A Unique Loss of Photosynthetic Functions and Unusually Low GC Content in the Minimized Plastids found in Holoparasitic Plants

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Abstract

The reduction of holoparasitic plants' plastomes is an intriguing phenomenon that we sought to explore via bioinformatic analysis of various DNA sequences. Our research supports that holoparasitic plants have a trend of unusually low proportions of GC content in their chloroplast genes compared to hemiparasitic and autotrophic plants. Plastid genes essential for sustaining life such as tRNA synthesis were present in each species of holoparasitic plant, whereas those related to photosynthesis were present in only a few species. We calculated dN/dS ratios of certain protein-coding genes with high mutation pressure that favored for holoparasitism which is vital for its functionality.

Significance

Parasitic plants can greatly damage and contribute to ecosystems. However, through understanding the causes of parasitism and the patterns of AT richness shown among them, scientists can predict potential parasite growth areas and manipulate the effects these parasites have on their environment. Interactions between holoparasitic plants and their hosts can have a major impact on other biotic factors within that ecosystem. Since holoparasitic plants do not have photosynthetic ability, they siphon nutrients from host plants. Due to deletion indels targeting guanine and cytosine nucleotide bases, the plant typically loses its capability for photosynthesis and demonstrates a reduced plastid genome, which can contribute to codon usage bias. Mutation is fundamental to evolution because it generates genetic variation on which

natural selection can act. By studying the lost genes in parasitic plants and the functions they lost, a genetic tree of evolution can be constructed to figure out the origin of parasites. Through studying the genes that were lost in holoparasitic plants and the associated functions lost with them, we can further understand the unique characteristics found in holoparasitic plants.

Introduction

Recent technological advances in high-throughput DNA sequencing have given biologists unprecedented access to many complete eukaryotic genomes (Goodwin, McPherson, and McCombie 2016; Shendure and Ji 2008; Chin et al. 2016). This allows a systematic investigation of genome evolution to understand its relation with the changes in morphology, physiology and biochemistry of the related organisms (Graur 2015; Lynch 2007). Due to their small sizes, the genomes of organelles like plastid and mitochondria play an important role in this research (Smith and Keeling 2013, 2015; Wicke et al. 2011; Daniell et al. 2016). Thousands of plastid genomes from various angiosperms are available in the public databases, and they are routinely used for plant systematics (Tonti-Filippini et al. 2017; Ruhfel et al. 2014). These plastomes show remarkable consistency in their architecture with lengths around 150 kbp, GC-content near 37% and about 110-120 genes. Genes in a typical plastid genome are organized in quadripartite manner consisting of two single copy regions separated by a pair of inverted repeats(Chen et al. 2020; Palmer 1985; Wicke et al. 2011). It has also been established that all plastid genomes are monophyletic originating from cyanobacteria (Timmis et al. 2004; Rodríguez-Ezpeleta et al. 2005; Sibbald and Archibald 2020).

Surprisingly the plastomes in a small subset of plants diverge substantially from the above consensus architecture (Chen et al. 2020; Wolfe, Morden, and Palmer 1992; dePamphilis and Palmer 1990). As an extreme example, the plastomes of *Balanophora laxiphora* and *Balanophora reflexa* are only 15.5 kb long with 19 genes and show record-setting AT-richness (H.-J. Su et al. 2019). Similar size reduction and AT-richness are also seen in the plastome of *Rhopalocnemis phalloides (Schelkunov, Nuraliev, and Logacheva 2019).* In general, the unusual plastomes are almost always from the parasitic plants, and therefore the relation between parasitism and genome architecture

is being investigated(Lyko and Wicke 2021; Wicke and Naumann 2018; Krause 2008). Parasitism in flowering plants evolved twelve times independently and comes in two forms - holoparasitism and and hemiparasitism (Nickrent 2020). The Santanales order, which includes both *Balanophora* and *Rhopalocnemis*, contains the largest number of parasitic genera and species. With increasing availability of complete plastid genomes from the parasitic plants of various orders, it will be interesting to identify common patterns in their unusual architectures.

