 *** | 0.00067 *** |
| Vitis             | 1.424 | 7018.3996 | 1.9674 | 0.161 | n.s. |
| L. chalcedonica   | 2.493 | 7023.2954 | 11.759 | 0.0006 *** | 0.036 * |
| L. flos-cuculi**  | 1.998 | 7018.7147 | 2.5976 | 0.107 | n.s. |
| L. abyssinica**   | 1.176 | 7017.5145 | 0.1972 | 0.657 | n.s. |
| node 7            | 1.355 | 7017.6766 | 0.5214 | 0.470 | n.s. |
| S. conoidea**     | 1.214 | 7017.4239 | 0.016 | 0.8993 | n.s. |
| node 8            | 1.029 | 7018.1340 | 1.4362 | 0.231 | n.s. |
| S. fruticosa      | 5.899 | 7029.2347 | 23.6376 | 0.000001 *** | 0.000007 *** |
| Atropa Na         | N*   | 7018.5259 | 2.22 | 0.136 | n.s. |
| Solanum Na        | N*   | 7018.0874 | 1.343 | 0.247 | n.s. |
| Lycopersicon Na   | N*   | 7021.3030 | 7.7742 | 0.0052 ** | n.s. |
| node 14           | 4.232 | 7020.2991 | 5.7664 | 0.016 * | n.s. |
| node 15           | 1.177 | 7018.1436 | 1.4554 | 0.228 | n.s. |
| node 19           | N*   | 7017.4737 | 0.1156 | 0.734 | n.s. |

*Branches with only non-synonymous substitutions.

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taxa (Fig. 3), most of the total substitution rate increase can be ascribed to non-synonymous substitutions. A generally elevated substitution rate for a whole genome such as the mitochondrial genome of some species of Plantago could be explained by, e.g., an increased amount of oxygen free-radicals [22], but elevated substitution rates in, and exclusive to, a specific gene are harder to explain. The very long branch leading to Silene conica and S. conoidea is puzzling in this context, because the rates of both synonymous and non-synonymous substitutions are very high, and there is no significant positive selection. The pattern is similar, but less extreme, in L. chalcedonica (Fig. 3). The length of the branches leading to Solanum/Leerspericon (Solanaceae), within the Fabaceae, to Vitis, and to Cucumis might also indicate elevated substitution rates, but this is much less striking than in the long Sileneae branches and in Oenothera (Fig. S1).

By comparing synonymous and non-synonymous substitutions, we were able to detect statistically significant positive selection at the gene level on three branches. However, we anticipate that the actual duration of the episodes of positive selection in the tree might be larger. The power of the tests employed here is relatively low, i.e., positive selection is difficult to detect even if it exists at many sites [7]. Recently, methods have been developed to detect positive selection on individual codons for specific branches (e.g., the branch-site likelihood method [23]). These methods are generally more powerful and their utilization has resulted in a marked increase in the number of published reports of positive selection [24]. However, simulations by Zhang [24] showed that the power of these methods comes at a cost in the form of high levels of false positives.

Under the assumption that substitutions in non-coding sequences are selection-wise neutral, elevated substitution rates in exon sequences compared to introns provide an indication of positive selection [25]. By comparing the branch lengths of the exon tree (Fig. 4A) and the intron tree (Fig. 4B) it is apparent that this is the case in S. fruticosa and probably also in Lychius chalcedonica. For example, the uncorrected pairwise base difference between S. fruticosa (Sf1) and S. schaffa is 0.25 in exons, but 0.05 in the introns, and for L. chalcedonica (exon: Lc1, intron: Lc3/Lc4 combined) and L. flavo-jovis these figures are 0.30 and 0.04, respectively. Finally, Oenothera flava (the only Oenothera species in this study to have introns) shows more variability in exons than in introns compared to Eucalyptus (0.26 and 0.18, respectively), although the difference is less striking for this taxon.

The clpP1 exon sequences that show the most extreme substitution rates are those of Silene conica and S. conoidea, but because they lack introns in the gene, the exon/intron comparison cannot be made. The variability in the gene is approximately an order of magnitude higher (synonymous and non-synonymous substitutions contributing roughly equally to the increase) than that of spacer-regions in the cpDNA of S. conica (see below). The pairwise base difference between Silene conica and Silene latifolia in intergenic spacers is on average 0.03 (rbcL/ac: 0.037, petA/petB: 0.033, psbE/petL: 0.026, sp20/sp21: 0.022, data from [18]), but the difference in the clpP1 gene is 0.31. Despite the very divergent sequences, the dN/dS ratios did not indicate positive selection acting within this group. Because ratios around 1 implicate absence of both positive and purifying selection, this indicates that the S. conica and S. conoidea sequences may have lost their function. However, the simulation experiment strongly rejected the hypothesis that the absence of stop codons can be explained by chance alone. Further support for this is given by the fact that Silene conica and S. conoidea have seven indels in the clpP1 sequence, all of lengths that are multiples of three. The existence of these indels that do not distort the reading frame is in itself strong evidence for maintained gene function. Finally, even if S. conica appears to have a somewhat elevated cpDNA substitution rate in general [10], it seems unlikely that the very high substitution rates in clpP1 would be the effect of lost function. Xing and Lee [26] found that alternative splicing could greatly relax selection pressure (measured as dN/dS). This effect was accompanied by a strong decrease in synonymous substitutions. Because we observe a strong increase in synonymous substitutions in our data (Fig. 5), this explanation too, seems unlikely in this particular case.

Whether there is a causal relationship between the increase in synonymous and non-synonymous rates in Silene conica/conoidea remains unclear. However, there are other indications that positive selection of clpP1 is correlated with increased synonymous substitution rates; Lychius chalcedonica and Oenothera elata also have elevated synonymous substitution rates (Fig. 3). Some of the other branches in the eudicot clpP1 tree (Fig. 2) have combinations of branch lengths and dN/dS ratios that indicate a more widespread occurrence of positive selection of the gene. In particular the branch leading to Solanum/Leerspericon (node 14 in Fig. 2A) that has a dN/dS ratio significantly higher than 1 before Bonferroni-correction (Table 2), but also the branches leading to node 6 (Fig. 2A), Medicago, Vitis, and Cucumis seem interesting targets for further investigations.

In a systematic search for positive selection in higher plants based on almost 140,000 embryophyte gene sequences from GenBank, very few cases of ω values above one were found when averaging over whole genes [27]. Only in two cases were ω>2, and both of these were sequence pairs of nuclear encoded genes [27]. This illustrates how unusual our findings are. The recent report on positive selection in the chloroplast gene rbcL [10] clearly shows that specific sites in that gene are under positive selection in a wide range of land plants. Our study gives indications that positive selection in the clpP1 gene might be widespread in flowering plants. In rbcL it is only a small proportion of the sites that appear to be under positive selection [10], whereas in clpP1 a very large proportion of the sites are affected.

In the present study, the rates of synonymous substitutions are rather conserved with respect to different taxa and genes, with the important exception of the species undergoing rapid evolution in the clpP1 gene (Fig. 3). None of the “normal” taxa or genes shows as high dS rates as the clpP1 gene from those three species. The degree of elevated dS rates also indicates an interesting pattern: the species with the strongest estimated positive selection has the least elevated dS rate and vice versa, i.e. the rate of non-synonymous substitutions are roughly the same among the three species.

Elevated evolutionary rates due to positive selection or relaxed selective constraints are often preceded by gene duplication [28]. We detected extra clpP1 gene copies only in Lychius chalcedonica and Silene fruticosa. Indeed, the completely sequenced chloroplast genome of Oenothera elata (NC_002693) contains only one copy of clpP1. Only one of the four clpP1 copies in L. chalcedonica is potentially functional (Lc1), i.e., the others contain stop codons or are incomplete. The intron-containing Lc4 fragment shows obvious signs of elevated substitution rates in clpP1, although less so than Lc1. The Lc3 copy, apparently a pseudogene, is less divergent than the other copies in Lychius chalcedonica. This does not seem to be an artifact due to missing data, because in the region where sequence information for Lc1, Lc2, and Lc3 overlap the uncorrected distance between Lc1/Lc2 and L. flavo-jovis (the “normal” Lychius species in this study) is 30.3%/34.6%, whereas between Lc3 and L. flavo-jovis it is 3.9%. Thus, in absence of a formal phylogenetic analysis of the clpP1 copies in L. chalcedonica, we may speculate that at least the duplication leading to Lc3 preceded the onset of positive selection. In S. fruticosa, Sf2 is
markedly less divergent than the two other copies, and thus also probably the result of an ancient duplication preceding the non-synonymous rates increase. In the region where sequence information for Sf1 and Sf2 overlaps the uncorrected distance between Sf1 and S. schafta is 22.6%, whereas between Sf2 and S. schafta it is 5.2%. Both these cases may thus agree with one of few documented cases where gene duplication precedes the onset of positive selection [29]. It may be that positive selection, under some circumstances, can be triggered by duplication rather than being an expected outcome of it.

The very large insertions (174 to 197 amino acids) found in the clpP1 exon 1 of Silene fruticosa (Sf1), Lychnis flo-cuculi, and L. abyssinica do not cause frame shifts or stop codons. To our knowledge, the effect of indel evolution has not been studied in relation to positive selection. It is possible that repetitive insertions are beneficial, because given that the repeats are in multiples of three nucleotides, they reduce the probability of stop codons, while possibly fostering new phenotypic variants.