Here we analyzed the plastid genomes from holoparasitic and hemiparasitic plants, and built a set of software tools to study GC content, lost genes and mutation rates. Our research supports that holoparasitic plants have a trend of unusually low proportions of GC content in their chloroplast genes compared to hemiparasitic and autotrophic plants. Plastid genes essential for sustaining life such as tRNA synthesis were present in each species of holoparasitic plant, whereas those related to photosynthesis were present in only a few species. We calculated dN/dS ratios of protein-coding genes of the holoparasitic and observed high mutation pressure. The tool built for this research are made publicly available in GitHub.

Results

GC Content:

The plastid genome sequences were downloaded from the NCBI Genbank database. The GC contents and genome sizes were computed for all sequences.

After graphing the plastid genome size in relation to the GC content of each analyzed species, a quadratic relationship was determined between the two variables. The plants of the *Balanophora genus* had the lowest GC content that ranged from 11.608% to 12.948% and also the smallest plastome sizes ranging from 14,624 bp to 16,056 bp. *M. kanehirai* with a GC content of 22.448% and plastome size of 25,740 bp and *H. visseri* with a GC content of 23.413% and plastome size of 27,233 bp indicated an increase in plastid genome size that corresponded with an increase in GC content. This relationship begins to reach the vertex with *L. madreporoides* (GC content 37.050% and 83675 bp), *C. chinensis* (GC content 37.613% and 86,927 bp), and *C.*

japonica (GC content 38.302% and 121,037 bp). The parasitic plants of the *Santulum* genus and *Phallecaria* genus, ranging from a GC content between 37.916% and 38.006% and a plastome size between 138,684 bp and 144,263 bp, begin to show an inverse relationship in which a larger plastome size indicates a lower GC content. Lastly, the plants of the *Krameria* genus that have a GC content ranging from 32.267% to 33.749% and plastome size ranging from 171,851 bp to 177,797 bp clearly indicate an increase in plastid genome size relates to a decline in the percentage of GC content.

Holoparasitic vs Hemiparasitic vs Nonparasitic **&** AT Rich vs GC Rich

Genus	Species	GC Content	Genome Size
Balanophora	***B. yakushimensis***	12.685%	14624 bp
	B. fungosa	12.637%	15130 bp
	B. reflexa	11.608%	15507 bp
	B. laxiflora	12.196%	15505 bp
	B. harlandii	12.948%	16056 bp
Mitrastemon	***M. kanehirai***	22.448%	25740 bp
Hydnora	***H. visseri***	23.413%	27233 bp
Cytinus	***C. hypocistis***	29.887%	19400 bp
Thismia	***T. neptunis***	30.891%	2156 bp
	T. alba	24.923%	10127 bp
Santalum	***S. boninense***	38.006%	144263 bp
	S. album	37.999%	144034 bp
Phacellaria	***P. glomerata***	37.930%	138684 bp

	P. compressa	37.916%	138903 bp
Krameria	***K. erecta*** ***K. lanceolata*** ***K. bicolor***	32.267% 33.749% 33.601%	177797 bp 171851 bp 172606 bp
Cuscuta (“photosynthetic holoparasite”)	***C. japonica*** ***C. chinensis***	38.302% 37.613%	121037 bp 86927 bp
**Lennoa	***L. madreporoides***	37.050%	83675 bp
Malus	***Malus domestica*** ***Malus sieversii***	36.572% 36.503%	159926 bp 160230 bp
Vitis	***Vitis vinifera*** ***Vitis stilbene***	37.384% 44.671%	160915 bp 160928 bp

Table 1. List of some holoparasitic, hemiparasitic, and nonparasitic plants along with their GC Content and genome sizes to outline the observable trends in base pair richness.

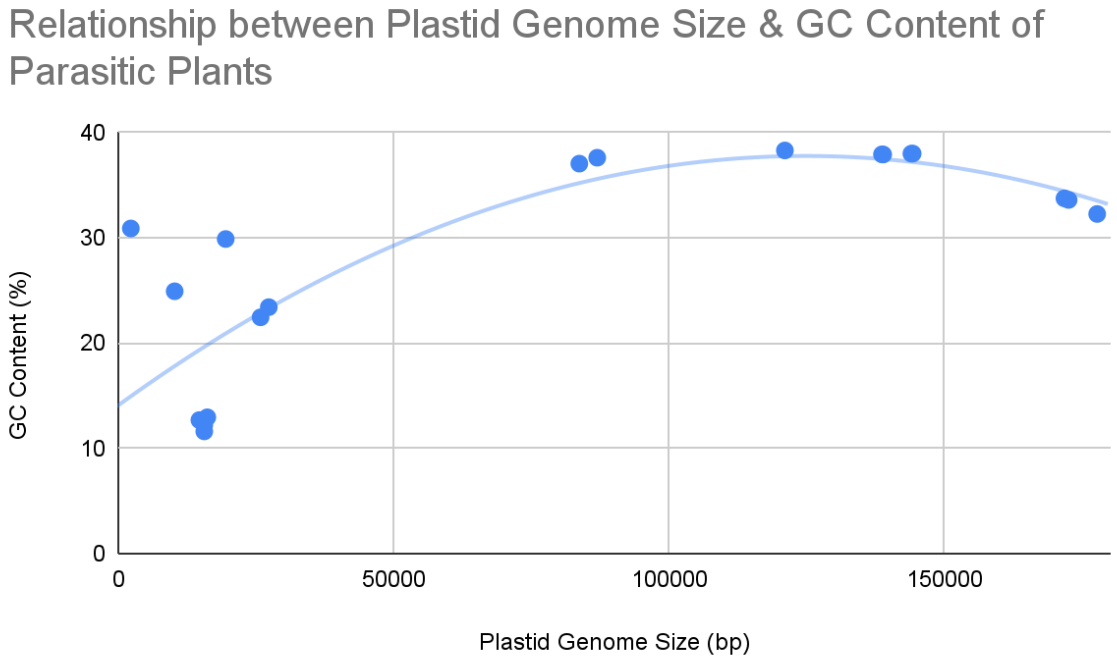

Fig. 1. Illustrates the relationship of Plastid Genome Size and GC Content (%) for the parasitic plants on the table above (all genuses except for the ones highlighted red) to demonstrate the quadratic relationship mentioned previously.

The holoparasitic genera (*Balanophora*, *Mitrastemon*, *Hydnora*, and *Cytinus*) were all deemed to have low GC content and smaller plastome sizes. The only exception to this finding was the holoparasitic plant in the genus of *Lennoa* that was found to be GC rich and have a relatively larger plastome size in comparison to other analyzed holoparasitic plants. Further studies can be done to determine what characteristics of the holoparasitic *Lennoa* genus could possibly cause this exception.

In order to contextualize the findings from parasitic plants, we also analyzed nonparasitic plants, including apple, grapevine, and potato plants in order to determine similarities and differences between these far less AT rich plants and our target plants. For the *Malus sieversii* with 36.503% GC richness *and Malus domestica* with 36.572% GC richness.. The *Vitis vinifera* with 37.384% GC richness and *Vitis stilbene* and 44.671% GC richness.These results further support that most holoparasitic plants are

considered to be AT rich, especially in comparison to hemiparasitic and nonparasitic plants.