Conclusions
In our study, four distantly related taxa or groups of taxa (Oenothera, Silene fruticosa, Silene conica/conoidea, and Lychnis chalcedonica/flos-cuculi/abyssinica) exhibit substitution rates in clpP1 exon sequences that are hitherto unprecedented in the chloroplast genome. We conclude that these high evolutionary rates are correlated with positive selection of clpP1 in the evolutionary histories of at least three of these four groups. In the case of Lychnis, this was probably preceded by a duplication of a segment including clpP1, psbB, psbT, psbN, and psbH, but only the clpP1 gene shows signs of positive selection. We cannot rule out the possibility of gene duplications as a causal agent in the other three cases, because duplicates may either be ambiguous, extinct, or undetected. One of the major aspects of the present results is that they indicate that positive selection may be accompanied by elevated synonymous substitution rates in some cases. If this indeed proves to be the case it will have far-reaching consequences for our ability to detect positive selection. Also, the fact that positive selection appears to have originated in at least three closely related lineages of Sileneae calls for caution when interpreting plastid data at population and phylogenetic levels (cf. [9]). The relationship between cpDNA duplications, increased substitution rates, positive selection, and indel evolution in the chloroplast genome needs further scrutiny, and plant clpP1 appears to constitute an excellent model system for such studies.

MATERIALS AND METHODS

-Taxon sampling
All seed plant clpP1 sequences on GenBank [30] as of May 15th 2006, as well as the nuclear clpP2-6 from Oryza and Arabidopsis, were downloaded; in addition, 21 species of Sileneae and four species of Oenothera were sequenced for the gene (Table S1). The sampling of Sileneae species follows that of Erixon and Oxelman [18], but Silene conoidea and Lychnis abyssinica were added after the observation that closely related taxa exhibited extremely high substitution rates. The inclusion of Oenothera in the study was deemed important because a survey of complete chloroplast genomes on GenBank revealed that Oenothera elata (NC_002693) lacks introns and shows signs of elevated substitution rates. The four species of Oenothera were chosen to represent different sections in the genus [31].

-DNA Amplification and Sequencing
All 21 Sileneae species (except Silene conoidea, Lychnis flo-cuculi, Lychnis flos-jovis, and Lychnis abyssinica) were amplified and sequenced in a continuous region from the end of rbcL to the beginning of petB corresponding to c. 18 kb in Spinacia. DNA preparation, amplification, sequencing, and general primers follow Erixon and Oxelman [18].

The clpP1 region in Oenothera was amplified with the PCR primers rps12-F and clpP/psbH-R6 (Fig. 5). For sequencing of this product, eight primers were constructed (Table S2). In addition, some specific primers were made for Silene fruticosa and Lychnis chalcedonica, either to amplify a specific sequence copy or to sequence through large insertions (Table S2).

-Alignment
The amino acid sequences from Arabidopsis and Oryza for all five nuclear members of the clpP1 gene family were aligned using ClustalW version 1.83 [32], with default settings, together with chloroplast clpP1 amino acid sequences. The nuclear sequences corresponding to the 21 first amino acids in the chloroplast clpP1 gene of Spinacia were excluded prior the analysis, because the alignment responded strongly even to small changes in the parameters. All sequences corresponding to the six last amino acids of Spinacia clpP1 were also excluded, due to extreme length variation both within clpP1 and among the other gene family members. The amino acids were only used in the alignment process; all analyses were done on nucleotide sequences.

Figure 5. The clpP1 Gene and Flanking Regions with Primer Sites. Approximate position of general primers for Sileneae [18] with reference to Spinacia. Boxes correspond to protein coding genes with their name and position in Spinacia. Boxes above the line denote genes that are transcribed from left to right, and those below are transcribed from right to left. Dotted lines represent introns.
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All non-clpP1 exon DNA sequences were manually aligned, using the principles of Oxelman et al. [33], in the sequence alignment editor Se-Al version 2.0a11 [http://evolve.zoo.ox.ac.uk/].

-Phylogenetic analysis
Bayesian phylogenetic analyses were performed with MrBayes version 3.1.2 [34]. For both the gene family data set (72 terminals) and a restricted eudicot clpP1 data set (38 terminals, see below), substitutions were modeled with the GTR+Γ model, which received highest AIC scores according to MrAIC.pl version 1.4 (http://www.abc.sc/~nylander/) together with PHYML version 2.4.4 [35]. The large data set ran for 50 million generations with six MCMC chains (temperature (τ) = 0.2) and four independent runs with trees sampled every 1000th generation. The eudicot data set ran for 20 million generations with four MCMC chains (τ = 0.2) and two independent runs with trees sampled every 1000th generation. The first 50% of the sampled trees were, in both analyses, discarded as burn-in.