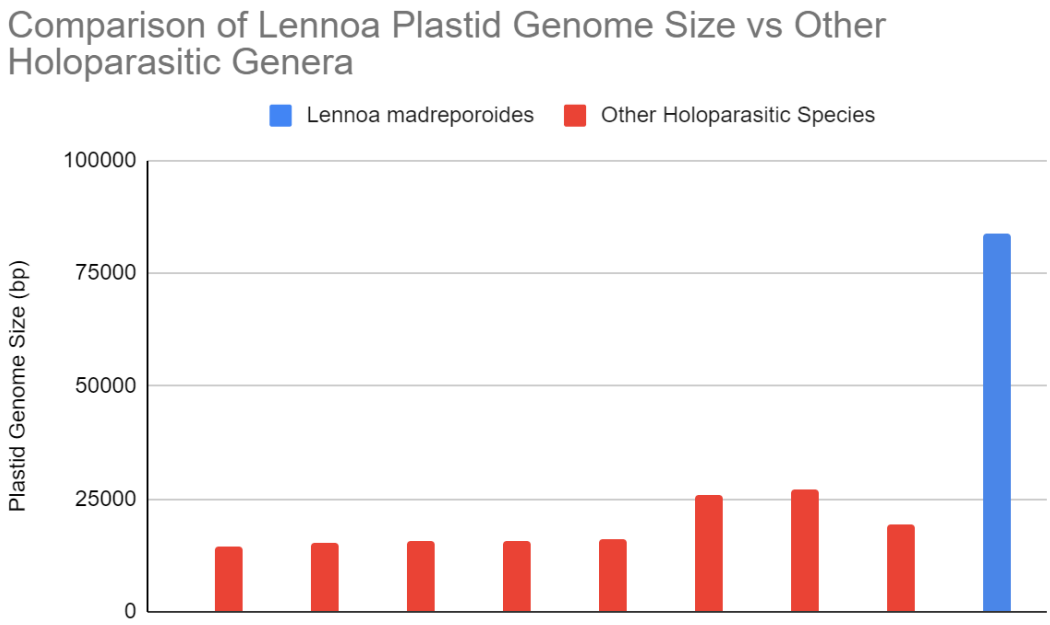

Fig. 2. There is a large difference between the *Lennoa madreporoides* genome size and those of other holoparasitic species

Lost Genes:

We analyzed the genomes of 9 different holoparasitic plant species (*Cuscuta reflexa*, *Viscum coloratum*, *Cassytha filiformis*, *Cuscuta gronovii*, *Orobanche crenata*, *Nuytsia floribunda*, *Orobanche gracilis*, *Viscum articulatum*, *Orobanche rapum-genistae*, and *Cytinus hypocistis*).

In general, the genes common amongst at least 7 plants were responsible for creating proteins essential to life function such as RNA-binding and macromolecule synthesis. Almost none of them were linked to photosynthetic processes. In contrast, the genes that were found in at most 4 plants were not crucial to life as the genes that occurred in at least 7 of the plants. In contrast, the genes that were found in at most 4 plants were not as essential as the genes that occurred in at least 7 of the plants. For example, ndhE and ndhG are both involved with the shuttling of electrons in

photosynthesis. Since photosynthesis does not occur in holoparasitic plants, these genes are not essential to function.

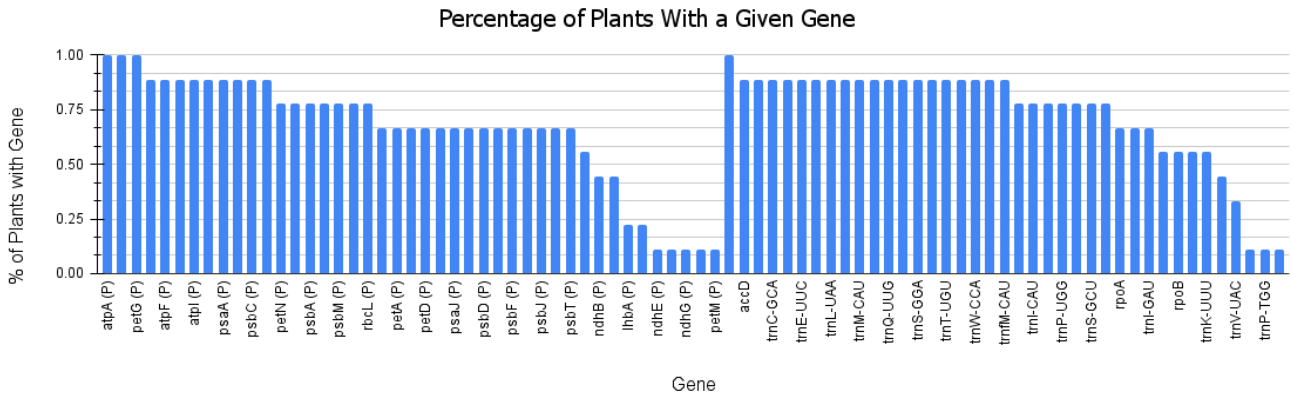

Fig. 3. Depicts a distribution of gene frequencies in a random sample of 9 holoparasitic plant species. A few genes are present in most of the plants and a few genes only present in 1 or 2 plants.

Mutation Rates:

In a broader sampling of holoparasitic plants, previous research identified 14 most conserved protein-coding genes in Balanophora. We chose 9 of them (*rps2, rps3, rps4, rps7, rps11, rps12, rps14, rps18, rps19*) to perform dN/dS tests on.