Bayesian analyses, with the same settings as above except that number of generations were 10 million, were performed on: a) a matrix of only third positions from the Eudicot data set, b) a matrix of Sileneae clpP1 intron sequences, and c) a matrix of Sileneae clpP1 exon sequences.

-Detection of positive selection
PAML version 3.14 [21] was used to calculate the non-synonymous (dN) and synonymous (dS) substitutions rates, and the ratio (ω) between them. To test for variation in ω among the branches, the likelihood for the data under a model with fixed ω (estimated from data) for the entire tree (m0) was compared to a model allowing for the branches to have different ω (m1). To test the null hypothesis that addition of a ω parameter for each branch does not increase likelihood of the data, a likelihood-ratio test was performed, where the test parameter is assumed to follow the χ2 distribution with the degrees of freedom equal the number of tree branches – 1. The null hypothesis of absence of selection on individual branches was tested by comparing the maximum likelihoods from the m1 analysis to maximum likelihoods for models with free ω for all branches except that the ω of the branch under consideration was set to 1 (m2). This test has one degree of freedom. Only branches with ω>1.0 were tested, but because these were detected a posteriori, the probabilities were Bonferroni corrected [7].

-Topologies used for estimates of dN/dS
Two different tree topologies were used for calculations of dN and dS values. The first topology (Fig. 2A) was based on the classification of the Angiosperm Phylogeny Group II [36] at the family level and on strongly supported within-family relationships published elsewhere [17–20,37,38]. The sistergroup relationship between Silene conica and S. conoides has not been published before, but is based on their very similar morphology, i.e., they belong to the section Conoimorpha Otth, which is characterized by a calyx morphology and a basic chromosome number, both of which are unique in Silene. Five Silene sequences were excluded from this analysis; these were identical or almost identical (≤4 bases different) to at least one of the non-excluded sequences. Only sequences with the entire clpP1 coding region were included in the analyses detecting for positive selection.

To explore the effect of the tree topology on the outcome a second substitution rate analysis was performed using the 50% majority-rule consensus phylogeny from the Bayesian analysis of third positions (Fig. 2B). This topology is incompatible with the first topology with respect to the relations within Fabaceae. Another substantial difference is in the level of resolution. Eight more branches are unresolved in the second phylogeny compared to the first and two relationships are resolved only in the second (in Oenothera and in Lychnis, see Fig. 2).

-Synonymous substitution rate comparison
In order to obtain an estimate of the synonymous substitution rates in the clpP1 exons in Sileneae we made pairwise comparisons between eleven Silene/Lychnis species and Heliosperma (outgroup). These estimates were compared with data from three other chloroplast genes (psbB, 1527 bp; cemA, 639 bp; petA, 963 bp). The reason for choosing these particular genes was that they are the only large genes present in the 18 kb fragment sequenced for all the twelve taxa [18], and when Goremykin et al. [39] compared the synonymous substitution rates for these genes of ten angiosperms relative to Ppaus, the average dS values were similar and clpP1 had the lowest value (clpP1, 0.36; cemA, 0.89; psbB, 1.00; petA, 1.53). We also made a pairwise comparison of dS values for the same genes from the complete chloroplast genomes of Oenothera elata and Eucalyptus globulus. PAML version 3.14 [21] was used for all estimates.

-Indirect test for loss of function
In the absence of expression studies, we conducted a simple test to evaluate if the observed open reading frames of sequences with highly elevated rates could have persisted by chance. Ten thousand sequences were generated with SeqGen version 1.3.2 [40] under the JC69 model of evolution using the Silene latifolia sequence as ancestral and a branch length of 0.25. The branch length was chosen to be considerably smaller than the longest branch observed in the substitution rate analysis, namely the one leading to S. conica/S. conoides, i.e., 0.47 (Figures 2A). A simple substitution model was chosen to simulate neutral evolution. Since the chloroplast DNA sequences have an AT-bias, the JC69 model will result in a conservative test, because an excess of A and T will increase the number of simulated stop codons simply because these are AT-rich (TAA, TAG, and TGA).

SUPPORTING INFORMATION
Figure S1 clpP gene family phylogeny. A) Bayesian consensus phylogram of all seed plant clpP1 sequences and the nuclear clpP2–clpP6 for Arabidopsis and Oryza, and B) without Oenothera and the long Sileneae branches. Numbers on nodes are Bayesian posterior probabilities (Bpp). Branches in bold have Bpp = 1.00. Only Bpp>0.90 are shown. Each * indicates a missing intron. Y indicates pseudogene. ? indicates incomplete gene (only exon 1) without stop codon.

Table S1 Plant taxa, vouchers for the sequences obtained in this study, and GenBank accession numbers

Table S2 Specific primers

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Author Contributions
Conceived and designed the experiments: PE BO. Performed the experiments: PE. Analyzed the data: PE. Contributed reagents/materials/analysis tools: PE BO. Wrote the paper: PE BO.
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