In Balanophora plastids, these 9 protein-coding genes with mutation pressure (dN/dS) greater than 1 suggest high mutation rates. As all 9 protein-coding genes were observed to have a positive or Darwinian selection pattern, that means there is significant evidence for genetic-code change in the Balanophora plastome, and that mutation rates are favored. Our findings support that these genes are compelling evidence that the plastid genomes of Balanophora are functional allowing this unique species to be holoparasitic.

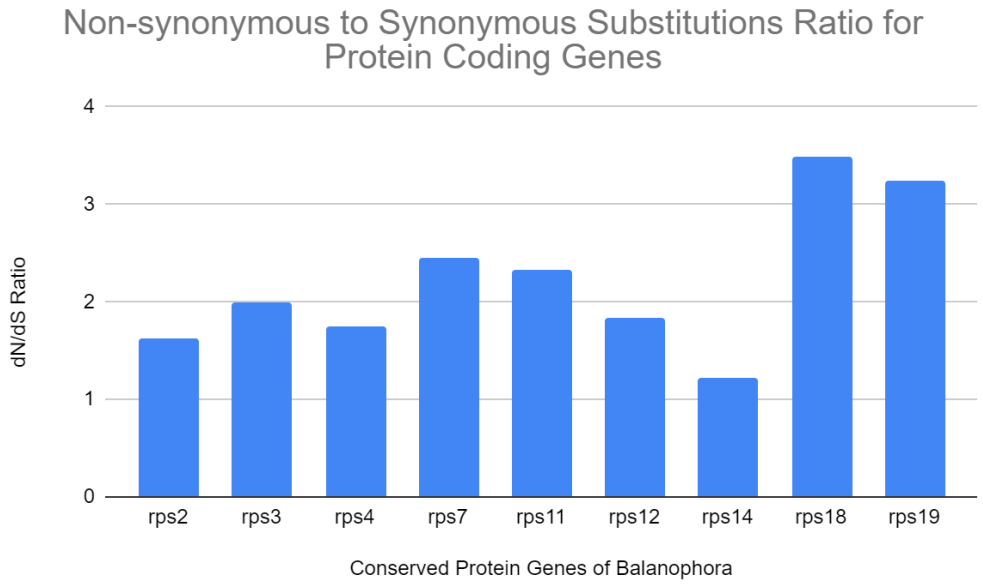

Fig. 4. Compares the dN/dS ratio of the 9 conserved protein coding genes of *Balanophora* to determine a broad overview of natural selection on holoparasitic plants

Analysis Tools:

We published a set of tools to help others continue similar investigations to analyze dN/dS ratio, lost gene analysis, and GC content calculation.
https://github.com/DHSCodingForMedicine/ScholarlyResearchProject2021-2022

Discussion

GC Content:

Since parasitism does not depend upon photosynthetic function, deleterious mutations may have accumulated without causing natural selection to eliminate the mutated organisms. This meant that the plastome decreased significantly in size, tending toward the reduced plastid genome. The Balphanora species had similar AT richness percentages around 88%, and the Thismia species had AT richness percentages around 86%.

Through analyzing our hypothesis on standard non holoparasitic plants, the *Malus sieversii*, *Malus domestica*, *Vitis vinifera*, *Vitis stilbene* all of which grow in similar environments demonstrate roughly similar AT richness percentages around 62%-63%.

This supports the theory that differences in AT richness could be correlated to differences in environments among plant species.

Past studies have found that GC content has a quadratic relationship with genome size, and this relationship is described in previous research (Šmarda et al. 2014). This is determined to likely be due to higher biochemical costs of synthesizing GC bases for larger genomes. The analysis of the plastomes for these various parasitic plants upholds this idea since the data demonstrated that smaller plastome sizes corresponded with lower GC content percentages, these two variables had a positive relationship up until a certain point of around 120000 bp. Then the values for GC content percentage begin to decrease while the size of the plastid genome continues to increase.

Also, the different characteristics between hemiparasitic and holoparasitic plants could indicate the disparity in their GC content and plastome sizes. The plastome sizes of the hemiparasitic plants are likely larger due to the plants' ability to photosynthesize. This would also explain the larger genome size of the *Cuscuta* genera since it is considered a photosynthetic holoparasite. However, because most holoparasitic plants do not need to photosynthesize, they can reduce the size of their genomes. This could also be a possible explanation as to why the majority of the holoparasitic plants have lower GC contents when relating it to the concept of higher GC content requiring a higher biochemical cost.

Lost Genes:

Taking a closer look at these results, we notice that most genes that had a low frequency of appearance in the species were photosynthetic genes. Holoparasitic plants don't have any use for photosynthesis since they receive most of their energy from their host through a structure called the haustorium unlike non-parasitic plants that convert energy from the sun into usable energy using the chloroplast. Therefore, the main purpose of the chloroplast, to make this energy for the plant, is now rendered useless. Thus, many mutations accumulate over generations within the chloroplast, particularly in those involved in photosynthesis, since those mutations don't affect the survival of the

plant, eventually leaving the chloroplast plastid genome to consist of many mutations and thus lost genes.

However, some genes in the chloroplast plastid which are necessary for the holoparasitic plants survival still exist with sparse mutations such as the tRNA genes because any plants which had harmful mutations often didn't live long to pass on their mutations to future generations, leaving behind only plants that didn't have those deleterious mutations. For example, the atpA and atpB genes are found in every species because they are essential for the survival of the plants by coding for the main energy carrying molecule, ATP. Another example of a gene that was sustained throughout many generations is the clpP gene that codes for a complex in the mitochondria, an organelle responsible for producing energy for the plant. So, essential genes like these are present in most of the plant species due to their importance or lack of photosynthetic importance.

Therefore, these results demonstrated and confirmed the theories that the holoparasitic plants, such as the *Balanaphora* presented in this paper, receive their energy from their host and so they lack function for the chloroplast. However, the exact mechanism through which these mutations accumulated is still unclear. Only identifying the genes that were present in each species isn't enough to determine the way those genes were lost. In order to do so, taking a look and comparing the DNA sequence to the ancestors of these holoparasitic species is necessary. Therefore, future experiments may be necessary in order to fully understand the root cause of the lost genes found in these AT rich holoparasitic plants.

Mutation Rates:

Since all of our dN/dS ratios that we calculated are greater than 1, which means it has exceeded unity, natural selection promotes changes in the protein sequence for the 9 protein coding genes we have analyzed within the *Balanophora* plastid. This shows that the 9 protein genes have a high mutation pressure in favor for holoparasitism and are vital for its functionality. There is clear evidence of saturation at synonymous sites leading to the *Balanophora* common ancestor, with $dS > 1.5$ for those genes (B. Su et al. 2022).

While our paper explores the mutation rate for *Balanophora*, similar research has been done on another holoparasitic plant, *Cytinus Hypocistis*. By evaluating the *C. Hypocistis* plastome and the dN and dS values for protein coding genes, it has been analyzed that the genres are mostly evolving under purifying selection and share similar selective intensity to *Malvales* autotrophic relatives (Roquet et al. 2016). This indicates that several of those protein coding genes in the *C. Hypocistis* plastome are functionally important, enabling it to be holoparasitic. However, there is previous research that shows natural selection does not promote certain protein coding genes for the *Cytinus* branch (rps3, rps11, and rps19). This relates to our research of genes that were only found in at most 4 holoparasitic plants (trnp-TGG, ndhE(P), ndhG(P), and petM (P)) by indicating how an accumulation of mutation rates that does not affect the plastid become lost genes.

Despite the common ancestor for *Balphanora* being *Santalales*, a flowering photosynthetic/hemiparasitic plant, evolutionary pressures and mutation rates have converged this species to become holoparasitic. Similarly, the common ancestor of *C. Hypocistis* is *Malvales,* a photosynthetic flowering plant. The photosynthesis capability of many flowering plants has been reduced by parasites. This indicates that further analysis of holoparasitic species' biotic factors must be considered to discover why these mutation rates are favored with highly selective pressures.

dN/dS ratio may not be the most accurate way to study mutation rates as there can be many discrepancies due to codon bias, alternative substitutions pathways, transition vs transversion mutation ratio that lead to inaccurate ratios despite proper method of calculating. A better way to evaluate mutation rates with a more limited number of errors through the McDonald Craigman Test. pN/pS ratio calculates the polymorphism within a species and is considered analogous to calculating dN/dS ratio. We are considering running tests to calculate the polymorphic changes that occurred to further help support our claim of high mutation pressures.

Materials and Methods

Obtaining Plastid Genomes:

Plastid genomes of nine different holoparasitic plant species (*Cuscuta reflexa*, *Viscum coloratum*, *Cassytha filiformis*, *Cuscuta gronovii*, *Orobanche crenata*, *Nuytsia floribunda*, *Orobanche gracilis*, *Viscum articulatum*, *Orobanche rapum-genistae*, and *Cytinus hypocistis*) [Group A] were downloaded from the NCBI database. Additionally, the plastid genomes of the following organisms were downloaded from the same source:

Holoparasitic -*Thismia* genus (*Thismia alba*, *Thismia neptunis)*, Balanophora genus (*B. yakushimensis, B. fungosa, B. reflexa, B. laxiflora, B. harlandii*), *Mitrastemon kanehirai*, *Hydnora visseri*, *Cuscuta* genus (*C. japonica and C. chinensis*), and *Lennoa madreporoides*

Hemiparasitic - *Santalum* genus (*S. boninense and S. album*), *Phallecaria* genus *(P. glomerata and P. compressa*), and *Krameria* genus (*K. erecta, K. lanceolata, K. bicolor*)

Nonparasitic - *Malus* genus (*Malus sieversii, Malus domestica), Vitis* genus *(Vitis vinifera, Vitis Stilebene),* and *Solanum tuberosum*

GC Content:

The genome sequences of *Thismia alba*, *Thismia neptunis*, *Balanophora laxiflora*, *Balanophora yakushimensis*, *Malus sieversii, Malus domestica, Vitis vinifera, Vitis Stilebene* and *Solanum tuberosum* were obtained from the GenBank database. The analysis of gene sequence ratios was performed via BioPython through BLAST, which was utilized with the obtained sequences. The methods of data collection primarily consisted of obtaining the GC-richness values using BioPython programming methods.

This same method was used for the analysis of GC content and genome size which included the Balanophora genus (*B. yakushimensis, B. fungosa, B. reflexa, B. laxiflora, B. harlandii*), Santalum genus (*S. boninense and S. album*), Phallecaria genus *(P. glomerata and P. compressa*), Cuscuta genus (*C. japonica and C. chinensis*), Lennoa genus *(L. madreporoides*), Mitrastemon genus (*M. kanehirai*), Hydnora genus

(*H. visseri*), Cytinus genus (*C. hypocistis*), and Krameria genus (*K. erecta, K. lanceolata, K. bicolor*). The Fasta files for the plastid genomes were downloaded from the NCBI GenBank database. The files were then run through the Pycharm IDE with the usage of BioPython methods to calculate the GC content and size of the plastid genomes.

Lost Genes:

Initially, we collected the genomes of 9 holoparasitic plant species (Group A) that represented holoparasitic plants as a whole. Using the NCBI database, we analyzed the same section of plastid DNA across all 9 plant species so we could identify whether a particular gene appeared in a given plant or not. We then implemented a simple filtering algorithm using Biopython to identify genes that occurred with a particular frequency.

Mutation Rates:

We first analyzed the protein-coding genes which were prevalent in Balanophora, so we chose 9 genes found in previous research (H.-J. Su et al. 2019) to analyze the mutation rates. Then we tested to see each dN/dS ratio using the dN/dS algorithm for the selected protein-coding genes as per Nei & Gojobori 1986 method with the derivative (per site conversion) adapted from Jukes & Cantor 1967, we used an algorithm incorporating the complete definition of dN/dS (Adelq). The algorithm consists of cleaning whitespace from each string, then plugging a string of each sequence into our method, then plugging that data into the formulas to find the dN/dS ratio.

$$d_N = -\frac{3}{4} ln\left(1 - \frac{4p_N}{3}\right) \qquad d_S = -\frac{3}{4} ln\left(1 - \frac{4p_S}{3}\right)$$

$$dN/dS = \frac{d_N}{d_S}$$

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